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CONTENTS

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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 and captions for illustrations 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.

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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 fully descriptive. 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.

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; good tables should not exceed the normal page width of the Journal, and authors should attempt to revise or rearrange data to fit this pattern.

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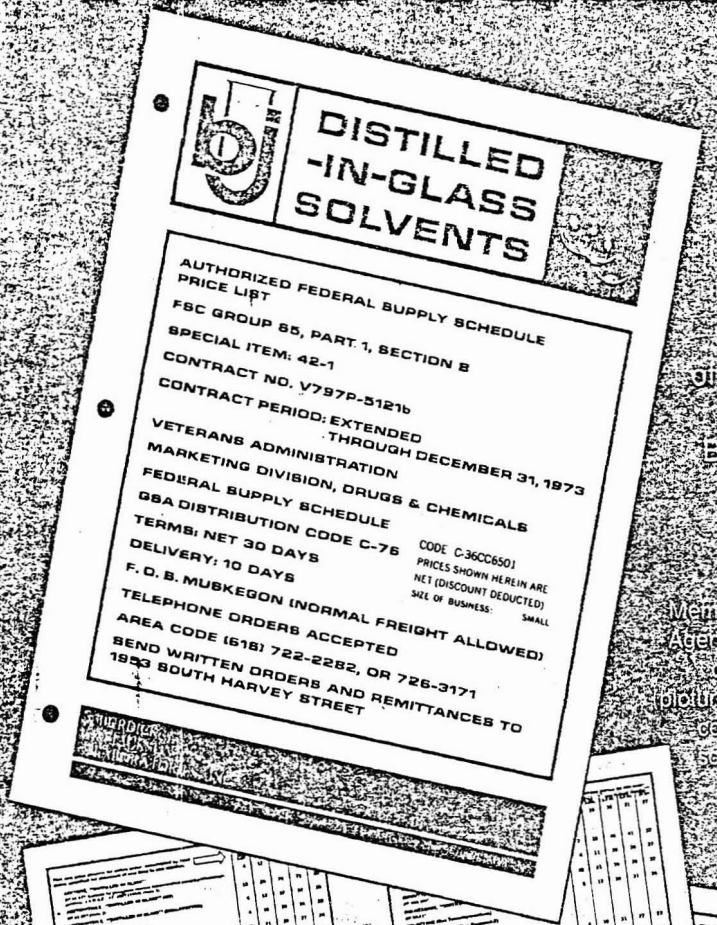

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References to journal articles must include the following information: Last names and at least one initial of *all* authors (*not* just the senior author); year of publication, enclosed in parentheses; title of journal, abbreviated according to accepted *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.

The abbreviation for the journal title should be repeated for each reference; the use of *ibid.* has been discontinued. *This Journal* will be referred to as *JAOAC*.

The compendium of methods of the Association should be listed as follows: *Official Methods of Analysis* (1970) 11th Ed., AOAC, Washington, D.C., with appropriate section numbers; the edition and year are, of course, subject to change.

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.

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.

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)
nm	nanometer(s) (millimicron)
′	foot (feet)
″	inch(es)
lb	pound(s)
oz	ounce(s)
ppm	parts per million
ppb	parts per billion
rpm	revolutions per minute
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
⅋	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.")

DAIRY PRODUCTS

Collaborative Study of the Quantitative Determination of Titanium Dioxide in Cheese

JOSEPH L. LEONE
Food and Drug Administration, 599 Delaware Ave., Buffalo, N.Y. 14202

A quantitative, colorimetric procedure for the determination of titanium dioxide has been subjected to collaborative study. The method incorporates ashing, dissolution of the ash in sulfuric acid, and color development using hydrogen peroxide. The spectrophotometric response is compared to a standard prepared similarly. The yellow-to-orange color is quite stable in the absence of Fe. Other interferences are Ni, Cu, Co, Mb, Vd, and Cr. A collaborative study of cheese containing 0.05, 0.10, and 0.4% titanium dioxide gave favorable results. The method has been adopted as official first action.

Titanium dioxide is often used as a whitener in the manufacture of cheese. Reports indicate the use of this material in New York State is a widespread industry practice. Current federal regulations have no provisions for the use of titanium dioxide in the manufacture of standardized cheeses. The use of the material and the absence of a regulation covering its use initiated the work on a quantitative method to meet any regulatory demands.

A quantitative method has been developed which incorporates ashing of the sample, dissolution of the ash in sulfuric acid to a known volume, and development of a yellow color with hydrogen peroxide. The spectrophotometric response is compared to a standard prepared similarly. Kolthoff and Sandell (1) state that the yellow-to-orange color given by acid titanium solutions with hydrogen peroxide is probably due to an ion such as $[TiO_2(SO_4)_2]^-$. The color of the oxidized solution is quite stable in the absence of iron. The following interferences should be noted, e.g., nickel, copper, cobalt, molybdenum, vanadium, and chromium, which produce colors that would lead to error (2). These minerals, if present in cheese, will be at the trace level. We found the standard curve to be linear for concentrations of 0.0 to 0.1 mg/ml which give absorbance values of 0.0 to 0.9.

Collaborative Study

Mozzarella cheese known and verified to be free of titanium dioxide was selected for the preparation of samples to contain 0.0, 0.05, and 0.1% titanium dioxide. A commercial product found to contain titanium dioxide was selected as a fourth sample.

Samples containing approximately 0.05 and 0.1% titanium dioxide were prepared by making a slurry with water, adding the appropriate amount of titanium dioxide, and blending in a Waring blender. The samples were then heated in a 55°C oven and periodically mixed by stirring to release the added water and to return the samples to their approximate original weight and consistency.

The 0.0% sample and the commercial sample were prepared by chopping and blending. The homogeneity of the prepared samples was assured by replicate analyses by the author. Prepared sets of the 4 samples were sent to 10 laboratories for analysis.

METHOD

Titanium Dioxide—Official First Action

(*Caution: See* 46.030.)

16.C10 *Standard Solution*

Titanium dioxide std soln.—0.1 mg/ml. Accurately weigh 50 mg TiO_2 and transfer to 250 ml beaker; add 15 g anhyd. Na_2SO_4 and 50 ml H_2SO_4. Add boiling chips, cover with watch glass, and heat to boiling on hot plate to dissolve. *Cool,* and cautiously add 100 ml H_2O with stirring. (Warm on steam bath if soln becomes cloudy.) Cool, transfer soln to 500 ml vol. flask contg 200 ml H_2O, and dil. to vol. with H_2O.

16.C11 *Preparation of Sample*

Weigh, to nearest 0.1 g, 10 g prepd sample, 16.191, into 100 ml Pt dish and char under IR lamp. Place in cold furnace and ignite at 850° to white ash.

Cool, add ca 1.5 g anhyd. Na_2SO_4 and 10 ml H_2SO_4, cover with watch glass, and bring to boil on hot plate to dissolve. Turn heat off and let cool on hot plate. *Cautiously* rinse cover, carefully add ca 30 ml H_2O, and mix with stirring rod to disperse any insol. salts. Heat on steam bath if insol. material forms cake on bottom of dish.

Transfer quant. to 100 ml vol. flask with aid of ca 40 ml H_2O. If soln is cloudy, heat on steam bath or in boiling H_2O bath to clarify. Cool, and dil. to vol. with H_2O.

16.C12 *Preparation of Standard Curve*

Transfer 0, 1, 2, 3, 4, and 5 ml TiO_2 std soln to sep. 5 ml g-s graduates (or vol. flasks) and dil. to vol. with H_2SO_4 (1+9). Add 0.2 ml 30% H_2O_2, mix, and det. *A* on recording spectrophtr in 1.0 cm cells from 650 to 325 nm against 0.2 ml 30% H_2O_2 in 5.0 ml H_2SO_4 (1+9). Det. *A* at max. ca 408 nm and prep. std curve.

16.C13 *Determination*

Transfer 3.0 ml sample soln to 5 ml g-s graduate (or vol. flask), dil. to vol. with H_2SO_4 (1+9), and continue as in **16.C12**, beginning "Add 0.2 ml 30% H_2O_2 . . ."

Det. mg TiO_2 in sample from std curve, and calc. as % TiO_2.

Results and Recommendation

The results of the 10 collaborators are presented in Table 1. The fortification levels of Samples A and B are approximate due to the difficulty of preparing an absolute spike level. Sample C is a verified blank and Sample D is a commercial product found in the market place, containing about 0.4% titanium dioxide.

Table 1. Results of collaborative study of titanium dioxide in cheese

	Sample			
Coll.	A 0.100	B 0.050	C 0.000	D Com. prod.
1	0.100	0.052	0.001	0.381
2	0.087[a]	0.047[a]	0.000	0.325[a]
3	0.102	0.048	0.000	0.380
4	0.099	0.053	0.003[a]	0.376
5	0.102	0.052	0.000	0.374
6	0.100	0.050	0.000	0.380
7	0.098	0.048	0.000	0.375
8	0.120[a]	0.057[a]	0.003[a]	0.426[a]
9	0.100	0.051	0.002	0.386
10	0.104	0.053	0.000	0.387
Mean ($\bar{X}$)	0.101	0.051	0.001	0.379
Std dev. (S)	0.008	0.003	0.001	0.024
Std error ($S_{\bar{X}}$)	0.003	0.001	0.0004	0.008

[a] Not within 99% confidence level.

$$\bar{X} = \frac{\Sigma x}{n}$$

$$S = \sqrt{\frac{\Sigma(X - \bar{X})^2}{n - 1}}$$

$$S_{\bar{X}} = \frac{S}{\sqrt{n}}$$

Results from Collaborators 2 and 8 were outside the 99% confidence level, but were included in the statistical evaluation. The positive results reported for Sample C appear to be due to instrumental error.

Comments received from the collaborators dealt mainly with technique. Some of these comments have been incorporated into the method. One collaborator suggested a 5-fold increase in the final volume of the color development step for the convenience of handling larger volumes. The author has no objection to this practice but refrained from including this suggestion in the method. This method is not rigid or empirical and lends itself to modification at the analyst's discretion.

Other collaborators commented on a time specification for charring and ashing the sample. This suggestion was not included in the method because the author felt that charring and ashing would vary among laboratories, depending on technique. Drying ovens can be substituted for the heating lamps, provided the sample is dried completely. A cold muffle furnace with gradual increase in temperature can also be substituted for the heating lamps.

Another collaborator suggested specifying a development time for the color reaction. The author

did not include this suggestion since color development is instantaneous and was found to be stable for at least 24 hr.

The Associate Referee recommends that the proposed method be adopted as official first action.

Acknowledgments

The author wishes to express appreciation to Edward G. Seibert, Food and Drug Administration, Buffalo, N.Y., for his guidance; to James R. Evans, Food and Drug Administration, Buffalo, N.Y., for his assistance in the statistical evaluation; and to the following collaborators who participated in this study:

Eugene A. Breault, John Gould, Jane Kaminski, Joanne Maguire, Carl A. More, E. Clifford Netz, and Michael Olson, Food and Drug Administration, San Francisco, Washington, D.C., Buffalo, Philadelphia, Dallas, Minneapolis, and New York, respectively

Wayne Chu and Raymond L. King, University of Maryland College of Agriculture, College Park, Md.

F. Kósikowski and Kunihide Masuyama, New York State College of Agriculture and Life Science, Cornell University, Ithaca, N.Y.

Jon L. Schermerhorn, State of New York Department of Agriculture and Markets, Albany, N.Y.

References

(1) Kolthoff, I. M., & Sandell, E. B. (1952) *Textbook of Quantitative Inorganic Analysis*, 3rd Ed., The Macmillan Co., New York, p. 706

(2) Furman, N. H. (Ed.) (1962) *Scott's Standard Methods of Chemical Analysis*, Vol 1, 6th Ed., D. Van Nostrand Co., Inc., Princeton, p. 1106

The recommendation of the Associate Referee was approved by the Referee and Subcommittee C and was adopted by the Association; *see* (1973) *JAOAC* 56, 397–398.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Collaborative Study of the Foss Milko-Tester Method for Measuring Fat in Milk

W. FRANK SHIPE and G. F. SENYK
Department of Food Science, Cornell University, Ithaca, N.Y. 14850

In a third collaborative study, the Milko-tester was compared with the Babcock method in 9 laboratories. In addition, 3 laboratories analyzed the samples by an ether extraction method. The standard deviations for these methods were +0.018, +0.032, and +0.044%, respectively. These deviations are based on triplicate determinations on 5 samples by each of the laboratories. The average values for all samples were 3.99, 3.96, and 3.88% for the Milko-tester, Babcock, and ether extraction methods, respectively. The standard deviation of differences between the Milko-tester and Babcock for all 9 laboratories was +0.077%.

The Foss Milko-tester was approved as official first action on the basis of the results of a 1968 collaborative study (1). Another collaborative study was made in 1971 (2) which included a comparison between the Mark II and automatic (MTA) Milko-testers. The results indicated that the 2 types of Milk-testers gave comparable results. Considerable variations were observed among laboratories, indicating a need for more careful calibrations of the instruments. The current study was undertaken to provide additional data on the reliability of these instruments.

The principle of the method and the operation of the Milko-testers were outlined in the previous reports (1, 2). Detailed operating instructions are given in the manufacturer's instruction book which can be obtained from Foss America, Inc., Rte 82, Fishkill, N.Y. 12524.

Collaborative Study

Samples were obtained from 9 individual cows of the Cornell University herd. They were brought direct from the barn and blended while still warm to yield 5 samples ranging from about 3.2 to 5.2% fat. Blends of the milk from 3 different cows were used to prepare Samples A, B, and C. Sample D was a blend of Samples A and B. Sample E was a blend of Samples A, B, C, and D. The samples were divided into the appropriate number of sample bottles. Stoppers were inserted carefully into the bottles and pressed to expel any air from the top. Bottles were chilled in ice water and packed in prechilled insulated boxes containing cans of frozen water. Samples were transported by car to the collaborators; all samples were still cold when delivered. Collaborators were instructed to test them within 2 days of delivery, and they all complied. They were asked to use the following procedure in preparing the samples:

Place samples in warm water bath (95–105°F) long enough to reach this temperature. Invert sample bottles 2 or 3 times and *then* remove stopper or cap from container. Mix by pouring gently from the original container into another container. Repeat this 4 times. *Immediately* after samples have been mixed take aliquots for methods to be used. For Mark II Milko-testers, obtain triplicate Milko-tester readings and then remove aliquots by pipet for Babcock tests. Do *not* mix all samples at once and then let them stand before performing Milko-tester analyses. However, all samples can be put in water bath at same time.

An effort was made to obtain collaborators who had the equipment to perform the Babcock, ether extraction, and Milko-tester official methods (3). Unfortunately, only 3 collaborators were equipped to perform the ether extraction method. There were 6 MTA or Mark III instruments and 3 Mark II Milko-testers. All collaborators with Milko-testers also performed the Babcock test. All collaborators were routinely performing fat analyses.

Results and Recommendation

A comparison of Milko-tester and Babcock results of 9 laboratories is shown in Table 1. The average difference of 0.03% between methods and the overall standard deviation of difference of 0.077% are considered acceptable for these samples which were blends of milk from only 9 cows. (According to the original Milko-tester study (1), the Milko-tester is considered properly calibrated

This report of the Associate Referee, W. F. Shipe, was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C and was accepted by the Association; *see* (1973) *JAOAC* 56, 397–398.

Table 1. Comparison of Milko-tester and Babcock results[a] obtained by 9 laboratories

Lab.	Milko-tester (M)	Babcock (B)	Diff. (M − B)	Std dev. of diff.
1	4.02	4.01	0.01	0.036
2	4.05	3.93	0.12	0.082
3	3.95	3.97	−0.02	0.051
4	4.07	3.95	0.12	0.110
5	4.05	4.01	0.04	0.030
6	3.89	3.87	0.02	0.057
7	3.97	3.98	−0.01	0.023
8	3.97	3.97	0.00	0.062
9	3.97	3.97	0.00	0.070
All samples	3.99	3.96	0.03	0.077

[a] Results represent averages of triplicate determinations on 5 samples, expressed as per cent fat.

Table 2. Comparison of Milko-tester and Babcock results[a] on 5 milk samples

Sample	Milko-tester (M)	Babcock (B)	Diff. (M − B)	Std dev. of diff.
A	3.21	3.20	0.01	0.055
B	4.07	4.05	0.02	0.062
C	5.06	4.95	0.12	0.101
D	3.62	3.64	−0.02	0.044
E	4.00	3.97	0.03	0.041
All samples	3.99	3.96	0.03	0.077
Samples A, B, D, E	3.73	3.72	0.01	0.052

[a] Results represent averages of triplicate determinations by 9 laboratories, expressed as per cent fat.

Table 3. Differences[a] between Milko-tester and Babcock values

Lab.	Samples A	B	C	D	E
1	2	0	7	−3	2
2	9	11	27	6	11
3	−6	−3	6	−5	0
4	7	15	30	3	5
5	5	0	5	1	7
6	−3	2	11	−2	1
7	2	−2	0	−4	0
8	−4	−1	10	−6	0
9	−5	0	11	−7	0

[a] Expressed in hundredth of per cent.

when the standard deviation of difference is not >0.10 for individual cow sample or not >0.06 for herd or composite sample.) However, the results from Laboratories 2 and 4 are not considered acceptable because of the large differences between methods. An examination of the results of these 2 laboratories indicated that their Milko-tester values for the samples with the highest fat content were significantly different from the rest of the laboratories. The effect of sample source on the Milko-tester − Babcock differences is shown in Table 2. As will be noted, the high fat sample (i.e., Sample C) showed the greatest difference. The exclusion of the results for Sample C greatly reduced the apparent difference between the 2 methods.

The results in Table 3 specifically show that the Milko-testers at Laboratories 2 and 4 were not properly calibrated, particularly for the higher fat levels. In fact, all but one of the laboratories reported higher values for Sample C by Milko-tester than by Babcock. This indicates that the instruments had not been carefully calibrated at the higher level.

In spite of the apparent inadequate calibration of some of the instruments, Milko-testers gave more reproducible results than the Babcock method, as illustrated in Tables 4 and 5. It also should be noted that the reproducibility did not seem to depend on the fat level. Therefore, if the Milko-tester is properly calibrated one can expect to obtain more reliable results with the Milko-tester than with the Babcock method.

The 3 laboratories that analyzed the samples by the ether extraction method obtained an average of 3.88% compared to the overall average of 3.99 and 3.96% for the Milko-tester and Babcock tests, respectively. The standard deviation for the ether extraction values was ±0.044%, compared to ±0.018 and 0.032% for the Milko-tester and Babcock tests, respectively. These results, plus those obtained in the previous study (2), do not justify recommending the ether extraction method as the preferred reference method.

The combined results of this study and the previous 2 studies (1, 2) indicate that one can obtain results with the Milko-tester that are comparable to results from the Babcock method. However, the variability among laboratories indicates the need for more careful calibration of the instruments.

It is recommended that additional studies be undertaken in an attempt to improve the calibration procedure.

Acknowledgments

The Associate Referee wishes to express his appreciation to the following participants for their contributions to this study:

Richard D. Aplin and Herbert E. Shepard,

Table 4. Comparison of standard deviations[a] between methods and analysts

Lab.	Method	
	Milko-tester	Babcock
1	0.016	0.016
2	0.016	0.045
3	0.010	0.036
4	0.035	0.026
5	0.024	0.032
6	0.010	0.018
7	0.009	0.043
8	0.007	0.036
9	0.008	0.036
All labs.	0.018	0.032

[a] Based on triplicate determinations on 5 samples.

Table 5. Comparison of standard deviations[a] between methods and samples

Sample	Method	
	Milko-tester	Babcock
A	0.017	0.014
B	0.019	0.030
C	0.015	0.038
D	0.017	0.041
E	0.019	0.036
All samples	0.018	0.033
Samples A, B, D, E	0.018	0.032

[a] Based on triplicate determinations by 9 laboratories.

Boston Regional Marketing Area, 230 Congress St., Boston, Mass.

Pravin A. Bhuva, John M. Moore, Leah R. Snow, and Michael J. Vaughn, Federal Milk Marketing Area Laboratory, 51 East St., Dedham, Mass.

Myron Brown, Dairy Herd Improvement Cooperative, Box 573, Cobleskill, N.Y.

Matthew Cacace and Rachel Cook, Pollio Dairy Corp., Campbell, N.Y.

Elmer George and Barbara Peat, State Food Laboratory, 1220 Washington Ave., Albany, N.Y.

Blakely Harris, Linda Austin, Nancy Gulyas, and Helen Wood, New York Dairy Herd Improvement Cooperative, Ithaca, N.Y.

Donald Hewlett, Dairy Herd Improvement Cooperative, Rte 44, Millbrook, N.Y.

Richard Lincourt, Breakstone Sugar Creek Foods, Walton, N.Y.

William Semerod and Diana Alcock, Dairymen's League Cooperative, 402 Park St., Syracuse, N.Y.

References

(1) Shipe, W. F. (1969) *JAOAC* 52, 131–138

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ENZYMES

Collaborative Study of a Test to Determine Whether Shucked Oysters Have Been Frozen and Thawed

EDITH GOULD
National Marine Fisheries Service, U.S. Department of Commerce, Milford, Conn. 06460

Six laboratories have collaboratively tested a method to determine whether shucked oysters have been frozen and thawed at any time. The method utilizes the differing electrophoretic migration rates of various forms of malic enzyme activity, some of which are not present in the tissue fluid until after the tissue has been frozen and thawed. The technique is enzymography, which combines electrophoresis and a specific histochemical medium. Five laboratories tested *Crassostrea virginica*, obtaining markedly different patterns for the unfrozen oysters compared with the frozen and thawed oysters. The sixth laboratory tested *C. gigas*, and was similarly successful in distinguishing between the storage treatments. In shucked oysters stored up to 3 weeks at 4°C, neither autolysis nor bacterial activity altered the enzymographic patterns. The method has been adopted as official first action.

Occasionally, shucked oysters that have been partially frozen during improper storage procedures are marketed as fresh-shucked oysters, although they have a shorter shelf-life and a greater ratio of fluid to solids than the unfrozen animal. At the request of the oyster industry (F. McGinnis, private communication, 1969), an objective test (1) was developed[1] to determine whether shucked oysters, fresh or spoiled, have been frozen and thawed, or superchilled (−2, −5°C) and thawed, at any time. The test has since been subjected to a collaborative study, the results of which are reported here.

Collaborative Study

Differing electrophoretic migration rates of variously soluble forms of malic enzyme (EC 1.1.1.40) are the basis for the test. A normally soluble form of malic enzyme (free ME) activity is present, but not always in detectable amounts, in the tissue fluid of unfrozen oysters. Additional, latent forms are solubilized by freezing and thawing the tissue, as they are by any gross disruption of cellular structure, such as homogenization. The solubilized latent forms are readily visualized by enzymography, the coupling of polyacrylamide gel electrophoresis, and a specific histochemical medium.

Because the method requires that the centrifuged tissue fluid (CTF) be obtained from whole oyster meats, the problem of uniform sampling arose, further compounded by the difficulty of arranging for the collaborating laboratories to conduct their studies simultaneously, on similarly fresh and on similarly stale samples. One laboratory, therefore, tested 36 coded shucked oysters (*Crassostrea virginica*), whose individual storage history was unspecified; 3 other collaborating laboratories were sent 4 lots (9 oysters each, for triplicate multiple samplings) of similarly coded oysters; and 2 more laboratories collected and treated their own test animals, one set of which was *C. gigas*. The 4 treatment variables were fresh-unfrozen, fresh-frozen/thawed, stale-unfrozen, and stale-frozen/thawed. Stale oysters were those that had been frozen and thawed or left unfrozen, and then stored at 4°C 3 weeks.

For detailed instructions see the first paper reporting the test (1). Keep these 3 requirements especially in mind: (*1*) Prepare the polyacrylamide formularies *according to specifications* in the method rather than purchasing them, so that the buffer systems may be made more sharply discontinuous than those in commercially available formularies. (*2*) Keep the gels *cool* during the electrophoresis, either by putting the entire bath assembly in a cold room or a refrigerator, or by circulating iced water through cooling coils. (*3*) Incubate gels in the histochemical medium *no longer than 30 min* in a dark place, and then rinse immediately. (*4*) Centrifuge only *whole* shucked oysters. (Never mince or homogenize the meats. Any mechanical disruption of the tissue will produce results similar to those obtained by freezing and thawing the tissue.)

[1] The test was developed at Atlantic Fishery Products Technology Center, National Marine Fisheries Service, Gloucester, Mass. 01930.

Table 1. Variables in test procedure for malic enzyme enzymography of oysters

Operation	Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5	Lab. 6
Speed of centrfgn, X g	20,000	2,500	same as 1	same as 1	14,500	same as 1
Electroph. voltage	1 mA/col./30 min +2 mA/col./2 hr	same as 1	same as 1	1 mA/col./30 min, +2 mA/col./15 min, +5 mA/col./75 min	1 mA/col./1 hr, 3 mA/col./2 hr	same as 1
Temp.	4°C	same as 1	room temp.	same as 1	same as 1	same as 1
Histochem. medium	mixed ≤1 hr before use, except *p*-nitro blue tetrazolium added just before use	same as 1	same as 1	mixed 24 hr before use, except *p*-nitro blue tetrazolium and phenazine methosulfate added just before use	same as 1	same as 1

Table 2. Results of collaborative study of malic enzyme enzymography to determine whether shucked oysters have been frozen and thawed

Lab.	Samples	Correct identifications	Per cent
1	12	12	100
2	12	12	100
3	12	0	0[a]
4	16	16	100
5	36	36	100
6	12	12	100

[a] Laboratory 3 performed the electrophoresis at room temperature. All other laboratories cooled the gels as specified in the method.

A few unavoidable minor changes in procedure at some of the laboratories proved the test to be gratifyingly flexible in some areas (Table 1). (Since the method was first published, the Associate Referee has found that the substrate concentration in the histochemical medium can vary substantially, and that 0.1*M* potassium malate, one-fourth the original concentration, produces a creditable pattern of activity on the gels.) One laboratory, however, neglected to cool the gels during electrophoresis (Table 1) and failed to get a pattern of enzyme-active bands on any gel (Table 2). Very little heat can partially inactivate most enzymes, and 3 hr of unrefrigerated electrophoresis will generate no small amount of heat.

Results and Recommendation

With one exception the collaborating laboratories were able clearly to distinguish between unfrozen oyster meats and those that had been frozen and thawed, solely on the basis of the malic enzyme (ME) enzymograms (Table 2). There were no failures other than in the single laboratory that did not cool the gels during electrophoresis, as specified in the method. Because malic enzyme activity is thermolabile, the latent forms more so than the free form (2), the lack of band deposition on the gels in this case was probably due to some heat-inactivation of the enzyme forms during electrophoresis. The unanimous success of the other collaborating laboratories, all of which either refrigerated the bath assemblies or cooled the gels by circulated ice water, supports this inference.

In the 5 successful laboratories the unfrozen oysters, whether fresh or aged (up to 3 weeks at 4°C), never produced more than a single band (free ME), which was often missing or very faint. The frozen and thawed oysters, by contrast, consistently produced a very broad band (at the site of free ME), plus 2 thinner accessory bands and a thin cathodal band. Figure 1 represents the collective results with *C. virginica;* the pattern for oysters superchilled (−2, −5°C) and thawed is identical to the frozen/thawed pattern, as described earlier (1). Work with *C. gigas* produced a single faint band for unfrozen oysters, and 1 broad band plus a thin cathodal band for frozen and thawed oysters.

Because the possibility of technical error should be guarded against, it is recommended that for any questionable lot of shucked oysters a few be frozen and thawed and tested against some that were untreated. If the resulting ME enzymograms are alike, the lot in question has indeed been frozen and thawed at some point, inadvertently or otherwise; if the enzymograms are markedly different, the lot has not been frozen and thawed at any time.

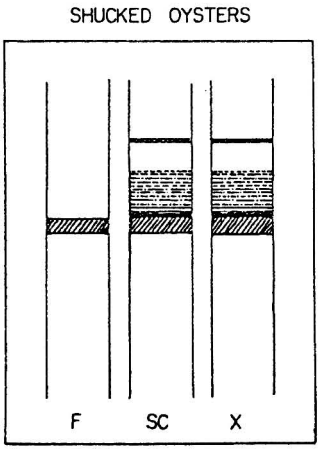

FIG. 1—Representation of malic enzyme enzymograms of centrifuged tissue fluid of oysters, *Crassostrea virginica*, frozen (X) or superchilled (SC) and thawed, and unfrozen (fresh, F).

The method has proved valid with both *C. virginica* and *C. gigas*, the 2 major oysters of American commerce. It can be used successfully either with fresh or with very stale oysters; neither autolysis nor bacterial action significantly alters either the fresh or the frozen pattern. On the basis of the clear-cut results obtained in the collaborative study and presented here, therefore, it is recommended that the method be adopted as official first action.

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Arthur Marin, New York State Food Laboratory, Albany, N.Y.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C and was adopted by the Association; *see* (1973) *JAOAC* 56, 398.

MEAT AND MEAT PRODUCTS

Moisture and Fat Analysis of Meat and Meat Products: A Review and Comparison of Methods

JULIO D. PETTINATI, CLIFTON E. SWIFT, and EDWARD H. COHEN[1]
Eastern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pa. 19118

Published analytical methods for moisture and fat analysis of meat and meat products and reviews of the methodology are surveyed. The methods are briefly described and characteristics such as time required for an analysis, accuracy, and precision are given. The discussion of instrumental methods includes methods which have not been fully developed for meat but which may become useful. Several of the considerations and limitations that are involved in moisture and fat analysis of meat are discussed. From the large number of moisture, fat, and combined methods available, the most promising were selected for a close inspection and compiled in two tables of data. The most useful rapid methods for meat industry quality control are the following: For moisture determination, high temperature mechanical convection oven drying, hot plate drying, moisture balance, and azeotropic distillation methods have the most advantages. For fat content determination, the modified Babcock procedures, X-ray transmission, specific gravity of heptane extracts, and determination of fat in the extract from azeotropic moisture analysis (a combined method) offer the most advantages.

The meat packing industry has an urgent need for rapid, relatively accurate, and simple methods for moisture and fat determination. Their availability underlies compliance with statutory requirements of regulatory agencies, quality control in manufacturing meat products, and good business management (1–4). Many methods currently available for analysis of moisture and fat content of meat and meat products were originally developed for other agricultural products and are not necessarily optimal for meats. In general, moisture and fat methods are chosen for either rapidity or accuracy, whereas obtaining both is the ultimate goal.

Desirable parameters for both ideal moisture and fat methods were outlined by Everson *et al.* (5), who indicated that the methods should perform as follows:

(*1*) Determine moisture in 15 min and fat in 30 min,

(*2*) Be applicable to the broadest range of unprocessed meats and formulated product,

(*3*) Be performable by any technical, and preferably nontechnical, employee with brief training,

(*4*) Use readily available apparatus of low initial investment and low cost per test,

(*5*) Have reasonable accuracy and good reproducibility, and

(*6*) Present few hazards easily controlled.

A profusion of analytical methods embodying varying numbers of these parameters can be found in the scientific literature. It was an objective of this review to delineate the salient features of reported methods of moisture and fat analysis, briefly using this framework of desirable parameters as the basis for comparison. It would be impossible to discuss all of these parameters for each method cited here; however, for those with some knowledge and experience in the analytical field, many of the features will be immediately obvious. It was another objective of this review to reduce available data on accuracy and precision to a uniform basis to permit comparison. The majority of methods cited reported results of comparison with official methods. In order to provide the desired uniform basis, results that were reported in many of the original publications were normalized. More specifically, differences in means between the experimental and standard method were determined to ascertain whether a tendency existed towards a constant positive or negative difference in relation to the standard method. One value of this is that a method capable of achieving acceptable precision, but which consistently yields low or high values, may be

[1] Present address: Office of Research and Monitoring, Environmental Protection Agency, Philadelphia, Pa. 19106.

shown to be useful if a constant factor can be applied to correct the results to agree with reference values. As a result of this treatment, three numerical values are shown in many of the method reviews: (*1*) average accuracy (amount lower or higher than reference method), (*2*) difference range, and (*3*) standard deviation calculated from the normalized differences by the standard formula, s.d. = $\pm\sqrt{\Sigma d^2/(n-1)}$.

Moisture Analysis

Meat tissue holds moisture in various states which were characterized by Joslyn (6) as follows: (*1*) a solvent for dispersion of crystalloids such as sugars, salts, and acids of low molecular weight or a dispersing medium for colloidal solutions of hydrophilic macromolecules such as proteins and gums; (*2*) adsorbed thinly as a mono- or polymolecular layer on tissue structural surfaces; and (*3*) in chemical combination as water of hydration. The energy required to free chemically and physically bound water and/or to force it through physical barriers such as cell walls and capillaries limits the diffusion of water from tissue. At the surfaces, solutes tend to concentrate at the evaporating surfaces and "case-harden" or seal the sample. After some portion of the moisture content has been driven off, the forces that hold the remaining water actually become stronger. At this point, the energy requirements are greater to drive off the last of the moisture. To merely apply higher temperatures can lead to inaccurate results through fat spattering, sample decomposition, and oxidation. Hence, the removal of water from tissue by heating is complicated. However, oven methods, though time consuming, are simple to perform, relatively reproducible, and useful as primary reference methods for evaluating new methods. For practical purposes, then, moisture content of meat and meat products can be arbitrarily defined as the quantities determined by the official oven methods. The variety of chemical and physical approaches that has been investigated as alternative methods will be related in the following sections.

Non-Instrumental Moisture Methods

Although moisture determination is one of the most commonly applied food assays, there are few reviews of the methodology and even fewer devoted to meat and meat products. Churchward (7) reviewed moisture methods for foods including meat. Methods involving electrical resistance measurement, vacuum and atmospheric ovens, and azeotropic distillation were discussed. The last of these methods was reviewed in depth and the historical development of the method was summarized. Churchward stated that the distillation method has many advantages and proposed the preferred conditions for the procedure.

Joslyn (6) presented a thorough review of moisture methodology and suggested the conditions that are important to the analyst concerned with food products.

Everson *et al.* (5), reviewing moisture methods applied to meats, selected azeotropic distillation for evaluation and comparison. Ethylene dichloride, toluene, and 2-octanol were compared as solvents for the distillations. 2-Octanol was recommended for a 10–15 min moisture determination. Results for 4 samples of meat product averaged 1.0% moisture lower than those by the official method. Normalized difference ranged from −1.4 to +2.2; s.d. = ±1.6% moisture.

Klima *et al.* (8) reviewed 9 current methods of moisture analysis applied to meat and meat products for time of analysis, accuracy, and simplicity of procedure. Methods reviewed were the Czechoslovakian standard procedure (105°C oven method), azeotropic distillation, electrical resistance, dielectric constant, nuclear magnetic resonance, fast neutron absorption, 170°C oven, dielectric heating, and moisture balance. Results of their own investigations to improve the oven method were also presented. They preferred drying samples at 150°C, although the meat industry in their country uses a 170°C oven temperature routinely. Heat transfer to the sample was improved by use of a thick metal plate for the floor of the oven and by using metal drying dishes. Heating coils in the ceiling of the oven reduced drying time further, and they reported a 20 min drying time for samples. This oven was used to analyze 150 meat samples in a comparison with their standard method. Mean moisture values were essentially identical; difference ranged from −0.66 to +0.72 and s.d. = ±0.33% moisture. A rapid moisture balance which would hold 8 samples on a circular platform was also developed; it allowed a moisture determination to be made in 20 min, or, when samples were done serially, results were obtained at 3½ min intervals.

The Swiss standard methods used for meat and meat products by the regulatory agencies of that country were reviewed by Wyler (9). Moisture methods he cited were 101°C oven, infrared radiation moisture balance, Karl Fischer titration, and azeotropic distillation. Karl Fischer titration was stated to be of no interest because both free and bound moisture were measured. Azeotropic distillation with tetrachloroethylene as the solvent was stated to be the Swiss standard method. Accuracy by this method was reported to be "1%", using 15 g samples distilled for 1 hr.

Pearson (10) reviewed methods for moisture determination in fresh sausage and evaluated 3 oven

procedures: heating of samples at 103±2°C to constant weight (4–6 hr), 135°C for 30 min, and 104°C (with vacuum) to constant weight (30 min). Reported mean moisture content values and difference ranges were 44.7% (−2.2 to +1.5), 44.5% (−2.3 to +1.8), and 46.9% (no difference range reported), respectively. The 135°C procedure was recommended for adoption as a rapid method.

Oven-Drying Methods

Four drying procedures for meat product samples were evaluated by Windham (11) in a study with 11 collaborators. In two of the procedures, samples were dried with air ovens and in the other two with vacuum ovens, as follows: (*1*) 16–17 hr drying in a 100–102°C mechanical convection oven, (*2*) 2.5–3.5 hr drying in a 125±5°C mechanical convection oven, (*3*) 16–17 hr drying in a 69–71°C vacuum oven, and (*4*) about 6 hr drying in a 98–100°C vacuum oven. The procedures were found to be satisfactory for a variety of meat products except for the last of these, which was not suitable for high fat products such as pork sausage. This study led to the adoption of several of the present official methods. Results reported by procedures 2, 3, and 4 were compared with those obtained by procedure 1. By procedure 2, 108 values averaged 0.16% moisture higher; normalized difference ranged from −0.97 to +1.76 and s.d. = ±0.33% moisture. By procedure 3, 101 values averaged 0.14% moisture lower; normalized difference ranged from −1.40 to +3.42 and s.d. = ±0.55% moisture. By procedure 4, 72 values of products other than those with high fat content, such as pork sausage, averaged 0.16% moisture higher; normalized difference ranged from −1.45 to +1.09 and s.d. = ±0.36% moisture.

Four methods of moisture determination on fresh meat samples were evaluated by Benne *et al.* (12). Results of vacuum oven-drying at temperatures of 50, 70, or 100°C for 6, 18, and 24 hr showed values which were generally low after 6 and 18 hr at 50°C. After 24 hr, values agreed well with results obtained by drying samples 6 hr at 100°C. Hot air oven at 100–105°C required 5 hr to dry high fat meat samples and 24 hr to dry lean samples. Azeotropic distillation using toluene and a Bidwell-Sterling receiver took 2 hr for a determination. Two of the 3 analyses they made were low compared with the 100°C vacuum oven, 6 hr method. Vacuum desiccator drying of samples over sulfuric acid, at room temperature, required 8–15 days. Even after 15 days results for 2 of the 3 samples were low.

Drying of meat samples by mechanical convection oven at 200°C was proposed by Perrin and Ferguson (13). They stated that after some experience with the method, an analyst could determine moisture with good results in 15 min. A 2600W mechanical convection oven with an inside volume of 1 cu. ft was used. Results for 25 samples, compared with results by the official method, averaged 0.1% moisture higher; difference (without normalizing) ranged from −0.4 to +0.5 and s.d. = ±0.02% moisture.

The International Organization for Standardization (14) adopted the following oven procedure for moisture determination of meat: Samples are dried at 103±2°C for 2 hr, cooled in a desiccator, and redried for 1 hr periods until the dried sample weight differs no more than 0.1% from the previous weight. Duplicate determinations were stated to differ no more than 0.5% moisture.

Moisture determination by the British standard method (15) for meat samples cited use of a 103°C oven. The procedure reported is as follows: 5–10 g of sample, minced twice through a grinder, is mixed with 3–4 times its weight of sand and dried 30 min in a 103±2°C oven; 5–10 ml of ethanol is added to the dried residue; ethanol is evaporated on a 60–80°C water bath and the residue is heated 2 hr in a 103±2°C oven. Heating, cooling, and weighing is repeated until 1 hr of heating does not reduce weight by more than 0.1% the weight of the test portion. Difference between duplicate determinations should not be more than 0.5 g moisture/100 g sample.

Six rapid methods of moisture analysis were compared to the official method by Cohen (16). Three methods were of the non-instrumental type: gravity convection oven at various temperatures, specific gravity, and hot plate. Ground beef and frankfurter samples, 5 g each, were dried and weighed in the oven experiment for up to 4 hr and at temperatures up to 200°C. Results obtained with 2 hr drying at 125°C averaged 0.1% lower moisture than by the official method; normalized difference ranged from −0.8 to +0.7 and s.d. = ±0.5% moisture. The specific gravity method was evaluated with 25–100 g samples homogenized 30 sec with cold anhydrous ethanol. Portions of the extracts were weighed in 10 ml volumetric flasks at 20°C. Results averaged 3.6% higher moisture than by the official method; normalized difference ranged from −4.0 to +4.8 and s.d. = ±3.9% moisture. The same products were also analyzed by the hot plate method. Samples of 10 g each were placed in aluminum dishes, on aluminum foil to prevent loss by spattering; these were heated on a hot plate which maintained 200°C at the heating surface for 45 min, cooled 10 min, and weighed. Results averaged 0.7% moisture higher than by the official method; normalized difference ranged from −0.9 to +0.7 and s.d. = ±0.7% moisture.

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.

Acetyl Chloride Reaction

A preliminary report of the acetyl chloride method adapted to a 1½ hr moisture analysis of meat products was made by Lórant and Pollak (17). Essentially, acetyl chloride was reacted with the moisture of the sample to form acetic acid and HCl. Ethanol was then reacted with residual acetyl chloride to form ethyl acetate and HCl, which was titrated with sodium hydroxide solution. The difference between this result and a blank indicated the amount of acetic acid generated and hence the amount of moisture in the sample. Results for 8 samples averaged 0.3% moisture lower than those by the Hungarian standard oven procedure and azeotropic distillation with toluene; normalized difference ranged from −0.7 to +0.6 and s.d. = ±0.4% moisture.

Saponification

A 30 min moisture determination for meat, using titration, was reported by Glass and Addis (18). Samples were mixed with anhydrous methanol, homogenized, and centrifuged. To a portion of the extract, methanolic sodium methoxide and ethyl acetate were added, and after tempering in a 50°C water bath, the solution was titrated with ethanolic hydrochloric acid. Of 8 samples analyzed, 6 were compared with results by an 18 hr, 108°C oven-drying method and 2 were compared with azeotropic distillation. Results averaged 0.6% moisture higher than reference method values; normalized difference ranged from −0.6 to +1.4 and s.d. = ±0.6% moisture.

Azeotropic Distillation

This technique has been applied for moisture analysis of many food products. The first review of its analytical application was published by Hoffman in 1908 (19). He referred to a German patent issued to him in 1901 on the distillation technique. Applications which he and other investigators made in analyzing cereal grains, hops, malt, butter, and cellulose were discussed. Applications reported by Marcusson (20) who analyzed oils, fats, and soaps with xylene as the solvent were also reviewed. Another early investigator of azeotropic distillation was Young (21) who published a series of papers on the characteristics of azeotropic mixtures. More recent reviews of the development and applications of the technique were presented by Othmer (22), Churchward (7), Fetzer (23), and Joslyn (6). The theory of azeotropic distillation is treated in detail by Carlson and Stewart (24).

Many of the early studies of azeotropic distillation for moisture distillation were conducted with low boiling solvents to prevent hydrolysis of carbohydrates, for example, levulose in molasses and fruit products (25, 26). As a result, the use of the higher boiling xylene was limited, and hence toluene was favored. Xylene is the preferred solvent for azeotropic distillation of meat samples because the higher boiling temperature and higher water-to-solvent ratio of the azeotrope allow moisture to be distilled more rapidly. Other solvents have been used in various procedures, such as cyclohexane and benzene for rapeseed oil (27) and isopropyl ether for fish (28). The use of liquids heavier than water was proposed by some investigators to prevent localized overheating in the boiling flask. With such solvents some samples will float on the surface within the boiling flask. Tetrachloroethylene was used for this reason by Phillips and Enas (29) and Wyler (9), and for its low flammability. The distillation procedure using this and other chlorinated hydrocarbons, however, requires special calibrated glassware, since water in the receiver tube will float upon the solvent; furthermore, the use of denser solvents prolongs distillation time.

A 4% mixture of *n*-amyl alcohol in toluene was used by Miller (30, 31) for moisture determination. The alcohol content was sufficient to prevent water films from adhering to the condenser inner surface. Similarly, Calderwood and Piechowski (32) used 2 drops of 95% ethanol at the end of xylene distillations for the same purpose.

Azeotropic distillation with toluene was compared to mechanical convection oven-drying at 100°C for 16–18 hr by Hill (33), using 25 g meat samples which had been ground 3 times. Averaged results were the same as by the reference method. For fresh meat, the range of difference was ±0.7 and s.d. = ±0.3% moisture. For frozen meat, the range of difference was ±1.3 and s.d. = ±0.6% moisture. Others who used toluene as the solvent were Kerr (34, 35), Benne *et al.* (12), Thompson and Corsi (36), Lunder (37), and Cohen (16). Cohen analyzed 10 g samples of ground beef, frankfurter, and pork sausage for an azeotropic distillation of 15 min to 1 hr. The analyses were determined to be complete when no additional water collected in the receiver upon continued distillation for intervals of 5 min. Results averaged 2.1% moisture lower than values obtained by the official method. Normalized difference ranged from −0.9 to +1.0 and s.d. = ±0.8% moisture.

An evaluation of 27 selected solvents for suitability in azeotropic distillation was reported by Cohen and Kimmelman (38). Ground beef, frankfurter, and pork sausage were analyzed for moisture content. Six of the solvents—ethylbenzene, cumene, 2-ethyl-1-hexanol, 1-heptanol, 1-octanol, and 2-octanol—yielded excellent results for 10 g samples using 15 min distillations. Some of the other solvents evaluated were found to be satisfactory for distillations of 30 min or more, such as nonane, *m*-xylene, and butyl ether. Results for the 3 solvents—cumene, 1-octanol,

and 2-octanol—for all 3 meat products, using 15 min distillations, were essentially the same; pooled results averaged 0.2% moisture lower than results by the official method. Normalized difference ranged from −0.7 to +1.3 and s.d. = ±0.5% moisture. When these same distillations were continued for 30 min, results averaged 0.2% moisture higher than those by the official method. Normalized difference range and standard deviation were the same as for the 15 min distillations reported above.

Instrumental Moisture Methods

Instruments of Potential Use for Meats

A large variety of instruments can be used to measure moisture content of products by the application of well known physical principles including measurement of the response of the water molecule to some form of applied energy. Some of the instrumental methods have been readily applied to food products of simpler composition than meat, such as products that are free of fat. Other instrumental methods are not applicable to meat because the moisture content range of meat is higher than the instrument can measure.

The operating range of various instruments for moisture determination is among the characteristics reviewed by Roth (39). Operating range is important because analysis of meat samples may require determinations ranging between 0 and 80% moisture. At the time of his review, the ranges for some of the instruments were as follows: electrolytic hygrometer 0–10%; resistance electrode and cobalt chloride colorimetry 0–20%; capacitance meter 0–25%; and conductivity meter 0–50%. All of these are too limited for meat analysis. Radio frequency absorption instruments were reported as being usable for up to 60% moisture and nuclear reflection instruments for up to 80%. However, neither of these latter 2 instruments measured water specifically. Microwave absorption instruments have a 90% moisture upper limit and therefore might possibly be developed for moisture analysis of meats. Two other methods, reviewed by Roth, which will analyze up to 100% moisture use moisture balances and Karl Fischer titrators. These 2 types of apparatus will be discussed later in this review.

Webb (40) reviewed instrumental methods that were applied to specific moisture analyses and could possibly be adapted for use with meat. In addition to a number of the methods discussed above, others using refractometry, coulometry, and nuclear magnetic resonance were discussed.

Moisture Balance

The Ohaus Model 770 moisture balance was compared to the official oven method by Solberg and Riha (41) and Cohen (16). The apparatus embodies a triple-beam balance, a shielded infrared lamp, and a moisture calculator which can be read to 0.1% moisture. In the former report, results for frankfurters and ground meat were compared to values obtained by the oven method. Results averaged 0.01% moisture lower than the reference method; difference ranged from −0.9 to +0.7% and s.d. = ±0.4% moisture. In the latter report, samples of 2 lots of beef, 1 frankfurter, and 1 pork sausage were analyzed and compared with the results by the official method. Results averaged 0.4% moisture higher than by the reference method. Normalized difference ranged from −0.3 to +0.4 and s.d. = ±0.3% moisture. Results with the Dynatronic infrared radiation moisture balance (oven-type) and the official oven method were also compared by Cohen, using 12 g samples of 3 types of meat product. The samples were dried at 100±10°C for 45–60 min periods. Results averaged 0.1% moisture higher than by the reference method. The range of normalized difference was ±0.6 and s.d. = ±0.5% moisture. Bloemer (42) evaluated the Brabender moisture balance. Bartels and Gerigk (43) evaluated a similar moisture balance, marketed both in Germany and in this country, the Ultra-X unit, which is discussed later in this review (see combined methods).

Karl Fischer Titrator

This technique was described by Mitchell (44) and applied to moisture content analysis of meat samples by Cook *et al.* (45). In a discussion of the determination of moisture in grain, the National Bureau of Standards (46) stated that the titrator was unsatisfactory because of the frequent restandardization that was necessary.

Refractive Index

This optical method of determining moisture content has been used in dried fruit analysis by Bolin and Nury (47) and in turkey meat analysis by Ning and Marion (48). The latter report revealed that a method depending on the refractive index of tissue slurries requires uniformity of type and preparation of samples. The results showed a difference between red and white muscle. Addis *et al.* (49) reported the refractive index of anhydrous isopropanol extracts of meat samples.

Infrared Absorption

Spectrophotometry of solvent extracts of food products other than meat has been reported by Gold (50) using methanol, by Rader (51) using dimethylformamide, and by Vornheder and Brabbs (52) using dimethylsulfoxide.

Gas Chromatography

Moisture determination in grain was discussed in a communication by the National Bureau of Stan-

dards (46). The gas chromatographic method used on methanol extracts was reported to overcome many of the shortcomings of earlier laboratory methods and provided a good reference method. The method was stated to be highly specific for water. The nature of the apparatus and equipment may limit the usefulness of the method to standardizing equipment used for field testing. Gas chromatographic procedures using methanol extracts were also reported for food products including dog food by Schwecke and Nelson (53), for fruits by Brekke and Conrad (54), and for meat samples by Addis *et al.* (49).

Nuclear Magnetic Resonance (NMR)

Steffa *et al.* (55) evaluated a wide variety of meats and meat products in a study of the possible use of NMR in moisture and/or fat content determination. The method did not appear promising because measurements were affected by differences in free or bound water content, whether the sample was beef or pork, and whether the sample was pre-chilled, or warmed after chilling. They also reported an indication that ionic constituents would affect measurements.

Radioactivity of Natural Potassium-40

Studies of the relationship of naturally occurring ^{40}K isotope to the composition of meat were reported by Kulwich (56) and Kirton *et al.* (57). The basis for the method is the measurement of radioactive counts primarily emitted from the lean portion of meat rather than the fat portion. Kulwich reviewed the known potassium content of various species and suggested that studies of ^{40}K content of lean, fat and bone, and different muscles could lead to estimates of lean content. The estimates would then serve to calculate moisture and fat content. Kirton *et al.* evaluated 20 pork and 15 lamb samples to estimate moisture, fat, and protein content by measuring ^{40}K counts. The results on pork, but not lamb samples, were reported to be promising.

Electrolytic Hygrometry

Preliminary results with a micro-procedure of electrolytic hygrometry, based on coulometry, were described in detail by Fraade (58) for food products. The instrument measured the current required to electrolyze water vapor introduced as a gaseous stream.

Fat Analysis

In the same respect that moisture content of meat is not neatly and exactly defined, fat extraction is not simply an extraction of fat per se. The factors that make this so will be apparent from the following considerations.

Solvent extractions of fat involve dissolving constituents of various solubilities. In addition, meat constituents such as phospholipids can be extracted. Also, factors that affect the analysis are particle size of the meat product, the product's previous processing treatment, the barrier effect of water, and the effect of colloidal and electrolytic constituents in the product.

While free lipids are readily solvent-soluble, that portion of the fat which is encapsulated in its native cell is more difficult to extract. If the product is of the emulsion-based type, fat is emulsified in the form of discrete globules as discussed in detail by Swift and Sulzbacher (59) and Swift (60).

The constituents of meat and meat products are structurally arranged in a highly organized fashion. Nonpolar fat solvents cannot rapidly penetrate polar or electrostatically bonded arrangements because such sites are similar to physical barriers. On the other hand, polar solvents, which can penetrate electrostatic barriers, may dissolve constituents other than fat. Thus care must be exercised in the choice of a solvent. Diethyl ether, for example, tends to extract water-soluble constituents unless it is absolutely free of water. Petroleum ether is preferred by many analysts because it extracts fewer nonfat components (61).

In an extensive study of chloroform extracts of the residues obtained from alcohol-ether extraction of nerve tissue, Brante (62) identified the presence of many constituents other than triglycerides including phospholipids, lecithin, choline, ethanolamine, inositol, and cholesterol. Free fatty acids were not tested.

Bloor (63) was the first to suggest that a polar solvent such as ethanol be added to a conventional lipid solvent such as diethyl ether to obtain more effective solvent penetration and more efficient extraction. The use of chloroform-methanol mixed solvent was reported by Folch *et al.* (64, 65) for extraction of brain lipids. Arnold and Hsia (66) reported higher estimates of fat from beef by using chloroform and tetrachloroethylene in a Soxhlet apparatus. However, samples were freeze-dried rather than oven-dried. Bligh and Dyer (67) homogenized fish tissue with chloroform-methanol solvent to form a miscible system. Water was then added to this system which resulted in a chloroform-lipid layer and an aqueous methanol layer. A number of other investigators used chloroform-methanol solvent for a variety of food products (68–78). Ostrander and Dugan (79) investigated fats from different parts of meat animals by precipitating protein with zinc acetate and extracting in a blender with chloroform-methanol solvent. Ciam and Dugan (80) developed a procedure using either diethyl ether or hexane to extract free lipids, and 95% ethanol or hexane-

ethanol to extract bound lipids. Hagen *et al.* (81) studied the extraction of lipids from beef samples. They evaluated 6 extraction solvent combinations (including diethyl ether, petroleum ether, mixed ethers, and chloroform), 3 drying procedures, and 2 methods of sample preparation. Chloroform-methanol extraction yielded the highest amounts of extracted fat. However, this extract also yielded the highest amount of phospholipid; samples which were acid-hydrolyzed yielded the lowest amount. Among the 6 different extraction methods, the phospholipid content ranged from 2.8 to 10.1% of the extracted solids.

Southgate (82) reviewed the use of chloroform-methanol for fat extraction of foods. In that report, the mixed solvent was distilled from the extract; the residue was then re-extracted with petroleum ether and shaken with sodium sulfate. The re-extraction with petroleum ether was not quantitative with some fats unless the residues were warmed into solution for several min. Bixby *et al.* (83) extracted liver samples by using the solvent mixture of the Mojonnier method in a Goldfisch extractor. The solvent mixture consisted of diethyl ether, petroleum ether, and ethanol, 5+5+2, respectively. Fat content by this method averaged 5.7% fat compared with 5.5% by Mojonnier method and 3.7% by extraction with diethyl ether in a Goldfisch extractor. The residue obtained by the 3-component solvent mixture was redissolved in diethyl ether. An average of 98% of the residue redissolved.

A number of these investigators used freeze-drying instead of oven-drying prior to extracting (66, 79, 81). This was done to avoid exposing the samples to heat when the fat extracts were to be further investigated for identification of constituents. It is possible that removal of the moisture barrier by this means would also leave tissue structure more porous than by oven-drying. Watts *et al.* (84) investigated this point by extracting fat by the Mojonnier method from fresh or freeze-dried ground beef and several other food products. The results were not significantly different: average yield from fresh product was 11.54% fat, and from freeze-dried product, 11.50% fat.

As the preceding discussion indicates, an evaluation of methods for fat analysis requires definition of the meaning of fat. For the present purpose of evaluating methods for industrial and regulatory use, fat is arbitrarily considered to be measurable as the ether-extractable material that is yielded by the official method.

Fat Determination Methods

Reviews of Fat Methods

Recent reviews of fat determination in meat products include one by Everson *et al.* (5) who discussed the Mojonnier method, a modified Babcock method, and the capacitance measurement procedure. Klima *et al.* (8) reviewed modified butyrometric methods extensively as well as a number of other methods. They discussed special Babcock bottles to contain up to 28 g of meat sample, the use of acetic-perchloric acids in place of sulfuric acid, alkaline digestion reagents, and pepsin enzyme digestion. They also reviewed refractometry, specific gravity, and capacitance measurements on solvent extracts of the fat, rapid rendering devices, potassium-40 content, and nuclear magnetic resonance. Of these methods, 2 were selected for further evaluation and results were compared with Soxhlet ether extraction. The methods were: (1) rapid extraction with carbon tetrachloride after mincing the samples in a top-driven homogenizer, followed by determination of specific gravity of the extract; and (2) butyrometric method using a 5 g cream bottle and 18*N* sulfuric acid which digested the meat sample in 25–30 min. Results for 31 samples analyzed by the specific gravity method averaged 0.3% fat lower than reference values. Normalized difference ranged from −1.9 to +2.9 and s.d. = ±1.0% fat. Results for 8 samples analyzed butyrometrically averaged 0.15% fat higher than reference values. Normalized difference ranged from −1.25 to +0.65 and s.d. = ±0.8% fat.

Whitehead (85) discussed 8 methods used for meat fat determination and described the specific gravity method developed at the Honeywell Corporation. Methods he reviewed included the Babcock, modified Babcock, capacitance, gamma-ray penetration, ultrasonic, empirical determination from moisture content, rapid rendering devices, and specific gravity.

Wyler (9) reviewed Swiss standard methodology for determining fat content in meat products. He stated that direct Soxhlet extraction is unsuitable for meat because bound lipids are not extracted from tissue. He also stated that butyrometric methods with either sulfuric or perchloric acid yielded inexact results and that the Mojonnier method required a special centrifuge. Standard methods, in his opinion, were those by which meat samples were digested with hydrochloric and sulfuric acids and subsequently were extracted with chlorinated hydrocarbons or with ether.

Smith (86) presented a broad survey of rapid methods for total fat determination in foods including meat and concluded that the most promising areas for further investigation are the volumetric, refractometric, and rapid extraction methods. Reviewed in detail were the butyrometric method and modifications of it, solvent extraction followed by refractive index measurement, specific gravity, infrared radiation and absorption, nuclear magnetic reso-

nance, capacitance, X-ray absorption, microwave absorption, rapid extraction, photometric, and ultrasonic methods. The review cited 106 references. Joslyn (87) presented a discussion of extraction methods for fat determination of food products including meat. The factors that affect solvent extraction of fat were reviewed. The chapter is well documented with 137 references. Pearson (10) reviewed methods of fat analysis for fresh sausage and evaluated 2 of these, namely, Soxhlet extraction with petroleum ether after drying at 100–105°C for 3–8 hr and the Gerber procedure on 2 g samples using a milk testing bottle (45 min method). The mean values and difference range obtained by the 2 methods, 34.1% (±5.9) and 34.8% (−5.4 to +4.9) fat, respectively, were not significantly different.

Extraction

Use of a series of 5 extractions with diethyl ether followed by partition in a separatory funnel having a fritted glass filter plate fused into it was suggested by Ernst (88) for meat samples. The procedure required 1.5–2 hr for a determination. Results for 23 samples averaged 0.2% fat higher than by the official method. Normalized difference ranged from −1.1 to +1.5 and s.d. = ±0.7% fat.

Windham (89) compared extraction of meat samples dried 6 hr with samples dried 16–18 hr at 100–102°C before Soxhlet extraction. Samples dried 6 hr were extracted with either ethyl ether or petroleum ether and samples dried 16–18 hr, with petroleum ether only. Mean values and difference range for 60 samples were: 24.2±0.5% fat for samples dried 6 hr and extracted with ethyl ether, 24.3±0.4% fat for samples dried 6 hr and extracted with petroleum ether, and 24.1±0.5% fat for samples dried 16–18 hr and extracted with petroleum ether.

Fat extraction by means of *n*-hexane or petroleum ether (boiling range 40–60°C) was selected for meat samples by the International Organization for Standardization (90). The procedure states that the solvent should be distilled from the extract and fat content should be determined gravimetrically.

Chromic acid oxidation of extracted fat was originally developed by Bloor (91) for determination of fatty acids. Paul (92) adapted the procedure for fat determination in small samples of tissue and O'Shea and Maguire (93) used it for 1.5 g samples of meat. The oxidation reaction was conducted on the fat extracted from the samples to keep chromic acid from reacting with other organic matter. The procedure required 2 hr for a determination. Pork, beef, and lamb samples, 34 determinations, were analyzed and compared with results by the official method. Results averaged 0.1% fat higher than the reference method. Normalized difference ranged from −1.0 to +1.6 and s.d. = ±0.6% fat.

Two British standard methods (94) for determination of total fat content of meat products both specify digestion of a minced sample with hydrochloric acid and gravimetric determination of the extracted fat. The analyst is directed to either digest a 3–5 g sample for 1 hr and, continuously or semicontinuously, extract the filtered residue with hexane or petroleum ether, or to digest a 2–3 g sample for 30 min and manually extract, 3 times, the digest with diethyl ether. For determination of the free fat of meat samples, the British standard method (95) cited Soxhlet extraction with hexane or petroleum ether for at least 6 hr of a 5–10 g minced sample dried as in the moisture determination and gravimetric determination of the extracted fat.

Modification in both the drying and extraction time of the official method for meat was evaluated by Cohen and Swift (96) to reduce time of analysis without loss of accuracy. Samples of ground beef, frankfurter, and pork sausage were dried for periods of 15–90 min and solvent-extracted for periods of 15 min to 16 hr. Replicate analyses were compared with results by the official method. Results showed that ground beef required 30 min drying time and 45 min extraction; frankfurter required either 30 min drying and 30 min extraction, or 45 min drying and 15 min extraction; and pork sausage required either 15 min drying and 30 min extraction, or 30 min drying and 15 min extraction. For these analyses recoveries were 99.2–100.5% of results by the official method. In each case, 15 min less drying time or 15 min less extraction time yielded fat recoveries of 95.8–98.6%.

Babcock Method Modifications

Emulsification of ground meat with Oakite brand household cleanser was proposed by Oesting and Kaufman (97). The emulsion that was formed was weighed into a Babcock bottle and the procedure was continued as in the usual Babcock method. Results of 20 analyses, compared with values obtained by the official method, averaged 0.04% higher fat content, difference ranged from −1.6 to +1.1 and s.d. = ±0.7% fat.

A modified Babcock procedure was evaluated as a rapid method for fat determination in meat by Windham (89, 98). The earlier report was a collaborative study of the method compared with ether extraction methods by 7 collaborators. Quadruplicate analyses on 60 samples averaged 0.01% higher fat with a difference range of ±0.64 and s.d. = ±0.4% fat. In the later report, Windham evaluated the same method along with 2 other rapid methods, perchloric-acetic acid modification of the Babcock and capacitance measurement. Results for 18 meat samples (4 different meat products) averaged 0.15% higher fat. No explanation was offered for this higher average difference compared to the previous report.

Normalized difference ranged from −0.6 to +1.2 and s.d. = ±0.4% fat.

Use of perchloric and acetic acids in place of sulfuric acid was proposed by Salwin *et al.* (99). Meat fat contents from 19 analyses were compared to values obtained by the official method. Results averaged 0.01% lower fat; difference ranged from −0.4 to +0.5 and s.d. = ±0.2% fat. However, Windham (98) and Krol and Meester (100) evaluated the method and found consistently higher values than those obtained by Soxhlet extraction. Windham reported finding 5% acid, calculated as acetic, in the fat layer so that fat content values had to be corrected by this amount; results for 18 meat samples averaged 1.1% higher fat. Normalized difference ranged ±1.0 and s.d. = ±0.5% fat.

The "Banco test", a modified Babcock procedure, was devised by Anderson *et al.* (101) to prevent charring of samples by sulfuric acid. Samples were treated with papain powder, sodium hydroxide, a solution containing urea, sodium carbonate, disodium phosphate and ethylenediaminetetraacetic acid in aqueous methanol, and a detergent. The procedure reportedly required 30 min for an analysis. It was evaluated by 4 laboratories, using 8 meat products, with the official method as reference. Results averaged 0.2% higher fat. Normalized difference ranged from −0.8 to +1.2 and s.d. = ±0.4% fat.

Another modification of the Babcock procedure was proposed by Whalen (102), who digested meat samples with hot hydrochloric acid and diluted the digest with dimethylsulfoxide in a procedure requiring 10 min. Results from 98 samples were compared with results obtained by the Mojonnier extraction method. Results averaged 0.17% lower fat. Normalized difference ranged from −1.3 to +1.6 and s.d. = ±0.6% fat.

Rapid Screening Devices

A number of devices have been developed for rapid screening of meat samples and have a limited acceptance in field inspection. One of these units is the "Fat-Alyzer" designed as an abbreviated Babcock procedure kit (103) to analyze a meat sample in 15 min.

In a rendering device made by the Univex Corporation (104, 105) the meat sample was heated between 2 electrodes, fat content and juices dripped into a flask, and fat column height was read directly in units of per cent fat. This unit was tested at our laboratories with unfavorable results.

The Hobart Mfg. Co. (106, 107) markets a similar rendering device, which was tested and reported by Bellis *et al.* (108). The unit utilized an inverted hot plate to render the meat sample in 15 min. Samples at 16 fat levels (14–29% fat content) were analyzed. Results reported showed that difference ranged from −0.49 to −4.10 with an average difference of 2.3% lower fat than values obtained by the official method.

Capacitance Measurement

Furgal (109) extracted fat from meat samples with *o*-dichlorobenzene and related the capacitance of the extract, corrected for temperature of extract, to fat concentration. Results for 20 samples of meat products averaged 0.2% higher fat than values obtained by the official method. Normalized difference ranged from −1.9 to +1.2 and s.d. = ±0.8% fat.

The above method was also evaluated by Everson *et al.* (5). In the 30 min analysis, 50 g samples of 20 meat and meat products were blended with 100 ml *o*-dichlorobenzene and also 5 g filter aid for 4 min, the blend was filtered, and the filtrate was measured for capacitance. Results averaged 0.6% fat higher than values obtained by the official method. Normalized difference ranged from −4.5 to +4.0 and s.d. = ±2.0% fat.

Another evaluation of this method was reported by Windham (98), who analyzed 18 meat samples. Results averaged 0.3% fat higher than values obtained by the official method. Normalized difference ranged from −0.7 to +1.5 and s.d. = ±0.7% fat.

Specific Gravity Measurement

Gipr and Lukashova (110) proposed determining fat content by measuring the specific gravity of solvent extracts of meat products. Trichloroethylene was used as the solvent and accuracy was reported to be within 1% of the Soxhlet fat extraction method. This was confirmed when Mahmood-ul-Hassan and Pearson (111) evaluated the procedure. Results for 8 samples of beef and 6 samples of pork averaged 0.1% lower fat for beef and 0.3% higher fat for pork than values by the official method. The range of normalized difference was ±0.6 for beef and −0.8 to +0.6 for pork; s.d. = ±0.5% fat for both meats.

Determination of fat content in meat by measurement of the specific gravity of the meat itself was developed by Whitehead (112). The apparatus measured weight, compacted volume, and temperature of 750 g samples. From these data, fat content was computed automatically after corrections were applied for animal species and the section of the animal sampled. The procedure was described as useful on the processing floor because measurements could be made in 30 sec on samples which contained no frozen mixture or foreign ingredients. Results on 69 samples of beef chuck averaged 0.06% higher fat than values obtained by the official method; range of difference was ±2.4 and s.d. = ±1.2% fat.

The above specific gravity apparatus was also evaluated by Malanoski and Greenfield (113) on meat samples of varied origins. Results on 56 sam-

ples averaged 1% fat lower than by the official method. Normalized difference ranged from −3.4 to +3.6 and s.d. = ±1.5% fat.

A method based on specific gravity was reported by Bittenbender (114) for measuring fat content of meat. Heptane extracts of samples were made and custom-made hydrometers were used to determine specific gravity at 34°C in a 15 min procedure. Temperature of the water bath was closely controlled because a variation of ±0.01°C in the extract influenced the accuracy by ±0.05% fat. Results on 17 meat samples averaged 0.3% fat higher than values obtained by the official method. Normalized difference ranged from −0.9 to +0.8 and s.d. = ±0.5% fat.

Refractometry

Fat determination of foods including meat was reported by various investigators (115–117). Samples were ground with monobromonaphthalene and sodium sulfate. The refractive index of the filtered extract was then read with a reported accuracy of ±0.5% fat.

The above procedure was modified and evaluated by Mahmood-ul-Hassan and Pearson (111) who used a 1+1 mixture of 1-bromonaphthalene and mineral oil as the extracting solvent. Results on 6 beef and 6 pork samples averaged 0.5% lower fat for beef samples and 0.7% lower fat for pork samples than values obtained by the official method. Normalized difference ranged from −1.0 to +0.5 for beef and −0.8 to +0.65 for pork; s.d. = ±0.6% fat for beef and ±0.5% fat for pork.

A refractometric procedure, a German standard method, was described by Rudischer (118) for fat analysis of meat samples. A mixture of perchloric and phosphoric acids and a low flame were used to digest a sample in 1-3 min. Without cooling the digest, 1-bromonaphthalene (or mixtures of mono- and dibromonaphthalene) was used to extract fat. Calcium carbonate and sodium sulfate were added to the extract which was then filtered, and the filtrate was read on a refractometer at a temperature of 50°C.

X-Ray Absorption

The Anyl-Ray Analyzer was reported to utilize a dental-type X-ray source to measure fat content of meat samples (119, 120). The instrument measured a 13 lb sample and was reported to yield values for fat content with a difference range of ±1.5 and s.d. = ±0.5% fat.

Reflectance Photometry

A photoelectric method of determining fat content was devised by Knudsen (121) for meat trimmings arranged on a conveyor band. As the meat was carried past a fluorescent tube, reflected light was measured by photoelectric cells and the signal was converted to indicate fat content. Results with the method are not available.

Combined Method for Moisture and Fat Determination

A combined method for moisture and fat content determination of oil seeds was proposed by Kaufman and Keller (27). Moisture was determined by azeotropic distillation with heptane, and fat content was determined gravimetrically after removal of solvent from a portion of the extract.

Azeotropic distillation using 2-octanol for moisture determination reported by Everson *et al.* (5) and their procedure for determining fat content by capacitance measurement of an *o*-dichlorobenzene extract of the sample were discussed earlier in this review. In a combined method for determining both moisture and fat content of the same sample, they reported that azeotropic distillation with a 1+7 mixture of 1-octanol and 2-octanol to determine moisture content, followed by capacitance measurement on the solvent extract obtained during the distillation, would yield the fat content in a procedure requiring 30 min. Using 15 g portions of 19 meat samples and 100 ml solvent for each assay, they compared results with values obtained by official methods. Results averaged 0.7% moisture lower and 0.3% fat lower than reference values. The range of normalized difference was ±1.9 for moisture and −2.1 to +2.9 for fat; s.d. = ±1.1% moisture and ±1.3% fat.

Wistreich *et al.* (122) also recommended azeotropic distillation as part of a combined method for moisture and fat content determination of meat samples. A special 2-compartment flask and 10 g samples were used. Water volume was read from a Bidwell-Sterling type receiver, and fat content was determined either gravimetrically after evaporating solvent from the extract, or by difference after drying and weighing the residue of the extracted sample. A distillation time of 2 hr was recommended. This procedure was later modified (36) so that a Florence flask with a center well was used to contain the sample and 250 ml solvent in place of the 2-compartment flask. Dry toluene was used for a distillation time of 30–90 min. Moisture and fat content values were obtained as with the earlier procedure.

Bartels and Gerigk (43) determined moisture and fat content in meat in a 2-part method by first determining moisture in a moisture balance (the "Ultra-X" unit) and then extracting fat from the residue with carbon tetrachloride. Samples were dried 15–25 min depending on type of meat product. The dried material was weighed and then extracted for 10–20 min. After extraction, the solvent was

evaporated from the residue by infrared radiation and fat was determined by difference. Results for 23 samples averaged 0.5% moisture lower than their reference method (4–6 hr drying in a 105°C oven) and 0.1% fat lower than Soxhlet petroleum ether extraction. Normalized difference ranged from −1.7 to +2.2 for moisture and from −1.7 to +1.8 for fat; s.d. = ±1.0% moisture and ±1.0% fat.

Moisture and fat content of meat samples were determined in a combined method by Davis *et al.* (123). The procedure made use of azeotropic distillation with *n*-butyl ether and a vacuum oven to dry the sample residue. Distillation time varied (2–2.5 hr) according to type of sample analyzed. After distillation, during which moisture and fat are removed, the sample residue was placed in a 140°C vacuum oven for 10–15 min and then weighed. Results for 46 samples averaged 1% moisture higher and 1% fat higher than values obtained by official methods. Normalized difference ranged from −1.7 to +1.3 for moisture and from −1.6 to +1.7 for fat; s.d. = ±0.8% moisture and ±0.8% fat.

An instrumental approach to develop a combined method for moisture and fat determination was proposed by Ben-Gera and Norris (124). Infrared absorbance of 2 g samples of comminuted meat was read in a spectrophotometer with a sample layer 2 mm deep. Moisture was determined by the difference in absorption at 1.725 and 1.800 μm and fat was determined by the difference at 1.650 and 1.725 μm. Accuracy was reported to be ±2.1% moisture and ±1.4% fat compared with values obtained by official methods. Technical problems of sample preparation and low sample transmittance were said to require further investigation.

Determination of moisture and fat content of meat by a combined method was also evaluated by Cohen and Kimmelman (125). Moisture content was determined by azeotropic distillation. Fat content was determined gravimetrically on an aliquot of the solvent extract. The suitability of 13 water-immiscible solvents was evaluated for the combined procedure. Ground beef, frankfurter, and pork sausage were analyzed by taking 10 g samples and 100 ml solvent in each determination. Moisture content of all three products was determined after 15–30 min of distillation. Recoveries of 95–100% of the moisture contents were obtained with a number of the solvents. Fat content was determined by taking 20 ml of the solvent extract in each case, evaporating the solvent by boiling under a stream of nitrogen (6–15 min), and weighing the residue. Of the 13 solvents evaluated, 95–100% recoveries of the fat contents were obtained with 4 of the solvents on ground beef samples and with 5 of the solvents on frankfurter samples after 15–30 min distillation; 95–100% recoveries were also obtained for pork sausage when distillation was continued an additional 15–30 min with 3 of the solvents. Optimum results for both moisture and fat content, for all 3 products, were obtained with the solvents *m*-xylene and cumene in a comparison with values obtained by official methods: (*a*) With *m*-xylene solvent, results averaged 0.6% moisture lower and 0.7% fat lower; range of normalized difference was ±0.9 for both analyses; s.d. = ±0.6% moisture and ±0.4% fat. (*b*) With cumene solvent, moisture content was estimated to be the same as by the official method and fat content was estimated to be 0.3% fat lower; the range of difference was ±0.7 for moisture and the range of normalized difference was ±0.5 for fat; s.d. = ±0.4% moisture and ±0.5% fat.

Evaluation of Methods

This survey reviewed the large number of methods available in order to identify the most promising, and to make an in-depth comparison of those which most closely meet the needs of the meat industry for quality control work. Data relevant to the selected methods were compiled and are shown in Tables 1 and 2. The moisture and fat methods were selected as useful for the following reasons: (*1*) simple to perform, (*2*) inexpensive, (*3*) rapid, (*4*) reasonably accurate and precise, and (*5*) applicable to a broad range of meat products. Also listed are the official methods to which they were compared.

Moisture Analysis

Method 1, AOAC official method 24.003(a), requires drying a meat or meat product sample 16–18 hr at 100–102°C in an air oven (mechanical convection preferred), cooling in a desiccator, and weighing to determine loss of weight as moisture. The method is indirect and is empirical. It is one of the reference methods that serves as the accepted standard for the meat industry and as the reference with which new methods are compared. It is by no means a rapid method. However, reproducibility of results is excellent.

Method 2, AOAC official method 24.003(b), requires drying a meat or meat product sample 2–4 hr, depending on product, in a mechanical convection oven at about 125°C. Similar to Method 1, it is an indirect and empirical method. However, it is more rapid than Method 1 and provides accuracy and reproducibility equivalent to Method 1.

Method 3, a modification of Method 2, uses a 125–150°C oven and is advantageous because the

Table 1. Comparison of methods of analysis for moisture content of meat

Method[a] No.	Type	Reference	Essential characteristic	Time required for a single analysis	Sample size,[b] g	No. of analyses[c]	Mean difference,[d] % moisture	Range,[e] % moisture	Std dev.,[f] % moisture	Cost of equipment[g] and training[h]
1	AOAC official	126, 11	100–102° air oven	16–18 hr	4–6	108	0	not available	0.25	>$250, 1
2	AOAC official	127, 11	125° mechanical convection oven	2–4 hr	4–6	108	0.2	−1.0 to +1.8	0.33	>$500, 1
3	Modified Method No. 2	16	125–150° gravity oven	2–4 hr	4–6	12	0	−0.3 to +1.0	0.7	>$250, 1
4	High temperature oven	13	200° mechanical convection oven	>15 min	25	25	0	−0.4 to +0.5	0.2	>$500, 1
5	AOAC official	128, 11	95–100° vacuum oven	5 hr	4–6	72	0.2	−1.5 to +1.1	0.4	>$150, 1
6	Low temperature vacuum oven	11	70° vacuum oven	16–18 hr	4–6	101	−0.1	−1.4 to +3.4	0.6	>$150, 1
7	Hot plate	16	200° at surface	30–45 min	10	24	0.7	−0.9 to +0.7	0.7	<$100, 1
8	Infrared radiation oven (Dynatronic)	16	IR heater, built-in balance	40–60 min	12	24	0.1	±0.6	0.5	>$600, 1
9	Infrared radiation lamp (Ultra-X)	43	IR lamp, built-in balance	15–25 min	2.5	23	−0.5	−1.7 to +2.2	1.0	>$400, 1
10	Infrared balance (Ohaus)	16	IR lamp, separate balance	30–45 min	10–20	24	0.4	−0.3 to +0.4	0.3	$100, 1
11	Azeotropic distillation (Si-Mo-Fat)	123	distillation with butyl ether	2 hr	10	46	1.0	−0.7 to +2.3	0.8	>$200, 2
12	Azeotropic distillation (AMIF)	5	distillation with 2-octanol	15 min	15	19	−0.7	±1.9	1.1	>$100, 2
13	Azeotropic distillation (Cohen)	38	distillation with various solvents	15 min 30 min	10 10	24 24	0	−0.7 to +1.3	0.5	<$100, 2

[a] Method identified by common name, type, author, or manufacturer.
[b] Sample size as reported in the reference.
[c] Number of analyses of samples reported in the reference cited.
[d] Mean difference is the average algebraic sum of differences between results by the experimental and reference methods, indicating average higher (+) or lower (−) results with the experimental method.
[e] Range of differences between results by the compared methods, after results by the experimental method were adjusted (made normal) to reference method results by adding or subtracting the method's mean difference to or from the individual differences.
[f] Standard deviation was calculated on normalized differences.
[g] Cost quoted is on a unit basis, unless noted differently.
[h] Training required to perform the method indicated as 1, 2, and 3 for brief, moderate, and thorough, respectively.

Table 2. Comparison of methods of analysis for fat content of meat

Method[a] No.	Method[a] Type	Reference	Essential characteristic	Time required for a single analysis	Sample size,[b] g	No. of analyses[c]	Mean difference,[d] % fat	Range,[e] % fat	Std dev.,[f] % fat	Cost of equipment[g] and training[h]
1	AOAC official	129, 89	Soxhlet or Goldfisch extraction	7 hr	3–4	240	0	±0.7	0.25	$75 per Soxhlet unit; $650 per Goldfisch unit, 2
2	Modified Method No. 1	96	rapid 125° drying and rapid extraction	¾–1¼ hr	3–4	27	0	−0.8 to +0.2	0.6	same as above
3	Modified Babcock	89, 98	modified Paley bottle, with or without centrifugation	20–30 min	9	240 18	−0.1 0.2	±1.0 −0.6 to +1.2	0.3 0.4	<$50 (for 6 samples), 1
4	Modified Babcock, $HClO_4$-HOAc	99	same as above	30 min	9	19	0	−0.4 to +0.5	0.2	same as above
5	Modified Babcock, HCl-DMSO	102	same as above	15 min	9	98	−0.2	−1.3 to +1.6	0.6	same as above
6	Modified Babcock (Banco)	100	same as above, Banco reagents	35 min	9	32	0.2	−0.8 to +1.2	0.4	$300, with centrifuge, 2
7	Rendering (Hobart)	108	inverted hot plate and collector	15 min	56.6 (2 oz)	64	−2.3	−4.1 to −0.5	not available	$195, 1
8	Rendering (Goss)	105	resistance heater and collector	15 min	15–25	not available	not available	±1.5	not available	<$100, 1
9	Moisture balance (Ultra-X)	43	CCl_4 extraction after drying	35–55 min	2.5	23	−0.1	−2.4 to +1.8	1.0	$1250, 1
10	Separatory funnel	88	extraction 5× with ether	1.5–2 hr	5	23	0.2	−1.1 to +1.5	0.7	<$50, 1
11	Infrared absorption	124	spectrophotometry	<5 min	1	34	not available	not available	1.4	>$3,000, 3
12	X-ray absorption (Anyl-Ray)	119	electronic unit	5 min	5902 (13 lb)	12	not available	±1.5	0.5	$5,000/year rental, 1
13	Specific gravity (Honeywell)	112, 113	semiautomatic weight and volume	3 min	750	69 56	0.1 −1.0	±2.4 ±3.4	1.2 1.5	$10,000 or rental, 1
14	Specific gravity of extracts	114	heptane extracts, hydrometers	15 min	20–40	17	0.3	−0.9 to +0.8	0.5	>$300, 2
15	Capacitance (Steinlite)	109	solvent extraction	20 min	50	20	0.2	−1.9 to +1.2	0.8	>$500, 2
16	Capacitance (AMIF)	5	distillation extract is measured	30 min	15	19	0.3	−2.1 to +2.9	1.3	>$500, 2
17	Extracted residue (Si-Mo-Fat)	123	micro vacuum oven	1–2 hr	10	46	−1.0	−2.6 to +0.7	0.8	>$200, 2
18	Distillation extract. residue (Cohen)	125	azeotropic distillation-extraction, evaporation	¾–1 hr (for both moisture and fat)	10	16 16	−0.7 (*m*-xylene) −0.3 (cumene)	±0.9 ±0.5	0.4 0.5	$75, 2

[a–h] Footnotes same as those of Table 1.

commonly available gravity convection oven is utilized. This oven is less expensive than a mechanical convection oven, required for Method 2 (AOAC Method 24.003(b)). Time for an analysis is the same as for Method 2 without loss of accuracy.

Method 4, the high temperature oven method, 15 min per determination, is rapid but it also requires the more expensive oven used in Method 2. Drying samples at 200°C may cause fat spattering and produce errors unless care is exercised.

Method 5, the vacuum oven procedure at 95–100°C, is accurate but requires 5 hr per analysis and the method is stated to be limited to lean meat samples because high fat samples will tend to spatter.

Method 6, the vacuum oven procedure at 70°C which provides results of fair accuracy with overnight drying, is not a rapid technique.

Method 7, drying of samples on a hot plate, yields results approaching, though not equalling, those by the official method. It may be preferred to the infrared radiation oven Methods 8 and 9 because a hot plate is commonly found in laboratories and more than one sample at a time can be put on a unit. A higher correction factor is required for the hot plate method than is necessary for Methods 8 and 9 and the reproducibility is intermediate.

Method 10, drying of samples by means of a moisture balance, gave good accuracy and better reproducibility than Methods 7–9. Multiple infrared lamps can be used for multiple sample analysis in conjunction with a single balance.

Methods 11, 12, and 13, azeotropic distillation, may be performed economically and have the added advantage that analysis requires only 15–30 min. Moisture content is measured directly as volume of water distilled from the sample and collected as condensate. This directness and relative simplicity has led to the widespread adoption of azeotropic distillation as a moisture method by various segments of the food industry. This, and the frequency with which reviews of moisture methods have concluded that azeotropic distillation has the most promise, led to the extensive evaluation of the technique in our laboratories (16, 38, 125). Another inherent advantage of azeotropic distillation is that the moisture receiver can be used indefinitely after an initial calibration.

Fat Analysis

Method 1, AOAC official method 24.005(a), requires: (*1*) drying a meat sample 6 hr at 100–102°C or 1.5 hr at 125°C; (*2*) ether extraction for 4–16 hr, depending on condensation rate; (*3*) drying the extracted fat for 30 min at 100–102°C; (*4*) cooling the fat and weighing. The method is a direct, gravimetric determination of fat content. It is not a rapid method but reproducibility of results with the method is ±0.25% fat.

Method 2 is a modification of Method 1 in which a sample is dried in a 125°C gravity oven in 30 min as efficiently as in the preceding procedure. Fat can then be ether-extracted in 45 min or less, depending on type of meat product, without loss of accuracy. This procedure reduces analysis time by 4¼ hr as compared with the official method.

Methods 3, 4, 5, and 6, the Babcock-type methods, have the advantage that an analysis can be made in 15–35 min. These are widely used, direct methods of analysis with a fair degree of accuracy. Concentrated sulfuric acid yields the most rapid digestion of meat samples but this occasionally leads to sample charring, which clouds the meniscus and affects accuracy. Use of dilute sulfuric acid minimizes charring but prolongs digestion time. Meat product samples containing spices cannot be determined accurately by these methods because the spice particles float at the interface between the 2 liquid phases. When sulfuric acid is replaced by mixed acetic and perchloric acids (Method 4), a correction is required for acetic acid dissolved in the fat column. In Method 5, sulfuric acid charring is eliminated by digesting the meat sample with hydrochloric acid. The constituents of the digested sample are then diluted with dimethylsulfoxide, which readily dissolves all digest constituents except fat. The problem of spice particles at the fat-aqueous interface remains and, in addition, there is a hazard to the analyst in working with dimethylsulfoxide. Method 6 utilizes a number of protein solubilizing agents in place of the customary acid and introduces less hazard to the analyst. Digestion of the sample is relatively rapid but the method is unduly complicated by the need for 3 separate water baths (55–60°C, 95–100°C, and boiling water bath).

Methods 7 and 8, which utilize fat-rendering devices, are relatively inaccurate. The devices do not provide a means for pressing the residue of rendered samples. Therefore, more fat content

remains unmeasured than if a press-cake were utilized. Use is limited to making crude approximations preliminary to laboratory analysis. Normal variation of meat type and processed meat product composition directly affects amount of fat drip.

Method 9 utilizes an infrared radiation moisture balance to remove moisture from a sample. Fat is extracted from the dried residue with carbon tetrachloride. The extracted residue is then dried with infrared radiation so that fat is determined by difference. The principle used in the Ultra-X method was evaluated at our laboratory with a moisture balance. Extraction was found to be incomplete. The method is fairly rapid but reproducibility is marginal.

Method 10, extraction by means of a modified separatory funnel, shows that since samples are not dried prior to extraction, the moisture barrier to ether extraction can be overcome by the physical action of vigorously shaking the sample and ether in the funnel. However, the procedure requires up to 2 hr for an analysis.

Method 11, spectrophotometric measurement of infrared absorption, may require further development for use as a rapid method. The technique is capable of very sensitive measurement but it is also very sensitive to interfering substances. The cost of present instruments would tend to limit its acceptance and use.

Method 12, X-ray transmission analysis, in the preliminary reports in the literature appears to have considerable potential as a rapid, simple, and nondestructive method for fat determination. A thorough evaluation of the instrument on a number of meats and meat products would benefit the interests of meat analysts. The instrument cost is high, and this factor would limit its use to large processors.

Method 13, semiautomatic specific gravity measurement, was reported to be rapid, simple, and nondestructive of the sample. Cost of the instrument tends to limit its use to the very large processor. It requires programming for use with each type of fresh meat being measured. Foreign ingredients, such as spices and frozen moisture, introduce error. From the two reports in the literature concerning the method, standard deviation values of ± 1.2 and $\pm 1.5\%$ fat indicate that accuracy of the method is marginal.

Method 14, specific gravity of the fat extracts by means of hydrometers in a 15 min procedure, has the elements of a good, rapid method. A fire hazard exists when heptane is used as the extractant. The cost of a tempering bath which will maintain a temperature of ± 0.01°C and a series of hydrometers is not excessive.

Methods 15 and 16, capacitance measurement of fat extracts by means of *o*-dichlorobenzene or 1+7 mixture of 1-octanol and 2-octanol, is fairly rapid but the solvents have a very unpleasant odor and the sensitivity of the method to temperature variation can lead to error. Only fair accuracy was reported.

Method 17, vacuum oven drying and weighing of the residue that remains after distilling off moisture and extracting fat from the meat sample with *n*-butyl ether, was reported to require 2–2.5 hr.

Method 18, gravimetric determination of fat in the extract obtained by azeotropic distillation, involves evaporation of solvent from an aliquot of the extract. Distillation for 15–45 min is required to determine moisture and to obtain a representative fat extract, depending on type of product. Gravimetric determination of fat in the extract is obtained by solvent removal in 6–15 min, depending on type of solvent. Accuracy of the results was good. Total time for determining both moisture and fat is between 30 min and 1¼ hr, depending on type of product. Evidence that azeotropic distillation prevails as the preferred method for combined moisture and fat determination is noted in the combined methods review section where 5 of the 7 procedures reviewed applied this technique.

Conclusions

Until careful comparative studies have been made of many of the methods evaluated above, a precise order of value cannot be given to some methods which now appear equally satisfactory. Methods presently available that meet the needs of the meat industry for rapid analytical methods and newly developed methods which may merit this status upon further investigation are as follows:

Moisture Analysis

Method 4, high temperature mechanical convection oven-drying, is one of the most rapid for moisture determination. Its accuracy and precision are quite satisfactory. The method should be more thoroughly evaluated, since data on only 25 samples were reported.

Method 7, hot plate drying with a 200°C heating surface, is not as rapid as Method 4 but it has a good degree of accuracy and it can be a useful and economical method for moisture analysis.

Methods 8, 9, and 10, drying by infrared radiation, require 15–60 min for moisture analysis. Method 10 provides results in 30–45 min with better reproducibility than Methods 8 and 9.

Methods 11, 12, and 13 yield moisture results by azeotropic distillation. Method 11 requires 2 hr for an analysis whereas Methods 12 and 13 require 15–30 min. The accuracy and precision of the results shown for Methods 11 and 12 are not as good as those of Method 13. This may be attributed to factors incidental to the method, such as cleanliness of glassware, or imperfections, such as scratches on the condenser inner surfaces.

Fat Analysis

Method 2, modified official method, is not one of the most rapid fat analysis methods, but good accuracy and a fair degree of precision can be obtained by drying samples 30 min in a 125°C gravity oven and extracting 45 min with ether.

Methods 3, 4, 5, and 6 are all modified Babcock-type procedures. These methods have been very commonly used for rapid analysis and will continue to be used in the future. The procedures are fairly rapid and simple, and provide a good degree of accuracy. Within this group of 4, Method 5 with a 15 min analysis time apparently is the most rapid, although its reproducibility is the poorest.

Method 11, infrared absorption spectrophotometry, will remain an expensive instrumental method but it is potentially a very rapid method as a combined procedure for determining both moisture and fat if it is further developed to improve accuracy.

Method 12, X-ray transmission analysis, is also an expensive instrumental method and even more rapid than infrared absorption, since no special preparation of the sample is required for the measurement. The method is limited to measuring fat content but analyses are very rapid and simple.

Method 13, semiautomatic specific gravity measurement of meat, appears to be the most rapid method for assaying fat content, but from the published results, it is one of the least accurate of the available methods.

Method 14, specific gravity of heptane extracts measured by hydrometers, is very advantageous for measuring fat only. It is fairly economical, rapid, and simple, and provides acceptable accuracy.

Methods 15 and 16, capacitance measurement of solvent extracts, are fairly rapid and simple methods for fat content determination but accuracy of results is poor.

Methods 17 and 18 permit fat analysis in combined methods after determining moisture content by azeotropic distillation. The procedures are most useful when both quantities are to be determined; otherwise many of the above procedures are more rapid for determining fat alone. Of these two, Method 18 is more rapid, accurate, and precise.

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PESTICIDE FORMULATIONS

Collaborative Study of a Gas-Liquid Chromatographic Determination of *d-trans*-Allethrin

VERNON J. MEINEN and DEAN C. KASSERA
McLaughlin Gormley King Co., 1715 Fifth St., S.E., Minneapolis, Minn. 55414

A gas-liquid chromatographic (GLC) method for the quantitative determination of *d-trans*-allethrin in technical materials and formulations was collaboratively studied. The samples are diluted with acetone and analyzed by GLC, using a flame ionization detector and a 5% OV-1 GLC column. A statistical evaluation of the matched pair technical samples shows an overall coefficient of variation of 0.34% and a coefficient of random error of 0.49%. An evaluation of the *F*-factor reveals no indication of systematic error contributions. A statistical evaluation of the results for 2 *d-trans*-allethrin formulations showed coefficients of variation of 4.98 and 1.98. An examination of the *t*-values revealed a small contribution to systematic error. The somewhat high results of sample C are believed to be a result of lower quality GLC resolution. The addition of a requirement for minimum GLC column performance to the method should increase the overall quality of the GLC resolution. The method has been adopted as official first action.

A preliminary study of the gas-liquid chromatographic (GLC) determination of *d-trans*-allethrin was reported at the 85th Annual Meeting of the AOAC in 1971 (1). At that time, a factor was incorporated into the method to correct for interference from MGK® 264. Further investigation of several manufactured lots of MGK 264 Synergist (frequently formulated with *d-trans*-allethrin) showed that no interference was present. As a result, the method was modified by eliminating the correction factor. Also, to insure satisfactory GLC resolution, a minimum requirement of theoretical plates/ft for the GLC column was added to the method, as a result of the collaborative study. The method given below includes both modifications.

This report of the Associate Referee, D. C. Kassera, was presented at the Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

METHOD

d-trans-Allethrin (*dl*-2-Allyl-4-hydroxy-3-methyl-2-cyclopentene-1 Ester of *d-trans*-2,2-Dimethyl-3-(2-methylpropenyl)-cyclopanecarboxylic Acid)—Official First Action

Gas Chromatographic Method

(*Caution: See* 46.041.)

6.C10 *Principle*

d-trans-Allethrin is dild in acetone contg dibutyl phthalate as internal std. Ratios of GLC peak hts of *d-trans*-allethrin and dibutyl phthalate in sample and std are compared for quant. detn. Method is applicable to both tech. *d-trans*-allethrin and various formulations of it. Not applicable to formulations contg large amt of MGK Repellent 874 (2-hydroxyethyl-*n*-octyl sulfide).

6.C11 *Apparatus and Reagents*

(a) *Gas chromatograph.*—Equipped with flame ionization detector and 4′ × 4 mm id glass column packed with 5% OV-1 (Analabs, Inc.) on 80–100 mesh Chromosorb W (HP). Operating conditions: temps (°)—column 165, injection port 230, detector 230; gas flows (ml/min)—N carrier gas 125, air 350–400, H 40–50; sensitivity—10^{-9} amp full scale, attenuation 4× for tech. material, 10^{-9} amp full scale, attenuation 1 for formulations. Before use, condition column 2–3 hr at 275° with N flow 50 ml/min. If necessary, vary column temp. or gas flow to attain retention times of ca 4 and 7 min for internal std and *d-trans*-allethrin, resp. Also vary detector sensitivity or injection vol. to attain ≥100 mm peak ht for each compd (ca 16 μg *d-trans*-allethrin). Theoretical plates/ft must be >200.

Calc. theoretical plates/ft (N) as follows: $N = 16(L^2/M^2 \times F)$, where L = retention of GLC peak in mm; M = mm peak baseline produced by drawing tangents to points of inflection of peak; and F = length of column (ft).

(b) *Internal std soln.*—4.0 mg dibutyl phthalate/ml acetone.

(c) *d-trans-Allethrin std solns.*—(*1*) *Soln A.*—Approx. 4 mg/ml. Accurately weigh ca 1.0 g *d-trans-*

allethrin (available from McLaughlin Gormley King Co., 1715 5th St, SE, Minneapolis, MN 55414) into 50 ml vol. flask and dil. to vol. with acetone. Pipet 20 ml this soln into 100 ml vol. flask, add 50 ml internal std soln by pipet, and dil. to vol. with acetone. Use this soln for detn of tech. material. (*2*) *Soln B.*—Approx. 1 mg/ml. Pipet 25 ml soln A into 100 ml vol. flask and dil. to vol. with acetone. Use this soln for detn of *d-trans*-allethrin in formulations.

6.C12 *Preparation of Sample*

(a) *Technical material.*—Accurately weigh sample contg ca 1.0 g *d-trans*-allethrin into 50 ml vol. flask and dil. to vol. with acetone. Pipet 20 ml aliquot into 100 ml vol. flask, add 50 ml internal std soln by pipet, and dil. to vol. with acetone.

(b) *Formulations.*—Accurately weigh sample contg ca 200 mg *d-trans*-allethrin into 50 ml vol. flask, add 25 ml internal std soln by pipet, and dil. to vol. with acetone. Pipet 25 ml aliquot into 100 ml vol. flask and dil. to vol. with acetone.

6.C13 *Gas Chromatography*

(a) *Technical material.*—Inject aliquots (ca 3 μl) std soln A until ratio of *d-trans*-allethrin:dibutyl phthalate peak hts varies <1% for successive injections. Repeat with sample soln, followed by duplicate injections of std soln. If peak ht ratios differ >±1% from previous std injections, repeat series of injections.

(b) *Formulations.*—Proceed as in (a), using std soln B. Repeat std injections after each series of 3 sample injections. If peak ht ratios differ >±1.5% from previous std injections, repeat injections.

6.C14 *Calculations*

(a) *Technical material.*—Calc. peak ht ratios for duplicate std injections before and after sample injections and average the 4 values. Calc. and average peak ht ratios for sample injections.

$$\%\ d\text{-trans-Allethrin} = (W' \times P \times R)/(W \times R'),$$

where W' and W = g std and sample, resp.; P = % purity of std; and R' and R = peak ht ratios of std and sample, resp.

(b) *Formulations.*—Calc. av. for all std peak ht ratios and for sample peak ht ratios.

$$\%\ d\text{-trans-Allethrin} = (W' \times P \times R \times 2)/(W \times R'),$$

where W' = g std in final diln.

Collaborative Study

Each collaborator received 4 samples to be analyzed for *d-trans*-allethrin by the proposed GLC method. Two samples (A and B) were prepared to follow Youden's procedure (2) for closely matched pairs. Samples C and D were D-TRANS® Intermediates. Single determinations were requested on each of the 4 samples. Of the 16 collaborative results received, 2 sets of results were rejected, one because of unsuitable GLC resolution and the other as a result of unsatisfactory reproducibility. Six collaborators used modifications of the suggested GLC column: 2 used stainless steel and 2 used approximately 5′ columns; 2 used a liquid phase other than that specified in the method. Collaborator 3 used 3% OV-7 and Collaborator 9 used 5% UC-W98. Adequate separation was obtained and the results from the last 2 collaborators were retained for statistical analysis. The remaining modifications were considered minor. The samples are described in Table 1.

The secondary standard was analyzed by using a recrystallized *α-dl-trans*-isomer of allethrin as a primary standard. Several determinations by 3 laboratories familiar with allethrin analysis by GLC support the 93.0% content of the secondary standard.

Results and Discussion

Table 2 gives the collaborative results for the 2 samples of technical *d-trans*-allethrin. Using the Dixon criterion (3), 2 outliers were excluded from the original data and 2 results were excluded after totaling the results for samples A and B. Sample A averaged 92.9% (range, 91.7 to 94.3) with a coefficient of variation of 0.67 and sample B averaged 90.2% (89.5 to 91.0) with a coefficient of variation of 0.50. The average difference between A and B was 2.7%, as opposed to the expected 3.0%.

From the differences between the samples, the standard deviation of random error, S_r, is 0.44 and the overall standard deviation, S_d, is 0.31. The low F-ratio and resulting negative value obtained for S_b^2 indicate no systematic error.

Table 1. Description of collaborative samples

Samples	Description	*d-trans*-Allethrin expected, %
A	Technical material	93.00
B	Technical material	90.00
C	D-TRANS Intermediate 1868	9.00
D	D-TRANS Intermediate 1864	1.25
E	Standard (secondary)	93.00

Table 2. Collaborative results for technical *d-trans*-allethrin (%)

Coll.	Sample A	Sample B	Diff. (D), A − B	Total (T), A + B	$Di - \bar{D}$	$Ti - \bar{T}$
1	94.6[a]	91.0	—	—	—	—
2	92.6	90.7	1.9	183.3	−0.76	+0.2
3	92.8	89.7	3.1	182.5	+0.44	−0.6
4	92.0	89.5	2.5	181.5[a]	−0.16	—
5	93.2	89.8	3.4	183.0	+0.74	−0.1
6	93.2	90.3	2.9	183.5	+0.24	+0.4
7	93.1	89.9	3.2	183.0	+0.54	−0.1
8	93.2	90.6	2.6	183.8	−0.06	+0.7
9	93.3	90.2	3.1	183.5	+0.44	+0.4
10	92.7	90.6	2.1	183.3	−0.56	+0.2
11	94.3	90.6	3.7	184.9[a]	+1.04	—
12	91.7	92.1[a]	—	—	—	—
13	93.2	89.6	3.6	182.8	+0.94	−0.3
14	92.5	90.0	2.5	182.5	−0.16	−0.6
Av.	92.9	90.2	2.66	183.1		
Std dev.	0.62	0.45				
Coeff. of var.	0.67	0.50				
S_d						0.31
S_r					0.44	
S_b						—[b]

[a] Excluded from statistical evaluation with 95% confidence on the basis of Dixon test (3).
[b] No evidence for systematical error.

Table 3. Collaborative results for technical *d-trans*-allethrin (%), using the official AOAC method

Coll.	Sample A	Sample B
10	94.1	90.8
13	93.4	90.4
14	94.0	92.0
Av.	93.8	91.1

Collaborators 10, 13, and 14, being familiar with the official AOAC method (4), were asked to analyze samples A and B, using the titrimetric procedure; *see* Table 3. The average for sample A was 93.8% (93.0% expected). The average for sample B was 91.0% (90.0% expected).

The collaborative results obtained for samples C and D are given in Table 4. One result is excluded from sample D, using the Dixon criterion (3). The 14 results from sample C averaged 9.23% (range, 8.83 to 9.80) with a standard deviation of 0.46 and a coefficient of variation of 4.98. The average per cent recovery was 102.6. Sample D averaged 1.26% (1.21 to 1.30) with a standard deviation of 0.024 and a coefficient of variation of 1.90. The probable reason for the higher coefficient of variation for sample C is a combination of questionable GLC resolution and high MGK 264 content. Unsatisfactory GLC column resolution will result in a poor separation of MGK 264 and *d-trans*-allethrin, which leads to high results because of a slight interference from the tailing MGK 264. The effect is not as noticeable in sample D because of the smaller amount of MGK 264 present. The t-value (2) for sample C was 1.88 and for sample D, 1.50. From the t-values it can be concluded, with 5% risk, that the difference between the average per cent found and the expected value is not a result of appreciable systematic error.

From the chromatograms submitted by collaborators, it was noted that columns of widely varying quality (150–650 theoretical plates/ft) were used. Usually poorer results could be traced to lower quality resolution. This was the case with one of the sets of results excluded from the statistical study. The second set of results was discarded as a result of very poor reproducibility (not within method requirements) which evidently was caused by instrumentation problems because the GLC resolution was excellent. Most of the outliers were from poorer quality chromatograms. One exception was Collaborator 12. His data showed excellent resolution and reproducibility, but his results were somewhat erratic. Of the 14 collaborators whose data were used in

Table 4. Collaborative results of determination of *d*-trans-allethrin (%) in formulations

Coll.	Sample C[a] Found	Sample C[a] Recd	Sample D[b] Found	Sample D[b] Recd
1	8.83	98.1	1.23	98.4
2	9.80	108.9	1.30	104.0
3	9.33	103.7	1.27	101.6
4	9.06	100.7	1.21	96.8
5	8.83	98.1	1.27	101.6
6	9.20	102.2	1.25	100.0
7	9.31	103.4	1.25	100.0
8	9.14	101.6	1.27	101.6
9	9.05	100.6	1.27	101.6
10	9.30	103.3	1.27	101.6
11	9.10	101.1	1.29	103.2
12	9.71	107.9	1.43[c]	—
13	9.50	105.6	1.25	100.0
14	9.10	101.1	1.23	98.4
Av.	9.23	102.6	1.26	100.9
Std dev.	0.46		0.024	
Coeff. of var.	4.98		1.90	
t-Value	1.88		1.50	

[a] D-TRANS Intermediate 1868 which contains 9.00% *d-trans*-allethrin, 30% MGK 264, 18% piperonyl butoxide, and 43% petroleum distillate.

[b] D-TRANS Intermediate 1864 which contains 1.25% *d-trans*-allethrin, 2.5% MGK 264, 2.5% Tropital®, and 93.75% petroleum distillate.

[c] Excluded from statistical evaluation with 95% confidence on the basis of Dixon test (3).

the statistical evaluation, 9 were rated as having excellent (>250 theoretical plates/ft), 4 as having good (ca 200), and one as having acceptable (<150) resolution. As a result of these observations, a minimum requirement of 200 theoretical plates/ft for the GLC column has been added to the method.

Each collaborator was asked to make 5 consecutive injections of the standard solution. Eight collaborators submitted the requested data, which are summarized in Table 5. Six of the 8 coefficients of variation are well under 1.0, which shows good reproducibility under varying circumstances. The relatively high coefficients of variation of Collaborators 4 and 11 probably are due to the small peak height measurement (under 100 mm) used.

In general, the collaborators commented favorably on the proposed method. For the most part, they found it to be rapid and easy to follow. Several collaborators suggested smaller sample weights to eliminate dilutions. One collaborator suggested that possibly the reproducibility requirements of injections were too stringent. Table 5 as well as the statistical analysis support the reproducibility requirements of the proposed method.

Table 5. Collaborative results for reproducibility of GLC injection

Coll.	Av. ratio[a]	Std dev.	Coeff. of var.
1	1.310	0.0055	0.42
2	1.360	0.0097	0.73
3	1.324	0.0055	0.42
4	1.286	0.016	1.40
6	1.387	0.0076	0.55
7	1.435	0.0064	0.45
9	1.327	0.0031	0.25
11	1.227	0.023	1.87

[a] Average of 5 successive injections.

Recommendations

The precision of the proposed method as evaluated by this collaborative study is acceptable within AOAC guidelines. The accuracy of the collaborative results agree well with the expected results. It is recommended that the proposed method, along with the modifications, for the gas chromatographic determination of *d-trans*-allethrin in technical materials and in formulations be adopted as official first action, as an alternative to 6.200–6.205.

In addition, it is recommended that the official final action mercury reduction method for the determination of pyrethrin II, 6.115, be modified, as follows, for application to refined pyrethrum extracts (reinstatement of 4.110, par. 3 (9th Ed., 1960)):

(a) Add the following phase to the beginning of paragraph 3, 6.115: "For crude pyrethrum exts, treat . . . ."

(b) Add the following paragraph after present paragraph 3:

"For refined pyrethrum exts, add 2 ml neut. alcohol and 20 ml H_2O and heat to dissolve acid. Cool, filter thru gooch if necessary, add 1–2 drops phthln, and titr. with 0.02*N* NaOH (1 ml = 0.00374 g pyrethrin II). Check normality of 0.02*N* NaOH same day as sample is titrd."

Data obtained in both the 1970 and the 1972 studies of the World Standard Pyrethrum Extract (*Pyrethrum Post*, April 1970; S. W. Head, The Pyrethrum Bureau, Nakuru, Kenya, private communication) support these changes.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were adopted by the Association; *see* (1973) *JAOAC* 56, 393–394.

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A. A. Carlstrom, Chevron Chemical Co., Richmond, Calif.

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Paul Korger, Wisconsin Department of Agriculture, Madison, Wis.

C. J. Lord, Cooper Technical Bureau, Berkhamsted, Herts, England

William McDermott, Union Carbide Corp., South Charleston, W.Va.

J. Mignard, Roussel UCLAF, Neuville, France

W. K. Miller, S. C. Johnson, Racine, Wis.

H. H. Poulsen, Kemikaliekontrollen, Lyngby, Denmark

R. T. Rossi, Shell Development Co., Modesto, Calif.

Eric Scheuneman, Canada Department of Agriculture, Ottawa, Ontario, Canada

Joe B. Sills, Fish and Wildlife Service, United States Department of Interior, Warm Springs, Ga.

R. Willemart, Roussel UCLAF, Romainville, France

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(2) Youden, W. J. (1967) *Statistical Techniques for Collaborative Tests*, AOAC, Washington, D.C.

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Collaborative Study of the Ultraviolet Spectrophotometric and Gas-Liquid Chromatographic Methods for the Determination of Trifluralin and Benefin in Formulations

LARRY G. HAMBLETON
Department of Biochemistry, Purdue University, W. Lafayette, Ind. 47907

Fourteen collaborators participated in a study of an ultraviolet (UV) spectrophotometric and a gas-liquid (GLC) method for the determination of trifluralin and benefin. In the UV method, trifluralin or benefin is extracted from the solid carrier or dissolved in *n*-hexane, if the formulation is liquid, purified by chromatography on Florisil, and determined by UV spectrometry at 376 nm. In the GLC method, the compound is extracted from the solid carrier or dissolved in acetone, if liquid, and determined by flame ionization GLC, using a column containing 5% DC-200 on 80–100 mesh Chromosorb W (HP). A statistical evaluation of the data by the technique of closely matched pairs is presented. The data show good correlation between the 2 methods and the methods seem to be reasonably accurate and precise. The ultraviolet and gas chromatographic methods have been adopted as official first action.

In 1963, Holzer, Scroggs, and Leary (1) reported an ultraviolet spectrophotometric method for the determination of trifluralin (2,6-dinitro-*N*,*N*-di-*n*-propyl-α,α,α-trifluoro-*p*-toluidine) in formulations. Since that time the method has undergone unpublished revisions. The ultraviolet (UV) method reported in this collaborative study was a compilation, by the Associate Referee, of unpublished methods supplied by Elanco Products Company. The methods supplied were for the analysis of specific formulations of trifluralin and benefin (*n*-butyl-*N*-ethyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine, Balan®). The method studied enables the analyst to use one method for all types of trifluralin and benefin formulations.

The gas-liquid chromatographic (GLC) method studied was that described earlier (2) with a few minor changes and with the inclusion in the method of the determination of benefin.

Collaborative Study

Five trifluralin and 3 benefin samples were sent to 18 collaborators; 13 collaborators reported results in time for the statistical evaluation of the data. One collaborator reported results too late to be included in the evaluation of the data. Four trifluralin samples and 2 benefin samples were sent as closely matched pairs, as discussed by Youden (3). The following samples were sent to collaborators: Pair 1—sample 1, 5% trifluralin granular and sample 2, 4.5% trifluralin granular; sample 3—Rose and Flora Preen containing 0.174% trifluralin, 1.0% Di-Syston® on a 7-8-5 fertilizer; Pair 2—sample 4, 44.5% trifluralin emulsifiable concentrate and sample 5, 44.0% trifluralin emulsifiable concentrate; sample 6—2.5% Balan granular; and Pair 3—sample 7, 19.4% Balan liquid concentrate and sample 8, 18.4% Balan liquid concentrate. In addition to the samples, each collaborator was supplied with trifluralin and benefin reference standards and also the diisobutyl phthalate internal standard. Samples 4 and 7 were diluted with 3.34 and 6.02% solvent, respectively, and identified as samples 5 and 8.

The collaborators were requested to make a single determination of each sample by the UV and the GLC methods and to also include their recording charts, when possible, along with their results and comments.

METHODS

Trifluralin (2,6-Dinitro-*N*,*N*-di-*n*-propyl-α,α,α-trifluoro-*p*-toluidine) and Benefin (*n*-Butyl-*N*-ethyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine)—Official First Action

Ultraviolet Method

6.C15 ***Principle***

Trifluralin or benefin is extd from solid carrier or dissolved in *n*-hexane if liq., purified by chromatgy on Florisil, and detd by UV spectrometry at 376 nm.

6.C16 ***Reagents***

(a) *Florisil.*—100–200 mesh. Test elution characteristics of Florisil by adding 5 ml std soln to prepd column. Proceed as in 6.C19. Elution vol. should be $\geq$80 ml but $<$100 ml. If elution vol. does not fall within this range, adjust H_2O content of Florisil by

trial and error to obtain proper elution (add H_2O to decrease elution time; dry at 130° to increase it.)

(b) *Std soln.*—1.25 mg/ml. Weigh 0.125 g trifluralin or benefin ref. std (Elanco Products Co., Indianapolis, IN 46206), into 100 ml vol. flask, dil. to vol. with *n*-hexane, and mix.

6.C17 *Preparation of Column*

Insert glass wool plug in bottom of 25 × 400 mm glass column with Teflon stopcock. Add, with constant tapping of column, 5 g anhyd. Na_2SO_4, stdzd Florisil, (a), to ht of 2″, and 5 g anhyd. Na_2SO_4. With stopcock open, add 50 ml *n*-hexane and let drain to top of column. Close stopcock.

6.C18 *Preparation of Sample*

(a) *Dry formulations (containing more than 1% trifluralin or benefin).*—Weigh sample contg 0.25 g trifluralin or benefin into Soxhlet extn thimble (33 × 80 mm), cover with glass wool, and ext with $CHCl_3$ 1 hr beyond time when no further color is extd. Quant. transfer ext to 200 ml vol. flask with $CHCl_3$, dil. to vol. with $CHCl_3$, and mix. Transfer 5 ml to r-b flask and evap. *just* to dryness on rotary evaporator.

(b) *Dry formulations (containing 1% or less trifluralin or benefin).*—Weigh sample contg 0.05 g trifluralin or benefin, ext, transfer to 200 ml vol. flask, and dil. as in (a). Transfer 25 ml to r-b flask and evap. *just* to dryness on rotary evaporator.

(c) *Liquid formulations.*—Weigh sample contg 0.12 g trifluralin or benefin into 100 ml vol. flask. Dil. to vol. with *n*-hexane and mix vigorously. Proceed as in 6.C19.

6.C19 *Determination*

Transfer 5 ml soln from (c) or residue from (a) or (b), with aid of *n*-hexane, to Florisil column. Transfer 5 ml std soln to second Florisil column. Wash sample into column with small portions *n*-hexane. Let each portion drain to top of column before adding next. Fill column with *n*-hexane, discarding eluate until band has moved ca ¾ length of column. Collect eluate contg trifluralin or benefin band (first yellow-orange band to elute) in 100 ml vol. flask. (If band requires >100 ml vol. to elute, replace vol. flask with r-b flask, evap., and transfer quant. to 100 ml vol. flask.) (*Caution: See* 46.011(a) and 46.061.) Dil. to vol. with *n*-hexane and mix. Det. A of sample and std solns in 1 cm cells at 376 nm against *n*-hexane as ref.

6.C20 *Calculations*

% Trifluralin or benefin

$$= (A \times \text{g std} \times F \times P)/(A' \times \text{g sample}),$$

where A and A' refer to sample and std solns, resp.; P = % purity of std; and F = 2, 0.4, or 1 for sample prepns (a), (b), or (c), resp.

Gas-Liquid Chromatographic Method

6.C21 *Principle*

Trifluralin or benefin is extd from solid carrier, or dissolved in acetone if liq., and detd by GLC.

6.C22 *Reagents*

(a) *Diisobutyl phthalate internal std soln*—Weigh 0.625 g diisobutyl phthalate (K&K Laboratories, Inc.) into 250 ml vol. flask, dil. to vol. with acetone, and mix.

(b) *Std soln.*—1.6 mg/ml. Weigh 0.16 g trifluralin or benefin ref. std into 100 ml vol. flask, dil. to vol. with acetone, and mix.

6.C23 *Apparatus*

(a) *Gas chromatograph.*—Equipped with flame ionization detector; capable of programmed column temp. from 135 to 190° at 8°/min. Approx. instrumental conditions: inlet 205°, detector 275°, N carrier gas 60 ml/min.

(b) *Column.*—5′ × ⅛ or ¼″ od, stainless steel or Pyrex glass tube packed with 5% DC-200, 12,500 cstokes (Analabs, Inc.) on 80–100 mesh Chromosorb W (HP). Condition newly prepd column at 230° overnight while purging with N carrier gas.

6.C24 *Preparation of Sample*

(a) *Dry formulations (containing more than 1% trifluralin or benefin).*—Weigh sample contg 0.16 g trifluralin or benefin into Soxhlet extn thimble (33 × 80 mm), cover with glass wool, and ext with acetone 1 hr beyond time when no further color is extd. (*Caution: See* 46.011(a) and 46.046.) Evap. to ca 60 ml on steam bath with stream of air directed into flask. Quant. transfer to 100 ml vol. flask with acetone. Dil. to vol. with acetone and mix.

(b) *Dry formulations (containing 1% or less trifluralin or benefin).*—Weigh sample contg 0.04 g trifluralin or benefin, ext, and evap. as in (a). Quant. transfer to 100 ml vol. flask with acetone and proceed as in 6.C25 without dilg, beginning, ". . . add 10 ml internal std soln . . ."

(c) *Liquid formulations.*—Weigh sample contg 0.16 g trifluralin or benefin into 100 ml vol. flask, dil. to vol. with acetone, and mix.

6.C25 *Determination*

Pipet 25 ml acetone soln, 6.C24(a) or (c), and 25 ml std soln, 6.C22(b), into sep. 100 ml vol. flasks, add 10 ml internal std soln, dil. to vol. with acetone, and mix.

Inject 2.5 μl trifluralin or benefin std soln and

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start temp. program to give symmetrical peak ca 70% scale deflection and retention time 5.5 min. Diisobutyl phthalate internal std peak appears ca 2 min after std peak. Repeat injection of std soln until ratio of trifluralin or benefin peak area to internal std peak area is reproducible.

Without changing conditions inject 2.5 μl sample soln.

6.C26 *Calculations*

Calc. areas of trifluralin or benefin and diisobutyl phthalate peaks. Divide area of trifluralin or benefin peak by area of diisobutyl phthalate internal std peak to det. ratio R.

% Trifluralin or benefin

$$= (R \times W' \times P)/(R' \times W \times F),$$

where R and R' = ratio for sample and std solns, resp.; W and W' = g sample and std, resp.; P = % purity of std; and F = 1, 1, or 4 for sample prepns (a), (c), or (b), resp.

Results and Discussion

Thirteen collaborators reported results for both the UV and GLC methods and these were included in the statistical evaluation of the methods (Tables 1 and 2). Collaborator 14 reported results too late to be included in the statistical evaluations. All the collaborators followed the methods closely with very little deviation. Collaborator 6 used an isothermal column temperature of 190°C and a column packed with 2% SE-30 on Diatoport S rather than the stated column packing and conditions. His recordings were not included with his reported data and, therefore, these conditions could not be evaluated. Collaborator 9 indicated that sample 4 leaked and did not analyze the sample for this reason. Two collaborators had problems in analyzing sample 3 due to interference from the Di-Syston in the sample. Collaborator 1 had to use peak height rather than peak area and Collaborator 9 had to use relative peak areas of reference standard to sample without the use of internal standard for the calculation. The collaborators apparently had little trouble following either the UV or the GLC method.

The data were subjected to Dixon's criteria for outliers (4) and the laboratories were ranked according to Youden's ranking criteria (3). Ten individual results from a total of 208 results were considered as extreme by the Dixon test and were not included in the statistical evaluation of the data. When the laboratories were ranked according to the ranking criteria, no laboratories fell outside the critical scores at the 5% level for the UV or the GLC method.

Some indication of the accuracy of the methods can be obtained by comparing the differences in the pairs found with the expected differences from the dilutions. From the 3.34% dilution of sample 4 and the average amount of trifluralin found, sample 5 should contain 1.50% less trifluralin than sample 4. The average difference found was 1.35% trifluralin for the UV method and 1.82% trifluralin for the GLC method. The dilution of sample 7 with 6.02% solvent gives an expected difference between samples 7 and 8 of 1.21% benefin. The difference found was 1.22 and 1.29% benefin, respectively, for the UV and GLC methods.

The average amount found and the estimates of standard deviations are given in Table 3. The means from the UV and GLC methods were compared by the t-test at the 5% level and did not show a significant difference for any of the sample pairs or for sample 3. Sample 6 did show a significant difference between means of the 2 methods. The estimate of the standard deviation of how successfully repeat determinations can be made in the same laboratory and the estimate of the standard deviation for the distribution of systematic error between laboratories is given by s_r and s_b, respectively. Both of these estimates are favorable for each of the methods. The standard deviation of the data is given by s_d. When s_d^2 for the UV method is compared to that for the GLC method, using the F-test, there is a significant difference at the 5% level for sample pair 3. The other samples and sample pairs did not show a significant difference.

Conclusions and Recommendations

Results obtained with the UV and GLC methods appear to agree favorably. The means and estimates of standard deviation given by both methods agree closely with each other and are considered acceptable. The average differences found between the laboratory-diluted samples and the expected differences are also acceptable. Both methods seem to be reasonably accurate and precise and are applicable to all types of trifluralin and benefin formulations.

It is recommended that (1) the UV method for the determination of trifluralin and benefin be

This report of the Associate Referee was presented at the Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Table 1. Collaborative results for the analysis of trifluralin (%) by the UV and GLC methods[a]

	Sample 1 (5% granular)		Sample 2 (4.5% granular)		Sample 3 (0.174%)		Sample 4 (44.5% emul. conc.)		Sample 5 (44.0% emul. conc.)	
Coll.	UV	GLC	UV	GLC	UV	GLC	UV	GLC	UV	GLC
1	4.44	4.46	5.25	5.41	0.177	0.178	45.0	45.0	42.4	41.2
2	4.45	4.77	5.31	5.26	0.160	0.142	43.7	48.7	41.8	44.2
3	4.48	4.52	5.62	5.63	0.208	0.168	45.5	46.2	43.6	43.4
4	4.56	4.47	4.70	4.50[b]	0.170	0.143	45.1	43.3	43.4	42.7
5	4.91	4.83	4.65	5.12	0.222	0.183	45.1	44.8	44.6	44.1
6	4.50	4.50	5.50	5.20	0.170	0.120	44.4	45.4	44.5	44.4
7	4.90	4.90	5.00	5.20	0.180	0.190	45.5	45.0	44.1	44.9
8	4.56	4.65	5.53	5.63	0.168	0.166	45.3	44.1	43.7	43.1
9	4.50	4.34	5.38	5.28	0.169	0.159	—	—	42.9	42.5
10	4.60	4.40	5.60	5.40	0.177	0.178	44.7	44.7	44.0	44.1
11	4.90	4.50	5.60	5.30	0.037[b]	0.043[b]	45.8	45.6	44.4	43.3
12	4.46	4.67	5.51	5.73	0.170	0.170	43.1	43.5	43.0	43.8
13	4.66	4.76	5.67	5.11	0.193	0.168	45.6	46.2	43.8	43.6
14[c]	4.64	4.67	5.63	5.52	0.170	0.163	43.7	45.4	44.5	43.1

[a] See text for description of samples.
[b] Extreme values omitted from further calculations on the basis of the Dixon test (4).
[c] Results received too late for inclusion in statistical evaluation.

Table 2. Collaborative results for the analysis of benefin by the UV and GLC methods[a]

	Sample 6 (2.5% gran.)		Sample 7 (19.4% liq. conc.)		Sample 8 (18.4% liq. conc.)	
Coll.	UV	GLC	UV	GLC	UV	GLC
1	2.53	2.52	20.3	18.7	19.1	19.9
2	2.63[b]	2.57	22.6[b]	22.4[b]	18.8	19.2
3	2.44	2.49	19.9	20.0	18.9	19.0
4	2.10[b]	2.48	20.1	19.8	19.2	18.8
5	2.48	2.49	20.5	19.4	19.4	17.6
6	2.50	2.50	20.6	21.3	19.4	19.2
7	2.50	2.50	20.3	20.0	18.7	18.8
8	2.50	2.50	19.9	19.8	18.9	19.0
9	2.46	2.55	19.8	20.0	18.6	17.2
10	2.40	2.50	20.0	19.4	19.0	18.6
11	2.40	2.40	20.1	20.1	19.1	18.9
12	2.39	2.66[b]	20.7	20.7	18.9	19.1
13	2.43	2.55	45.3[b]	41.2[b]	18.8	17.0
14[c]	2.44	2.54	19.8	20.2	18.9	19.4

[a-c] See footnotes, Table 1.

Table 3. Statistical evaluation of the collaborative results for the UV and GLC methods[a]

	Av. found, %		s_r precision		s_b systematic		s_d	
Pair	UV	GLC	UV	GLC	UV	GLC	UV	GLC
1 (trifluralin)	4.92	4.96	0.31	0.22	$-s_b$	$-s_b$	0.23	0.17
2 (trifluralin)	44.2	44.4	0.59	1.07	0.59	0.58	1.02	1.34
3 (Balan)	19.6	19.4	0.19	0.46	0.19	0.34	0.33	0.71
Sample 3 (trifluralin)	0.180	0.164					0.018	0.020
Sample 6 (Balan)	2.46	2.50					0.048	0.042

[a] Results of a single determination on each sample by each collaborator (Collaborators 1–13).

adopted as official first action; and (*2*) the GLC method for the determination of trifluralin and benefin in formulations be adopted as official first action.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were adopted by the Association; *see* (1973) *JAOAC* 56, 393–394.

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Collaborative Study of a Gravimetric Method for Mercury in Formulations Containing Chlorinated Pesticides

JAMES E. LAUNER
Laboratory Services, Oregon Department of Agriculture, Salem, Ore. 97310

A gravimetric method for mercury was modified and collaboratively studied with formulations containing ~6% phenylmercuric urea and ~7% captan in one test pair and ~2% phenylmercuric urea and 30% lindane in another test pair. Mercury is determined by precipitation and weighing cupric propylene mercury iodide ($Cu\ pn_2HgI_4$), following refluxing with dilute HI-H_2SO_4 solution containing I_2. Single determinations on 4 samples by 11 collaborators showed that the standard deviation estimation of random error was 0.063 for the captan pair and 0.034 for the lindane pair. Standard deviation estimates of systematic error were 0.061 and 0.013, respectively. In the preliminary study, the method showed close agreement with other accepted methods for 15 formulations. Lindane, captan, dieldrin, heptachlor, and zineb do not interfere; however, the method is not applicable to chloro- or nitrophenolic compounds nor to materials not decomposed by the digestion mixture. The method has been adopted as official first action.

The titrimetric method for mercury in pesticide formulations, 6.A25–6.A28 (1), is both simple and accurate for most mercury compounds, including moderately chlorinated materials such as phenylmercuric chloride, ethylmercuric chloride, or mercurous chloride; however, due to formation of nonionized mercuric chloride which is not titrated by standard thiocyanate solution, the method produces low results in the presence of highly chlorinated compounds such as lindane or heptachlor. In addition, some laboratories object to its use by less experienced operators because of the hazard of substances reacting violently with the oxidizing mixture. The gravimetric method described here was reported by Walton and Smith in 1956 (2) and is a modification of an analytical method used by the Canada Department of Agriculture.

In a preliminary study, analytical results for 16 formulations were compared by the gravimetric method and either the titrimetric method or the HCl-H_2S method (3). Test items consisted of the following: 2 were undiluted 1970 collaborative samples; 5 were these materials spiked with 21% captan, 23% dieldrin, 23% heptachlor, 23% zineb, and 22% maneb, respectively; 2 mixtures contained 40% lindane; and the remaining formulations were mercuric nitrate, phenylmercuric acetate, methylmercuric-8-hydroxyquinolate, Ceresan L, Ceresan M, Panogen 15, and Semesan Bel.

Close agreement between methods was found for all samples except Semesan Bel. As Walton and Smith (2) explained, the gravimetric method gives high results with chloro- and nitrophenolic compounds due to formation of waxy phenols which are insoluble in the washing solvents. Maneb causes slightly lower but still acceptable results, however, since the titrimetric method produces close agreement between formulations with or without maneb or zineb, the gravimetric method seldom would be used on these mixtures.

METHOD

MERCURY

Gravimetric Method—Official First Action

(Applicable in presence of large amts Cl-contg materials; not applicable to chloro- or nitrophenols nor to materials not decomposed by digestion mixt.)

6.C01 ***Reagents***

(a) *Dilute sulfuric acid.*—Add 30 ml H_2SO_4 to H_2O in 100 ml vol. flask, cool, and dil. to vol. with H_2O.

(b) *Sodium sulfite soln.*—10%. Dissolve 10 g Na_2SO_3 in H_2O in 100 ml vol. flask and dil. to vol. with H_2O.

(c) *Ammonium citrate soln.*—pH 7.0. *See* 2.037(a).

(d) *Precipitating reagent.*—Add .20 ml 1,2-propanediamine (Eastman Kodak Co., practical grade) to 100 ml 1*M* $CuSO_4$ soln. Store in g-s container.

(e) *Wash soln.*—Add 1 g KI and 2 ml pptg reagent to 1 L H_2O.

6.C02 ***Preparation of Sample***

(a) *Solns.*—Mix thoroly and weigh, by difference, sample (max. 5 g) contg 0.02–0.08 g Hg into 125 ml Erlenmeyer.

(b) *Dusts.*—Mix thoroly and, using glass weighing dish, weigh sample as in (a). Transfer thru powder funnel into 125 ml ₹ erlenmeyer.

6.C03 *Determination*

(*Caution:* Conduct detn in well ventilated hood.)

Add to sample in following order: 5 ml *ethylene glycol*, swirling to thoroly suspend solids, 4 g KI, 10 ml dil. H_2SO_4, 0.4 g I, and 2 glass beads. After thoro mixing, connect straight-tube H_2O-cooled condenser and, with low flame, heat to slight boil so that liq. condenses in lower portion of condenser. Swirl occasionally, avoiding excessive heat and crystn of large amt I in condenser. Reflux 1 hr and, while cooling flask in H_2O bath, immediately wash warm condenser with heavy stream of ca 25 ml H_2O. (If dye or I persists in condenser, loosen by reheating flask contents, without H_2O in condenser, until liq. refluxes slightly beyond adhering material. Wash condenser again with ca 25 ml H_2O, and cool flask.) Disconnect condenser and wash connections directly into flask. Add ca 2 ml 10% Na_2SO_3 dropwise with swirling, until I color slightly lightens. (Excess I must be present.) Neutze soln with NH_4OH, using pH test paper, until very slightly alk. (pH 7.0–7.3). Cool, and filter with vac. thru retentive paper (S&S Blue Ribbon, or equiv.) in buchner into 400 ml beaker. Wash flask and paper thoroly, keeping total filtrate <150 ml. Add 50 ml NH_4 citrate soln, bring mixt. just to boil, and stir in 5 ml pptg reagent. Cool and let stand ≥2 hr (preferably overnight); filter thru medium porosity glass crucible, previously dried at 105° and weighed. Transfer ppt with wash soln, and wash with same soln several times. Rinse I from ppt with ca 25 ml alcohol in 5 ml portions (some samples may require up to 50 ml) until filtrate is colorless. (Let alcohol stand few min with occasional swirling after each addn before applying suction. Ppt should be suspended in liq. each time.) Wash ppt with 15 ml $CHCl_3$ in 5 ml portions, suspending ppt each time as above until dye and pesticides are completely removed. Finally wash with 5 ml alcohol, dry 30 min at 105°, cool, and weigh.

Wt Hg = wt ppt × 0.218.

Collaborative Study

The collaborative test was designed according to the analysis of a closely matched pair, as described by Youden (4). The following samples were sent to collaborators along with a copy of the method: sample 1, 6.3% phenylmercuric urea containing 6% captan; sample 2, 5.9% phenylmercuric urea with 7.5% captan; sample 3, 2.1% phenylmercuric urea containing 30% lindane; sample 4, 2.0% phenylmercuric urea with 30% lindane. Samples 1 and 2 were prepared from collaborative samples 1 and 2 used in the 1970 mercury study (5) by dilution with captan dust. Samples 3 and 4 were derived from dilution of a commercial mixture of phenylmercuric urea and lindane with kaolin. Each sample was to be analyzed only once to avoid misinterpretation of data. Collaborators were to familiarize themselves with the method on known samples before proceeding with the unknown samples. They were also cautioned that rinsing the iodine and dye from the precipitate requires care and cannot be rushed.

Results and Recommendation

Table 1 shows the results obtained from the captan-phenylmercuric urea pair. None of the data could be rejected by the Dixon test (6). Table 2 shows results for the lindane-phenylmercuric urea pair. Again, none of the data could be rejected by the Dixon test.

Some indication of precision may be observed by comparing the difference between the pairs found and the theoretical differences by dilution. The average difference between samples 1 and 2 was 0.25%, whereas 0.23% was the expected difference. For samples 3 and 4, a 0.07% difference was found; 0.06% was expected.

This collaborative study indicates a 97.5% recovery of mercury compared to average data from samples analyzed by the titrimetric method 2 years ago (5). The Associate Referee believes that closer agreement between the 2 methods can be reached after the analyst becomes thoroughly proficient with the gravimetric method.

One collaborator suggested adding sodium sulfite as a solution, 2 others remarked that no color change was detected when sodium sulfite was added, and one also suggested that the precipitate be washed with a larger volume of alcohol and chloroform. These comments were incorporated into the final method.

The method is not applicable to chloro- or nitrophenolic compounds nor to materials not decomposed by the digestion mixture, i.e., Mercurochrome and Memmi; however, it fills a need for a method to determine mercury in the presence of chlorinated pesticides.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was adopted by the Association; *see* (1973) *JAOAC* 56, 393–394.

Table 1. Collaborative results for analysis of phenylmercuric urea: 3.5% mercury containing 7% captan

Coll.	Mercury, % Sample 1	Mercury, % Sample 2	Diff. (D), (1 − 2)	Total (T), (1 + 2)	$Di - \bar{D}$	$Ti - \bar{T}$
1	3.73	3.34	0.39	7.07	+0.14	+0.02
2	3.68	3.44	0.24	7.12	−0.01	+0.07
3	3.60	3.41	0.19	7.01	−0.06	−0.04
4	3.78	3.47	0.31	7.25	+0.06	+0.20
5	3.65	3.46	0.19	7.11	−0.06	+0.06
6	3.74	3.39	0.35	7.13	+0.10	+0.08
7	3.61	3.50	0.11	7.11	−0.14	+0.06
8	3.70	3.51	0.19	7.21	−0.06	+0.16
9	3.61	3.29	0.32	6.90	+0.07	−0.15
10	3.60	3.30	0.30	6.90	+0.05	−0.15
11	3.45	3.29	0.16	6.74	−0.09	−0.31
Av.	3.65	3.40	0.25	7.05		
S_d						0.107
S_r (random error)					0.063	
S_b (systematic error)						0.061

Table 2. Collaborative results for analysis of phenylmercuric urea: 1.2% mercury containing 30% lindane

Coll.	Mercury, % Sample 1	Mercury, % Sample 2	Diff. (D), (1 − 2)	Total (T), (1 + 2)	$Di - \bar{D}$	$Ti - \bar{T}$
1	1.21	1.10	0.11	2.31	+0.04	−0.06
2	1.21	1.15	0.06	2.36	−0.01	−0.01
3	1.25	1.17	0.08	2.42	+0.01	+0.05
4	1.21	1.22	−0.01	2.43	−0.08	+0.06
5	1.19	1.16	0.03	2.35	−0.04	−0.02
6	1.24	1.16	0.08	2.40	+0.01	+0.03
7	1.25	1.20	0.05	2.45	−0.02	+0.08
8	1.22	1.16	0.06	2.38	−0.01	+0.01
9	1.18	1.11	0.07	2.29	0.00	−0.08
10	1.22	1.16	0.06	2.38	−0.01	+0.01
11	1.24	1.06	0.18	2.30	+0.11	−0.07
Av.	1.22	1.14	0.07	2.37		
S_d						0.036
S_r (random error)					0.034	
S_b (systematic error)						0.013

Since the present study shows that the gravimetric method described compares favorably with the official first action titrimetric method, 6.A25–6.A28, it is recommended that the method be adopted as official first action for the determination of total mercury in formulations containing other chlorinated pesticides.

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This report of the Associate Referee was presented at the Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

ATTENTION

Abstracts of papers or reports to be presented at the 87th Annual Meeting of the AOAC, Oct. 9–12, 1973, MUST be submitted on standard, preprinted forms. The forms are available, on request, from the AOAC Editorial Office.

Since 1971 the Abstract Bulletin has undergone two changes. The abstract submitted by the author is now reproduced directly by a photographic process; it is no longer edited and retyped in the Editorial Office. This new procedure makes it imperative that authors provide an informative, carefully prepared abstract typed on the special form, plus 2 additional copies (carbon copies or photocopies). This form includes general rules for preparing satisfactory abstracts. Abstracts not fulfilling these requirements will be returned to the author for correction, if time permits. Improperly prepared abstracts received too late to allow this will not appear in the Abstract Bulletin. The Abstract Bulletin itself is a new size—4 × 9″, the same size as the AOAC program.

In addition to the abstract, five copies of *all* contributed papers and Associate Referee reports must be submitted to the AOAC office. The manuscripts should be double-spaced throughout. Unless otherwise specified, all manuscripts received will be considered for publication in *JAOAC*, pending satisfactory review. Each Associate Referee should also send one copy of his report to his General Referee.

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Write for standard abstract forms (one required for each presentation). Mail your abstract in early.

DEADLINE for receipt of abstracts—August 17, 1973.

Infrared Analysis of Carbaryl Insecticide: Modification of the Extraction Procedure to Accommodate Liquid Suspension Formulations

WILLIAM H. McDERMOTT
Research and Development Department, Union Carbide Corp., P.O. Box 8361, South Charleston, W.Va. 25303

The extraction procedure for the official first action infrared analysis for carbaryl in formulations, 6.206–6.208, has been modified by the addition of anhydrous granular sodium sulfate to make the method applicable to liquid suspension formulations of carbaryl. A limited collaborative study of the modified method yielded a standard deviation of ±1% at the 40% level and a coefficient of variation of ±2%. The modification has been adopted as official first action.

When the infrared method of analysis for carbaryl in formulations was adopted as official first action by the AOAC in 1967, only 4 commercial formulations were used in the analytical study (1, 2). These formulations were 2 dusts, a wettable powder, and a sprayable powder.

The purpose of this investigation was to modify the extraction procedure of the official method to make it applicable to aqueous slurry formulations of carbaryl. Three formulations were available for this study: SEVIMOL®-4 carbaryl insecticide and 2 experimental carbaryl aqueous suspension formulations. SEVIMOL-4 is a formulation containing 4 lb carbaryl/gal. molasses-water mixture. The experimental formulations contained 4 and 2 lb carbaryl/gal., respectively, suspended in water.

If these formulations are extracted with chloroform by the procedure in the official method, an emulsion results and infrared analysis is impossible.

In several methods of analysis for pesticide residues in moist commodities the sample is intimately mixed with a chloroform solvent and anhydrous sodium sulfate. The sodium sulfate is used to absorb water and coagulate the natural products of the commodities. This technique has been adapted for the extraction of carbaryl from carbaryl suspension formulations. The rest of the method is the same as the official method with 2 exceptions. Only the 5.75 μm band is used for quantitative measurement. This change permits the analysis of a smaller sample and, therefore, less water must be absorbed by the sodium sulfate. The absorbance of the carbaryl is measured at 5.75 μm with an I_o measured at 5.3 μm. This change eliminates the error caused by extraneous materials which affect the absorbance at 6.1 μm and therefore the baseline from 5.3 to 6.1 μm.

Some specialized sample handling techniques are specified to simplify the analysis. The sample is added to a measured amount of solvent in a flask. This prevents the sample from adhering to the glass walls of the flask. The sample is weighed and transferred to the extraction flask with a special hypodermic syringe to facilitate handling of a viscous slurry.

To test the applicability of this modified extraction technique a limited collaborative study has been performed. Samples and analytical grade carbaryl were sent to 11 laboratories for analysis and 9 collaborators submitted results.

METHOD

The changes given below incorporate the modifications necessary to make the official first action infrared method for carbaryl applicable to liquid formulations.

Heading, change caution to read: "*(Caution: See* 46.018, 46.040, 46.041, and 46.056.)."

6.206 *Apparatus and Reagents*

(b)(*1*) Line 3, after "Corp." add "PO Box 1906, Salinas, CA 93901."

(b)(*3*) *2.5 mg/ml.*—Transfer 0.25±0.01 g carbaryl, weighed to nearest 0.1 mg, to 250 ml g-s erlenmeyer. Pipet 100 ml $CHCl_3$ into flask, stopper, and swirl to dissolve.

(c) *Shaking machine.*—Wrist-action shaker (Burrell Corp., 2223 Fifth Ave., Pittsburgh, PA 15219, or equiv.).

(d) *Hypodermic syringe.*—1 ml, glass barrel with rubber-tipped plastic plunger (1 ml B-D Glaspak Tuberculin Discardit syringe supplied by Becton, Dickinson, and Co., Rutherford, NJ 07070, is suitable). Disposable syringe may be used repeatedly. Wash with H_2O and acetone or MeOH, air-dry, and

lubricate rubber plunger tip with silicone stopcock grease.

6.207 *Extraction*

(c) *Liquid suspensions.*—Following steps must be performed in order described as any deviation can cause erroneous results due to faulty sample transfer and incomplete extn: Place ca 20 g Na_2SO_4 in 250 ml g-s erlenmeyer. Pipet 100 ml $CHCl_3$ into flask. Vigorously shake sample bottle. Draw appropriate vol. sample into hypodermic syringe without needle. Use ca 0.5 ml sample for carbaryl 4 lb/gal. and ca 1.0 ml for carbaryl 2 lb/gal. Wipe outside of syringe with paper towel and weigh syringe and contents to nearest 0.1 mg. Add sample to erlenmeyer by slowly depressing syringe plunger. Do *not* allow syringe or sample to touch sides of flask. Sample must drop into $CHCl_3$. Reweigh syringe and calc. sample wt by difference. Stopper flask and shake vigorously 30 min on mech. shaker.

6.208 *Determination*

(a) and (b): Change calculations to read as follows:

Calc. % carbaryl by wt: $(A \times B' \times P)/(A' \times B)$, where P = % purity of carbaryl.

(c) *For liquid suspensions.*—Transfer sample soln to NaCl cell and scan. If Perkin-Elmer Model 21 spectrophtr is used, set instrument as follows: cell, 0.5 mm compensated with $CHCl_3$; range, 5.2 to 6.0 μm; resolution, 960 (program); speed, 2; gain, adjusted (ca 5). Repeat scan with std soln 6.206(b)(3). Measure A of carbaryl peak at 5.75 μm, using A at 5.3 as 0 point.

Calc. % carbaryl as in (a).

Table 1. Repetitive analysis of carbaryl insecticide formulations by 3 methods

Sample	Carbaryl, % by wt		
	AOAC infrared	Colorimetric	Saponification-evolution
Carbaryl, 2 lb/gal.	24.8±0.2	24.7	25.2±0.0
	25.1±0.0		
	24.9±0.1		
	Av. 24.9±0.2		
Carbaryl, 4 lb/gal.	46.5±0.3	46.8	47.2±0.1
	47.3±0.3		
	Av. 46.9±0.4		
SEVIMOL-4	42.6±0.1	43.1	42.5±0.1
	42.7±0.1		
	Av. 42.6±0.1		

Results and Recommendation

Table 1 presents the results of the repetitive analysis of 3 carbaryl formulations. The samples were analyzed in duplicate on successive days by the infrared method, using the modified extraction procedure. Each sample was analyzed in duplicate by the saponification-evolution method (3). The colorimetric method was also used to analyze each sample once (4). The maximum deviation from the mean between duplicates in the infrared method was ±0.3% at the 50% level and ±0.2% at the 25% level. The maximum deviation from the mean between assays on successive days was ±0.4 and ±0.2%, respectively. These deviations are the same as those found in the original collaborative study of the infrared method (1). The results of the colorimetric and saponification-evolution methods are included for comparison because it was not possible to obtain liquid suspension formulations whose carbaryl content was known exactly. The maximum deviation from the mean between methods is ±0.3% at both the 50 and the 25% levels.

Table 2 presents the collaborative results, using the modified extraction procedure for the analysis of SEVIMOL-4 carbaryl insecticide.

Each collaborator was sent a sample of the same batch of a commercial SEVIMOL-4 carbaryl insecticide formulation and a sample of carbaryl, analytical grade. Each collaborator was asked to analyze the sample in duplicate and report both results.

Preliminary results and questions by the collaborators indicated that the procedure in the

Table 2. Results of the collaborative analysis of SEVIMOL-4, using the modified extraction procedure

Coll.	Carbaryl, % by wt			
	1	2	Mean	Range
1	41.74	41.46	41.60	0.28
2	42.25	41.95	42.10	0.30[a]
3	41.20	41.50	41.35	0.30
4	41.93	41.08	41.51	0.85
5	43.35	43.90	43.63	0.55
6	42.40	42.20	42.30	0.20
7	42.60	42.40	42.50	0.20[a]
8	40.70	40.40	40.55	0.30
9	40.64	40.81	40.73	0.17

Mean of all laboratories = 41.81
Std dev. = ±0.95
Coeff. of var. = ±2.27

[a] Deviated from the recommended sample transfer technique.

method did not adequately describe the sample transfer. This part of the method was rewritten and each collaborator was asked to repeat the analysis, following the rewritten sample transfer procedure. Seven of the 9 collaborators complied with this request. The other 2 used their own sample transfer procedures.

The standard deviation found in this study was ±0.95% at the 40% level. The coefficient of variation was ±2.27%. These are approximately the values expected of an infrared method of analysis.

The following comments were made by collaborators. Collaborator 2 recommended that the method emphasize that vigorous shaking was necessary. This has been done. Collaborators 6 and 7 did not think that a syringe was necessary for sample transfer.

It is recommended that the modified extraction procedure for liquid suspension formulations be included in the official first action infrared method, 6.206–6.208.

Acknowledgments

The Associate Referee wishes to express his appreciation to the following collaborators and their associates for their cooperation and participation in this study.

William B. Buffaloe, North Carolina Department of Agriculture, Raleigh, N.C.

L. J. Burton, Department of Agriculture, Calgary, Alberta, Canada

A. A. Carlstrom, Chevron Chemical Co., Richmond, Calif.

Ernest A. Epps, Louisiana State University, Baton Rouge, La.

Alan R. Hanks, Texas A&M University, College Station, Tex.

J. E. Launer, Oregon Department of Agriculture, Salem, Ore.

J. P. Minyard, Jr., Mississippi State Chemical Laboratory, State College, Miss.

Julius C. Prinzo, Agway Inc., Ithaca, N.Y.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was adopted by the Association; *see* (1973) *JAOAC* 56, 393–394.

SEVIMOL is the registered trademark of the Union Carbide Corp. for an insecticide containing Sevin carbaryl insecticide and molasses.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Collaborative Study of the Nonaqueous Copper Colorimetric and Silver Titrimetric Methods for the Determination of Malathion in Technical Grade Malathion and in Malathion Formulation

RICHARD S. WAYNE
American Cyanamid Co., P.O. Box 400, Princeton, N.J. 08540

The nonaqueous copper colorimetric and the silver titrimetric methods for the determination of malathion were studied collaboratively. The study involved wettable powders, emulsifiable concentrates, dusts, and the technical grade product. The mean of all of the results indicates that both procedures will give comparable values for each sample type used in the study. Agreement between replicates in individual laboratories was excellent. Agreement between laboratories was satisfactory; coefficients of variation ranged from 1.3 to 4.8% for the samples studied. The 2 methods offer improved reliability and ease of operation when compared to the official first action colorimetric method, 6.265–6.269. The methods have been adopted as official first action.

One of the important factors in maintaining the continued success and acceptance of malathion for use in the world health area is the availability of reasonably accurate methods of analysis that can be implemented with instrumentation that is generally available in laboratories throughout the world. Two new methods, nonaqueous copper colorimetry (1) and silver titrimetry (2), for the analysis of technical malathion and malathion formulations were presented last fall at the Joint AOAC-NACA-CIPAC Symposium. In both cases, the methods eliminated the partitioning step and the critical timing requirements called for in the official first action colorimetric method, 6.265–6.269 (3–6). They employ instrumentation commonly available in most laboratories and are reasonably accurate when compared to results obtained by high precision gas-liquid chromatography.

Collaborative Study

The nonaqueous copper colorimetric and silver titrimetric methods were studied by 15 collaborators. Each collaborator received a sample of technical grade malathion, 2 wettable powders (WP), an emulsifiable concentrate (EC), and a dust.

Two standards, a cryoscopically analyzed malathion (99.3%) and a standard potassium *O,O*-dimethyl phosphorodithioate (99.0%), were provided, as well as the 8% copper naphthenate color reagent. Each collaborator was requested to provide all of the raw results on prepared data sheets. Provision was made for comments and for a description of the instrument used in the analysis. Replicate results were requested on 2 separate weighings for each of the samples and the standard.

Results

All of the results reported are presented in Tables 1 and 2. The distribution of results is shown in Fig. 1. The comparative precision obtained within each laboratory for each sample type is shown in Table 3. The results from Collaborator 14 were omitted from the calculations for the colorimetric method because of excessive water contamination in the solvents used in the analysis and the resulting lack of accuracy. Results from Collaborator 3 for the technical material and Collaborator 5 for the 50% wettable powder were omitted from the calculations for the silver titrimetric method; in each case the deviation between replicates was considered excessive when compared to the deviation between replicates obtained by the other laboratories. The coefficients of variation ranged from 0.71 to 1.99% for the colorimetric method and from 0.50 to 1.8% for the silver method. As expected, the silver titrimetric method showed a better level of precision than the colorimetric method. As a general rule, volumetric procedures are more precise than spectrophotometric methods because of the poorer precision of the instrumentation involved in the latter type.

The estimates of variance for each sample type are presented in Table 4. These estimates are composed of contributions from within-laboratories replication and from the degree of agreement between laboratories. The coefficients of variation ranged from 2.11 to 4.8% and 1.34 to

Table 1. Collaborative results for the determination of malathion (%) by the nonaqueous copper colorimetric method[a]

Coll.[b]	Tech.	57% EC	50% WP	25% WP	4% Dust
1	94.83	63.43	50.74	25.05	4.15
	94.60	63.31	50.11	23.84	4.19
Av.	94.72	63.37	50.43	24.44	4.17
2	93.85	59.34	49.31	25.00	4.01
	94.59	58.47	49.59	24.65	4.04
Av.	94.22	58.91	49.45	24.83	4.03
3	90.67	58.11	44.78	21.87	3.71
	90.67	56.25	45.14	23.16	3.71
Av.	90.67	57.18	44.96	22.52	3.71
4b	92.55	55.21	49.74	24.75	3.96
	94.41	57.76	48.98	24.89	3.66
Av.	93.48	56.49	49.36	24.82	3.81
4c	95.50	59.14	49.53	24.34	4.05
	95.62	59.62	49.77	24.96	4.02
Av.	95.56	59.38	49.65	24.65	4.04
6	95.06	55.84	49.54	24.42	3.96
	—	56.70	49.02	23.85	3.78
Av.	95.06	56.27	49.28	24.14	3.87
7	94.29	58.38	48.72	24.33	3.92
	94.29	58.49	49.18	24.32	3.91
Av.	94.29	58.44	48.95	24.33	3.92
8	93.91	57.94	49.14	24.39	3.91
	93.51	58.21	49.08	24.32	3.90
Av.	93.71	58.08	49.11	24.36	3.91
9	97.06	59.37	50.20	24.76	4.16
	95.20	58.20	49.36	24.89	4.15
Av.	96.16	58.79	49.78	24.83	4.16
10	96.7	59.5	50.6	25.2	4.2
	96.8	60.6	50.4	25.2	4.2
Av.	96.75	60.05	50.5	25.2	4.2
11	92.88	58.94	48.93	24.65	3.93
	93.65	58.43	49.44	24.50	3.92
Av.	93.27	58.69	49.19	24.58	3.93
12	90.80	55.90	44.89	24.92	4.53
	89.04	55.27	48.01	23.67	4.31
Av.	89.92	55.59	46.45	24.30	4.42
13	91.48	56.70	47.82	24.00	3.77
	91.37	56.38	47.35	23.81	3.80
Av.	91.43	56.54	47.59	23.91	3.78
14[c]	86.66	59.13	140.51	36.09	3.19
	108.33	55.07	212.37	36.57	3.90
Av.	97.49	57.10	176.44	36.33	3.55
15	94.61	59.19	48.82	25.12	4.04
	95.05	58.81	49.32	25.25	4.01
Av.	94.83	59.00	49.07	25.19	4.03
Overall av. (except Coll. 14)	93.86	58.13	48.84	24.44	4.00

[a] EC=emulsifiable concentrate, WP=wettable powder.
[b] Results not reported by Collaborators 4 (4a set) and 5.
[c] Results not included in overall average.

Table 2. Collaborative results for the determination of malathion (%) by the silver titrimetric method[a]

Coll.[b]	Tech.	57% EC	50% WP	25% WP	4% Dust
1	94.88	60.61	50.33	25.28	3.85
	95.36	59.77	49.98	24.47	3.85
Av.	95.12	60.19	50.16	24.87	3.85
2	93.6	57.2	45.0	25.3	4.09
	94.2	57.4	54.3	24.8	4.06
Av.	93.9	57.3	54.15	25.05	4.08
3	89.5	57.1	48.1	24.0	4.11
	92.3	56.4	48.8	24.3	3.85
Av.	90.9[c]	56.75	48.45	24.15	3.98
4a	94.95	58.84	50.41	25.14	4.07
	94.95	58.91	50.44	25.14	4.07
		58.96	50.20	25.10	
				25.13	
Av.	94.95	58.90	50.35	25.13	4.07
4b	93.3	58.70	50.09	24.92	4.05
	94.0	58.53	49.78	24.93	4.04
Av.	93.65	58.62	49.94	24.93	4.05
4c	94.7	59.21	49.93	25.13	3.99
	93.8	58.76	50.10	25.01	3.91
Av.	94.25	58.99	50.02	25.07	3.95
5	98.61	56.92	53.18	26.12	3.93
	97.98	58.22	47.95	25.79	3.94
Av.	98.30	57.57	50.57[c]	25.96	3.94
7	95.63	59.15	50.81	25.79	4.11
	96.33	60.19	50.77	25.59	4.11
Av.	95.98	59.67	50.79	25.69	4.11
8	93.85	58.04	50.09	25.10	4.07
	95.02	59.01	49.94	25.28	4.06
			49.60	25.08	4.09
Av.	94.44	58.53	49.88	25.15	4.07
9	96.19	58.37	49.59	24.17	3.94
	96.83	58.49	50.16	24.38	3.90
Av.	96.51	58.43	49.88	24.28	3.92
10	94.0	60.5	51.6	25.4	4.0
	94.9	60.5	51.3	25.4	3.8
Av.	94.45	60.5	51.45	25.4	3.9
11	95.47	60.16	49.94	24.72	3.84
	95.49	60.08	49.18	24.61	3.94
Av.	95.48	60.12	49.56	24.65	3.89
14	93.7	56.5	50.4	24.5	4.09
	94.3	57.7	50.0	24.2	3.96
Av.	94.0	57.1	50.2	24.35	4.03
15	95.46	57.77	49.34	24.92	3.93
	94.99	58.59	49.24	25.07	4.01
Av.	95.23	58.18	49.29	25.00	3.97
Overall av.[d]	95.10	58.63	50.32	24.98	3.99

[a] EC=emulsifiable concentrate, WP=wettable powder.
[b] Results not reported by Collaborators 6, 12, and 13.
[c] Results not included in overall average.
[d] Except values with footnote [c].

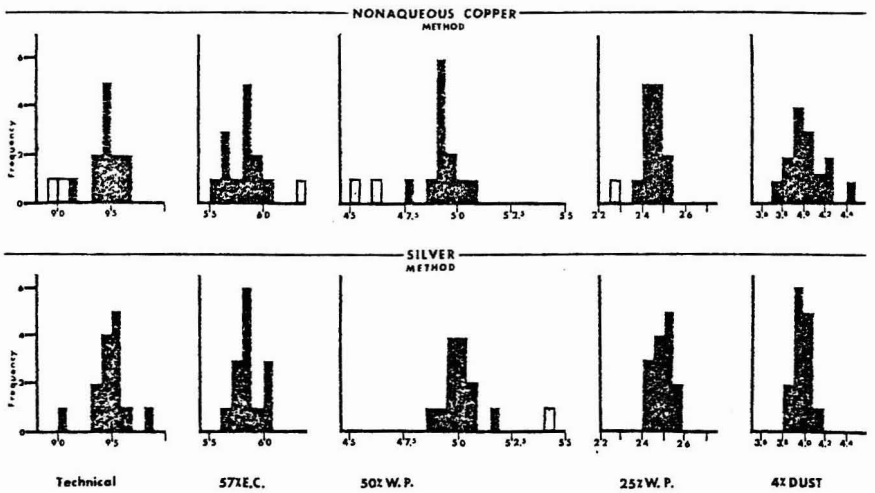

FIG. 1—Distribution of collaborative results for the determination of malathion by the nonaqueous copper colorimetric and silver titrimetric methods.

Table 3. Statistical analysis of the collaborative replicate results

Statistic	Tech.	57% EC	50% WP	25% WP	4% Dust
Copper Colorimetric Method					
Std dev.	0.67	0.74	0.68	0.45	0.079
Coeff. of var.	0.71	1.27	1.39	1.84	1.99
No. of collaborators	13	14	14	14	14
No. of replicates	26	28	28	28	28
Silver Titrimetric Method					
Std dev.	0.48[b]	0.49	0.28[c]	0.20	0.071
Coeff. of var.	0.50	0.84	0.55	0.81	1.8
No. of collaborators	13	14	13	14	14
No. of replicates	26	29	28	31	29

[a] All collaborators except No. 14.
[b] All collaborators except No. 3 (results of No. 3 = 5.9× std dev.).
[c] All collaborators except No. 5 (results of No. 5 = 18.8× std dev.).

Table 4. Statistical analysis of the collaborative results obtained between laboratories

Statistic	Tech.	57% EC	50% WP	25% WP	4% Dust
Copper Colorimetric Method					
Std dev.	2.0	2.01	1.53	0.66	0.19
Coeff. of var.	2.11	3.45	3.12	2.72	4.80
No. of collaborators	14	14	14	14	14
Silver Titrimetric Method					
Std dev.	1.28	1.19	1.30	0.51	0.08
Coeff. of var.	1.34	2.03	2.58	2.05	2.06
No. of collaborators	13	14	13	14	14

2.58% for the copper and silver methods, respectively. The total frequency distributions of results for the colorimetric and silver methods are shown in Fig. 2. All of the sample types used in the study have been normalized as percentages of the obtained average. The respective coefficients of variation for these distributions are 3.36 and 2.05%.

In order to estimate the variance associated with comparisons between laboratories, the contribution of the variance associated with the replicates within laboratories must be removed. The variance contribution to the study as a result of comparisons between laboratories is presented in Table 5. The combined coefficient of variation for the silver method is ±1.9%, compared to ±3.1% for the colorimetric procedure. If the contribution of the 4% dust is eliminated, the combined coefficient of variation is ±2.6%. It would appear that the colorimetric method is more difficult to perform than the silver method in the case of the 4% dust. However, the significance of ±0.2% in the absolute value in this case is a relatively minor consideration.

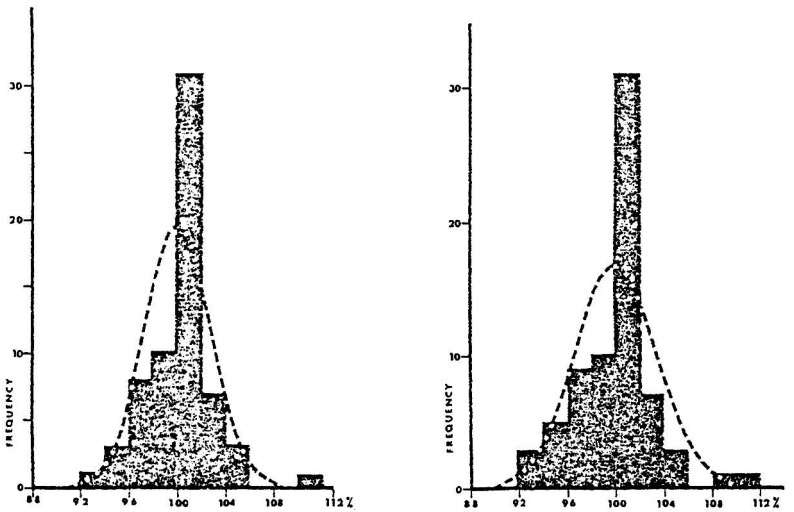

FIG. 2—Distribution of collaborative results for the determination of malathion by the nonaqueous copper colorimetric (left) and silver titrimetric (right) methods (all data normalized).

Table 5. Variance contribution to the collaborative results between laboratories

Coeff. of var.[a]	Tech.	57% EC	50% WP	25% WP	4% Dust
Copper Colorimetric Method					
γb	2.11	3.45	3.12	2.72	4.80
γw	0.71	1.27	1.39	1.84	1.99
$\gamma b^2 - \left(\frac{\gamma w^2}{2} + \frac{\gamma w,s^2}{2}\right)$	3.65	10.54	8.22	5.15	20.51
$\sqrt{\gamma b^2 - \left(\frac{\gamma w}{2} + \frac{\gamma w,s^2}{2}\right)}$	1.91	3.24	2.87	2.27	4.53
Silver Titrimetric Method					
γb	1.34	2.03	2.58	2.05	2.06
γw	0.50	0.84	0.55	0.81	1.8
$\gamma b^2 - \frac{\gamma w^2}{2}$	1.67	3.77	6.51	3.87	2.62
$\sqrt{\gamma b^2 - \frac{\gamma w^2}{2}}$	1.29	1.94	2.55	1.97	1.62

[a] γb—between laboratories (Table 4), γw—within laboratory (Table 3), and $\gamma w,s$—within laboratory coefficient of variance of standards (1.05).

It should be recognized that an additional variable is introduced into the colorimetric method because the standard variance is of the same magnitude as the sample. The silver method relies on a standard whose value can be obtained with a great deal of precision because of the volumetric technique involved.

Examination of the frequency distribution for each sample type used in the study, as represented by Fig. 1, indicates that some laboratories have obtained results that are introducing a marked skewness into the overall data. These are represented by the shaded portions in Fig. 1. The statistical analysis of selected data and the results omitted from the calculations are summarized in Table 6. The mean of all of the results indicates that both procedures will give comparatively the same values for each sample type used in the study. Good agreement is also obtained when compared to results obtained in this laboratory by gas-liquid chromatography, as seen in Table 7.

The overall distribution for the selected data in the nonaqueous copper procedure is represented by Fig. 3. The coefficient of variation for this distribution is ±2.84%, and ±1.45% if the 4% dust as a sample type is not taken into account. The overall coefficient of variation for the silver titrimetric method is ±1.85% (Table 6) for the selected data. The comparative distributions are shown in Fig. 4.

If one examines the data (5) on which the official first action recommendation for the colorimetric method was based, one finds that the nonaqueous copper and silver titrimetric methods perform equally as well as the aqueous copper

Table 6. Statistical analysis of selected collaborative results obtained between laboratories

Statistic	Tech.	57% EC	50% WP	25% WP	4% Dust
Copper Colorimetric Method					
Coll. omitted[a]	(3,12)	(1)	(3,12)	(3)	
Av.	94.46	57.72	49.36	24.58	4.00
No. of collaborators	12	13	12	13	14
Std dev.	1.55	1.44	0.93	0.41	0.19
Coeff. of var.	1.64	2.49	1.88	1.67	4.80
Silver Titrimetric Method					
Coll. omitted[a]			(2)		
Av.	95.10	58.63	50.00	24.98	3.99
No. of collaborators	13	14	12	14	14
Std dev.	1.28	1.19	0.81	0.51	0.08
Coeff. of var.	1.34	2.03	1.63	2.05	2.06

[a] Omitted on basis of the Dixon test at >95% confidence level (7).

Table 7. Comparison of the average results from the collaborative study with results from gas-liquid chromatography

Method	Tech.	57% EC	50% WP	25% WP	4% Dust
Copper colorimetric	94.46	57.72	49.38	24.58	4.00
Silver titrimetric	95.10	58.63	50.00	24.98	3.99
Gas-liquid chromatography	94.3	56.9	50.1	24.8	4.1

AOAC method. The overall coefficient of variation for the AOAC collaborative study (5) was estimated to be ±2.09%, which compares favorably to the values of ±1.76 and ±1.82% found in the present study for the nonaqueous copper and silver titrimetric methods, respectively.

The standard malathion and the standard potassium *O,O*-dimethyl phosphorodithioate provided in this collaborative study were analyzed by one collaborator, using the derivative mode silver titrimetric procedure. The results confirmed the given values provided for the study. Similarly, the comparative results for both standards by the colorimetric method indicated that they were equivalent.

Comments from Collaborators

Some collaborators indicated that the nonaqueous copper colorimetric method was tedious and required a large amount of glassware. The availability of anhydrous solvents was also indicated as a problem. The addition of a surfactant (2% dodecylbenzenesulfonate) to the ethanolic 1*N* NaOH was suggested for the copper method in order to reduce the gelatinous character of the precipitate when ethyl acetate is added. In one case, the official first action AOAC colorimetric method was preferred. Others indicated that the nonaqueous copper colorimetric method was simpler to perform than the silver titrimetric procedure. One collaborator experienced difficulty with the derivative end point as well as the titration to a specific millivoltage. The use of a cellulose column specified in the silver titrimetric method also appeared to be tedious.

Discussion

In the nonaqueous copper colorimetric method some water can be tolerated in the solvents used for the analysis. Excessive water, however, will result in turbid solutions, especially at the reading step. This condition is easily recognized and can be corrected. As a general rule, a nonpolar solvent should be used for the initial extraction or dilution of malathion in which case the source of contamination can only arise from ethyl acetate, ethanol (absolute alcohol denatured), or the sodium hydroxide used in the analysis. If acetonitrile is used, it should be treated as outlined in the previous study (5). The use of a surfactant in the base treatment step does eliminate the gelatinous character of the precipitate on subsequent addition of the ethyl acetate. It also eliminates the small floc of sodium acetate appearing on the surface of the solution after centrifuging. This is of particular concern in that lower results will occur if care is not taken and some of the floc is transferred into the reading step during pipetting. The improvement looks promising and probably will be included as soon as the overall effects can be determined.

The silver titrimetric procedure employs a working end point at a specific millivoltage in the range where the electromotive force is changing at a fast rate. In order to obtain reasonably stable readings and to avoid a positive bias, the titration must be conducted at a rate no faster than that prescribed (2). The readings will show some instability, as they generally do with precipitation titrations in the region of the end point. The derivative mode technique requires a fairly good automatic titrimeter; recent changes in design,

This report of the Associate Referee was presented at the Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

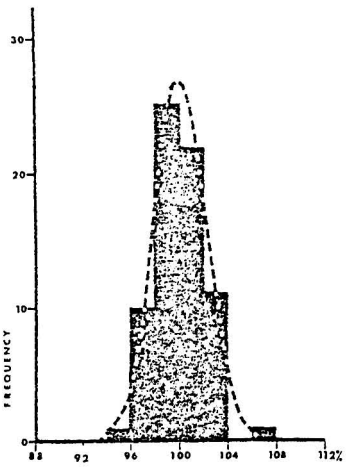

FIG. 3—Distribution of selected collaborative results from the silver titrimetric method (all data normalized).

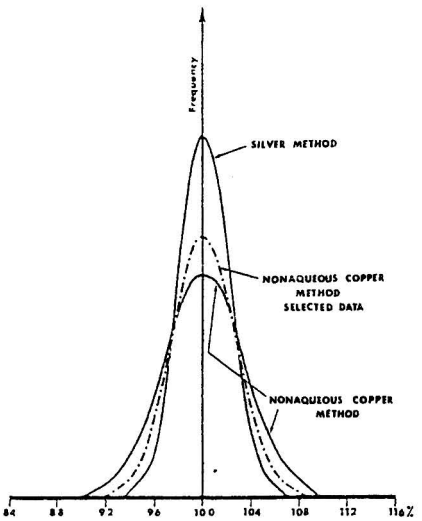

FIG. 4—Comparison of calculated curves for the nonaqueous copper colorimetric and silver titrimetric methods (normal distribution assumed).

particularly in the incorporation of high-impedence input stages, have improved the quality of the results obtainable.

The treatment of the technical product with the cellulose column eliminates the interference from any free *O,O*-dimethyl phosphorodithioic acid. In the case of the concentrate, it is used primarily to remove the surfactant present in the concentrate which results in a poorly defined end point if it is present in the titration step. The use of the cellulose column is of less importance in the case of the powders and dusts, since most of the interferences are retained on the excipients during the extraction with a nonpolar solvent.

The inherent weakness in the first action AOAC method (5) is the rapid fade rate of the colored complex in the aqueous phase during the partitioning step; a 15 sec delay results in a 4% loss of chromophore and a 2 min delay results in a 40% loss of chromophore. Temperature was also found to be a key factor. In addition, the stability of the colored complex in the cyclohexane is relatively poor because of the presence of strong acids in the system. All of these factors can be compensated for by exact timing, temperature control, and reproducible shaking techniques, as demonstrated by the collaborative study (5). However, general experience over the years has shown that the AOAC method is difficult (8) to implement properly in many laboratories, resulting in poor accuracy and precision, particularly when the assay is conducted relatively infrequently or by inexperienced operators. In the case of the 2 methods used in the present collaborative study, no inherent weakness in methodology has been demonstrated. There is every reason to expect that these procedures will withstand the test of time.

It should be recognized that this study was conducted with a high quality commercial product. Some preliminary work has already been completed and reported (1, 2) on the value and validity of these methods applied to malathion-containing products which were of considerably lower quality. However, the applicability of these methods as well as alternative methods of analyses as applied to all sample types of malathion produced throughout the world has yet to be determined.

Conclusion and Recommendation

A collaborative study of the nonaqueous copper colorimetric and silver titrimetric methods for the determination of malathion in technical grade malathion and malathion formulations has been satisfactorily completed. The study involved an examination by 15 collaborators of wettable powders, an emulsifiable concentrate, a dust, and a technical grade product. Statistical analysis indi-

cates that both procedures yield data essentially equivalent in quality to those obtained by the aqueous copper colorimetric method reported in the collaborative study which resulted in adoption of that method as official first action by the AOAC. Practical experience indicates that the exacting requirements called for in the AOAC method are difficult to implement properly in many laboratories, frequently resulting in poor accuracy and precision. In contrast, the 2 new methods do not have this inherent weakness in methodology. They offer improved reliability and have considerably more relaxed requirements than called for in the AOAC method. The methods are reasonably accurate and are only surpassed by some methods employing high precision gas-liquid chromatography.

It is recommended that the nonaqueous copper and silver titrimetric methods be adopted as official first action.

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A. Bevenue and S. Tanaka, Department of Agriculture, Honolulu, Hawaii

L. J. Burton, Canada Department of Agriculture, Calgary, Alberta, Canada

W. Brewbaker and C. Colton Carr, Michigan Department of Agriculture, E. Lansing, Mich.

A. A. Carlstrom, Chevron Chemical Co., Richmond, Calif.

M. B. Goette, J. Woehst, F. Stanford, and J. Miles, Center for Disease Control, Technical Development Laboratories, Savannah, Ga.

R. Grorud and L. A. Koehler, State Laboratories Department, Bismarck, N.Dak.

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E. Hayes and W. Bontoyan, Environmental Protection Agency, Beltsville, Md.

A. H. Hofberg, Geigy Agricultural Division, Ardsley, N.Y.

S. Horeis and G. Gildsdorf, Nebraska Department of Agriculture, Lincoln, Neb.

D. Kovachic and D. L. Campbell, Canada Department of Agriculture, Ottawa, Ontario, Canada

J. A. Neil and J. R. Perrin, Swift Agricultural Chemicals Corp., Bartow, Fla.

J. Prinzo, Agway Chemical Division, Ithaca, N.Y.

R. M. Speth and H. Specht, Nevada Department of Agriculture, Reno, Nev.

R. Wesley and W. R. McCaskill, Clemson University, Clemson, S.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was adopted by the Association; *see* (1973) *JAOAC* 56, 393–394.

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Collaborative Study of a Gas-Liquid Chromatographic Method for the Analysis of Ametryne, Prometone, and Terbutryn[1]

ARTHUR H. HOFBERG and ROBERT T. MURPHY
Agricultural Division, Ciba-Geigy Corp., Ardsley, N.Y. 10502

A gas chromatographic method for the determination of ametryne and terbutryn in wettable powder formulations containing about 80% active ingredient and prometone in liquid formulations containing about 25% active ingredient was collaboratively studied, using a matched pair scheme. The ametryne was extracted from the powder with chloroform containing dieldrin as an internal standard and chromatographed on Carbowax 20M, using a flame ionization detector; the same method was used for terbutryn. The sample containing prometone was dissolved in chloroform containing dieldrin and chromatographed in the same manner. Two samples of each pesticide were analyzed, using peak height measurements, with the following results: ametryne (14 collaborators)—1.63% overall coefficient of variation, 0.68% coefficient of variation for the random error, and 1.05% systematic error; prometone (15 collaborators)—2.27, 1.47, and 1.21%; and terbutryn (15 collaborators)—0.95, 0.51, and 0.56%, respectively. The method has been adopted as official first action.

Gas-liquid chromatographic (GLC) procedures involving extraction of pesticide from formulated products with a solvent containing an internal standard, followed by peak height measurement, were previously collaboratively studied with atrazine and prometryne (1, 2). The methods were adopted as official first action in 1970 (3) and as official final action in 1971 (4). The purpose of the present study was to determine whether the GLC procedure used for these compounds could be applied to formulations containing ametryne (2-(ethylamino)-4-(isopropylamino)-6-(methylthio)-*s*-triazine), prometone (2,4-bis(isopropylamino)-6-(methoxy)-*s*-triazine), and terbutryn (2-(*tert.*-butylamino)-4-(ethylamino)-6-(methylthio)-*s*-triazine).

The collaborative test for closely matched pairs described by Youden (5) was followed. Each collaborator was sent the technical herbicide to be used as the standard, technical dieldrin for use as an internal standard, a formulation (sample 1), and a 4.98% dilution of sample 1 with inert ingredients (sample 2). The 3 samples 1 were as follows: a wettable powder formulation containing about 80% ametryne, a liquid formulation containing about 25% prometone, and a wettable powder formulation containing about 80% terbutryn. The pesticides in the wettable powder formulations were extracted from the powder with chloroform containing dieldrin as an internal standard, while the liquid formulation was diluted with the same solvent. The extracted pesticides were detected by flame ionization GLC, using a column containing 3% Carbowax 20M on 80–100 mesh Gas-Chrom Q. Comparison of peak height ratios (pesticide to dieldrin) of the unknown samples with the corresponding peak height ratio of a standard solution containing known amounts of pesticide and dieldrin was used for quantitative measurement. The separation of ametryne, prometone, and terbutryn from dieldrin is shown in Fig. 1.

For uniformity and convenience, the method for the 3 herbicides studied has been combined with the official final action methods for atrazine, Diazinon, and prometryne. The combined method is given below.

METHOD

6.C05 *Standard Solutions*

(Caution: See 46.041.)

(a) *Dieldrin internal std soln.*—Weigh 2.0±0.02 g tech. dieldrin and dissolve in ca 200 ml $CHCl_3$. Dil. to 250 ml with $CHCl_3$ and mix well. Std should be ≥90% pure and contain no impurities eluting at retention time of pesticide being detd.

(b) *Aldrin internal std soln.*—(For Diazinon®.) Weigh 4.0±0.1 g tech. aldrin into 600 ml beaker. Slurry with 400 ml acetone to dissolve and filter thru paper into 1 L vol. flask, washing with several 100 ml portions acetone. Dil. to vol. and mix well. Std should be ≥90% pure and contain no impurities eluting at retention time of Diazinon.

(c) *Pesticide std soln.*—Accurately weigh 125 mg tech. pesticide of known purity (Ciba-Geigy Corp.,

[1] In current nomenclature, ametryne, prometone, and prometryne should be ametryn, prometon, and prometryn.

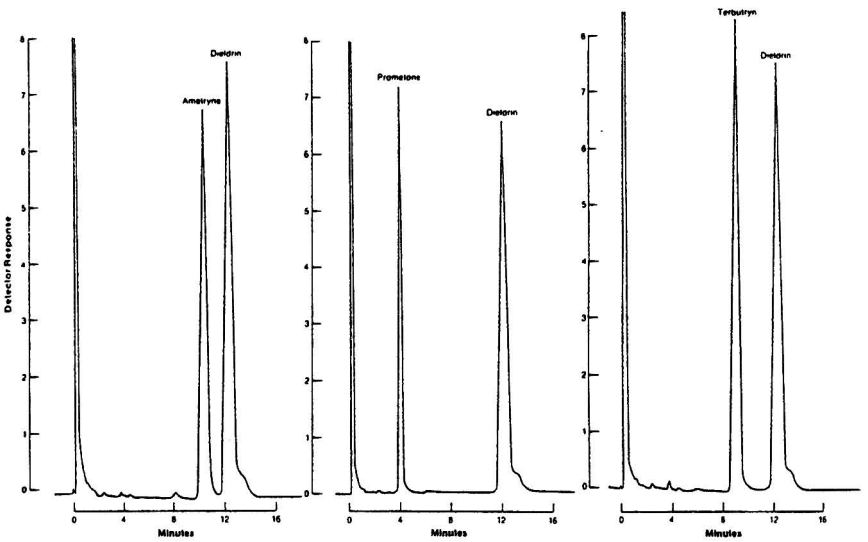

FIG. 1—Gas chromatograms of ametryne, prometone, and terbutryn, with dieldrin as internal standard.

Ardsley, NY 10502) into 2 oz round bottle with Al-lined screw cap. Pipet in 25 ml dieldrin internal std soln, (a), and shake mech. 30 min. For Diazinon, use 125 mg std, 4 oz bottle, and 50 ml aldrin internal std soln, (b), and shake well.

6.C06 *Preparation of Sample*

Accurately weigh ca 150 mg of 80% wettable power of ametryn, atrazine, prometryn, or terbutryn, or 500 mg prometon liq. formulation into 2 oz round bottle with Al-lined screw cap. Pipet in 25 ml dieldrin internal std soln and shake mech. 30 min. Let insol. materials settle or centrf. portion of ext to obtain clear soln.

For Diazinon, accurately weigh sample contg ca 110 mg into 4 oz Al-lined screw-cap bottle. Pipet in 50 ml aldrin internal std soln, cap, and shake.

6.C07 *Gas Chromatography*

For pesticides except Diazinon, use instrument equipped with flame ionization detector and 6′ × 0.25″ (od) (1.8 m × 4 mm (id)) glass column packed with 3% Carbowax 20M (Applied Science Laboratories, Inc.) on 80–100 mesh Gas-Chrom Q. (For Diazinon, use 10% silicone DC-200.) Condition 24 hr at 240° with N or He at ca 40 ml/min. Column should have ≥2000 theoretical plates (*sec* 6.C11).

Operate at following conditions: inlet 240°; column: Diazinon 190±10°, atrazine and prometryn 200±10°, prometon and terbutryn 200±20°, ametryn 215±15°; detector 204°; N or He carrier gas 80–100 ml/min; air and H 80–100 ml/min; attenuation varied so that peak hts of pesticide and internal std are 60–80% full scale. Retention times: prometon 3–5 min, Diazinon 5–6, atrazine 5–7, prometryn 6–8, terbutryn 8–10, ametryn 8–12, dieldrin 9–15, aldrin 10–12. (Ametryn and dieldrin peaks must be resolved. Prep. new column if variation of flow rate or temp. does not resolve peaks. Resolution may be improved by increasing column temp.)

6.C08 *Determination*

Inject 3 μl aliquots std soln until peak ht ratio of pesticide:internal std varies ≤1% for successive injections. Then make duplicate injections of sample followed by duplicate injections of std. Peak ht ratios of stds must be within ±1% of first accepted std values or repeat series of injections. Repeat for addnl samples.

6.C09 *Calculations*

Calc. peak ht ratios for both duplicate std injections preceding and following sample. Average the 4 values (R'). Calc. and average peak ht ratios of the 2 samples (R).

% Pesticide = $(R/W) \times (W' \times P/R')$, where W and W' = mg sample and std, resp.; and P = % purity of std.

This report of the Associate Referee, A. H. Hofberg, was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Results and Discussion

Collaborators were requested to perform only one determination on each sample, as described in the method. Two of the 18 responding collaborators had chromatographic difficulties and their results were rejected. Data from the remaining 16 collaborators were used in the study.

The raw data and scans returned by the collaborators were rechecked. We agreed in every case with the peak height measurements reported by the collaborators, but recalculation with a desk computer showed that the results reported by collaborators deviated more than 0.3% from the calculated values as follows: ametryne—4 collaborators, prometone—2, and terbutryn—6. The rechecked data were used for statistical evaluation, since the differences were caused by deviations from the requested calculation procedure, use of too few decimal places, or minor mathematical errors.

Ametryne

Table 1 shows the results for the matched pair study for the 16 collaborators. The results from Collaborators 5 and 41 are out of line for unknown reasons and were rejected after application of the Dixon test (6). Recalculation of the data after rejection of the results from Collaborator 41 reduced the overall standard deviation from 2.08 to 1.33, while the standard deviation for random error was not significantly changed (from 0.73 to 0.72). The standard deviation of the systematic error, S_b, was reduced from 1.38 to 0.79. Rejection of the results from both Collaborators 5 and 41 showed no further significant reduction in the overall standard deviation (from 1.33 to 1.28). However, the random error, S_r, was reduced from 0.72 to 0.53. No significant change in the systematic error, S_b, was found (from 0.79 to 0.82). Average values after rejection of these outlying results were 78.39±0.69% for sample 1 and 74.39±0.67% for sample 2.

An indication of the accuracy of the method may be obtained by comparing the average values reported for the pairs by the collaborators with results obtained by different internal procedures used by Ciba-Geigy for the analysis of ametryne samples. Results of 78.71% were obtained for sample 1 and 74.10% for sample 2 with a difference of 4.61% vs. the 3.99% obtained by the collaborators. Based on the 4.98% dilution of sample 1, a difference of 3.92% was expected between the samples.

Table 1. Collaborative results for determination of ametryne (%) in matched pair samples

Coll.	Sample 1	Sample 2	Diff.	Total
3	78.17	74.60	3.57	152.77
4	78.35	73.55	4.80	151.90
5[a]	(78.45)	(71.65)	(6.80)	(150.10)
6	78.50	74.06	4.44	152.56
23	78.98	74.89	4.09	153.87
24	80.29	76.90	3.39	157.19
28	78.71	74.50	4.21	153.21
30	76.94	74.54	2.40	151.48
37	78.63	74.60	4.03	153.23
38	78.85	73.44	5.41	152.29
39	76.78	73.67	3.11	150.45
40	78.50	74.47	4.03	152.97
41[b]	(74.35)	(68.99)	(5.36)	(143.34)
44	77.42	72.87	4.55	150.29
48	77.86	73.71	4.15	151.57
51	79.45	75.71	3.74	155.16
Av.	78.14	73.88	4.26	152.02
S_d	1.34	2.37		2.08
S_r			0.73	
S_b			1.38	
Av. excluding Coll. 41	78.39	74.21	4.18	152.60
S_d	0.90	1.21		1.33
S_r			0.72	
S_b			0.79	
Av. excluding Colls. 5 and 41	78.39	74.39	3.99	152.78
S_d	0.94	1.02		1.28
S_r			0.53	
S_b			0.82	

[a] Rejection with 95% significance level for Dixon test.
[b] Rejection with 99% significance level for Dixon test.

Prometone

Table 2 shows the results for the matched pair study for the 16 collaborators. The results from Collaborator 48 are out of line for unknown reasons and were rejected after application of the Dixon test (6). There is no change in the overall standard deviation of 0.62 with this rejection, but the value for S_r is reduced from 0.65 to 0.40. No evidence of systematic error, S_b, was initially found, while a value of 0.33 was obtained after rejection of the results of Collaborator 48. Average values after rejection of this outlying result were 27.26±0.53% for sample 1 and 25.63± 0.32% for sample 2.

The average values reported for the pairs by the collaborators were compared with results obtained by different internal procedures used by

Table 2. Collaborative results for determination of prometone (%) in matched pair samples

Coll.	Sample 1	Sample 2	Diff.	Total
3	27.88	26.18	1.70	54.06
4	26.78	25.51	1.27	52.29
5	26.60	25.25	1.35	51.85
6	26.85	25.35	1.50	52.20
23	27.06	25.69	1.37	52.75
24	27.84	25.10	2.74	52.94
28	27.27	26.03	1.24	53.30
30	28.24	25.57	2.67	53.81
37	27.21	25.26	1.95	52.47
38	26.86	25.92	0.94	52.78
39	26.25	25.19	1.06	51.44
40	28.24	26.16	2.08	54.40
41	26.81	25.47	1.34	52.28
44	28.06	26.01	2.05	54.07
48[a]	(26.10)	(25.88)	(0.22)	(51.98)
51	26.89	25.80	1.09	52.69
Av.	27.18	25.69	1.54	52.83
S_d	0.68	0.45		0.62
S_r			0.65	
S_b				—[b]
Av. excluding Coll. 48	27.26	25.63	1.62	52.89
S_d	0.64	0.37		0.62
S_r			0.40	
S_b			0.33	

[a] Rejection with 90% significance level for Dixon test.
[b] No evidence of bias.

Table 3. Collaborative results for determination of terbutryn (%) in matched pair samples

Coll.	Sample 1	Sample 2	Diff.	Total
3	79.42	75.63	3.79	155.05
4	79.51	75.48	4.03	154.99
5	80.40	76.65	3.75	157.05
6	80.25	77.10	3.15	157.35
23	79.94	75.67	4.27	155.61
24	79.90	76.32	3.58	156.22
28	80.41	76.24	4.17	156.65
30[a]	(77.48)	(73.44)	(4.04)	(150.92)
37	80.13	76.61	3.52	156.74
38	79.99	76.51	3.48	156.50
39	79.12	76.25	2.87	155.37
40	80.85	76.26	4.59	157.11
41	79.85	76.51	3.34	156.36
44	79.18	74.24	4.94	153.42
48	79.80	75.27	4.53	155.07
51	80.19	76.56	3.63	156.75
Av.	79.78	75.92	3.86	155.70
S_d	0.77	0.96		1.16
S_r			0.39	
S_b			0.77	
Av. excluding Coll. 30	79.93	76.09	3.84	156.02
S_d	0.48	0.72		0.76
S_r			0.41	
S_b			0.45	

[a] Rejection with 99% significance level for Dixon test.

Ciba-Geigy for the analysis of prometone samples; 27.63% was obtained for sample 1 and 25.93% for sample 2 with a difference of 1.70% vs. the 1.62% obtained by the collaborators. Based on the 4.98% dilution of sample 1, a difference of 1.36% was expected between the samples.

Terbutryn

Table 3 shows the results for the matched pair study for the 16 collaborators. The results from Collaborator 30 are out of line for unknown reasons and were rejected after application of the Dixon test (6). The value for S_r does not change significantly with the rejection of these results (from 0.39 to 0.41); however, S_b is reduced from 0.77 to 0.45. The overall standard deviation, S_d, is reduced from 1.16 to 0.76 with this omission. Average values after rejection of this outlying result were 79.93±0.36% for sample 1 and 76.09±0.55% for sample 2.

Analysis of terbutryn samples by different procedures used by Ciba-Geigy yielded results of 79.69% for sample 1 and 76.08% for sample 2 with a difference of 3.61% vs. the 3.84% obtained by the collaborators. Based on the 4.98% dilution of sample 1, a difference of 3.98% was expected between the samples.

Evaluation of the chromatograms submitted by collaborators in all 3 studies showed that column preparation is a serious problem, since the number of effective plates/ft of the columns used ranged from less than 100 to more than 600. Supplying collaborators with prepared column packing material may be a solution to this problem and will be considered for future studies.

The detailed review of the chromatograms and raw data was found to be essential for the success of the studies. Without the rejection of data, based on chromatographic difficulties, and the correction of minor mathematical errors, an acceptable method might have been rejected.

Recommendation

The GLC method as presented is rugged, accurate, and within the accepted limits of AOAC methods.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was adopted by the Association; *see* (1973) *JAOAC* 56, 393–394.

It is recommended that the gas chromatographic method for the determination of ametryne, prometone, and terbutryn in formulations be adopted as official first action.

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J. B. Audino, California Department of Agriculture, Sacramento, Calif.

A. Bevenue, University of Hawaii, Agricultural Department, Honolulu, Hawaii

W. H. Brewbaker, Michigan Department of Agriculture, E. Lansing, Mich.

L. J. Burton, Canada Department of Agriculture, Calgary, Alberta, Canada

D. L. Campbell, Canada Department of Agriculture, Ottawa, Ontario, Canada

A. A. Carlstrom, Chevron Chemical Co., Richmond, Calif.

J. Figgins, Ciba-Geigy Corp., McIntosh, Ala.

G. S. Grimes, Office of Indiana State Chemist and Seed Commissioner, Lafayette, Ind.

C. R. Guertal, Ohio Department of Agricultural Laboratories, Reynoldsburg, Ohio

P. E. Irwin, Virginia Department of Health, Richmond, Va.

H. P. Jensen, Ciba-Geigy Corp., Vero Beach, Fla.

N. R. Page, Clemson University, Clemson, S.C.

J. R. Perrin, Swift Agricultural Chemicals Corp., Bartow, Fla.

E. Pinzer and W. Simon, Ciba-Geigy Corp., Ardsley, N.Y.

B. Riley, Ciba-Geigy Corp., St. Gabriel, La.

R. Suter, Ciba-Geigy, Basel, Switzerland

L. Ullrich, Colorado Department of Agriculture, Denver, Colo.

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Collaborative Study of Methods for the Analysis and Control of AG-Chlordane and Its Formulations

MARSHALL A. MALINA
Velsicol Chemical Corp., 341 E. Ohio St., Chicago, Ill. 60611

Four methods for the analysis of AG-chlordane and its formulations were submitted to a collaborative study. Fifteen laboratories, including 5 CIPAC laboratories, participated in this study. The infrared method for the analysis of the content of the 2 chlordane isomers was precise, with a coefficient of variation of 0.015. The gas chromatographic method for the analysis of the heptachlor content also yielded good precision with a standard deviation of 0.16. The infrared assay method for granular formulations exhibited excellent accuracy and precision, with a coefficient of variation of 0.067 and an error of only +0.25% relative. The gas chromatographic assay method for emulsifiable concentrates exhibited poor accuracy and precision and was found unacceptable. The first 3 methods have been adopted as official first action.

AG-Chlordane, a product developed by Velsicol Chemical Corp., is a high purity chlordane containing, typically, 95% of the α- and γ-isomers of chlordane. Five methods for the analysis and control of AG-chlordane were reported at the joint AOAC-NACA-CIPAC Symposium held at the 86th Annual Meeting of the AOAC ((1972) *JAOAC* 56, 942–947).

Four of these methods, infrared analysis of the α- and γ-isomers in AG-chlordane, gas chromatographic analysis of heptachlor content of AG-chlordane, infrared assay of AG-chlordane in granular formulations, and gas chromatographic analysis of AG-chlordane in emulsifiable concentrate formulations, were collaboratively studied.

The 3 methods that were successfully studied are given below:

METHODS

AG-Chlordane (Octachloro-4,7-methanotetrahydroindane)—Official First Action

(Not applicable to tech. chlordane or its formulations)

α- and γ-Isomers in Technical Products—Infrared Method

6.C27 *Apparatus and Reagents*

(a) *Infrared spectrophotometer.*—Double beam, with matched NaCl cells, 0.1 and 0.2 mm.

(b) *Std soln.*—Into tared 10 ml vol. flask, weigh 1.00±0.05 g ref. std α-chlordane and 0.38±0.02 g ref. std γ-chlordane (Velsicol Chemical Corp.), dissolve in CS_2, and dil. to vol. with CS_2.

6.C28 *Preparation of Sample*

Melt entire sample in 100° oven and mix. Weigh 1.5±0.02 g into tared 10 ml vol. flask, dissolve in CS_2, and dil. to vol. with CS_2.

6.C29 *Determination*

(a) *α-Chlordane.*—Fill ref. cell with CS_2. Scan std and sample solns from 750 to 710 cm^{-1} (13.3 to 14.1 μm), using 0.1 mm NaCl cells. Construct baseline from 738 to 715 cm^{-1} (13.6 to 14.0 μm) and draw line from midpoint of max. A at 725 cm^{-1} (13.8 μm) to intersect baseline.

(b) *γ-Chlordane.*—Fill ref. cell with CS_2. Scan std and sample solns from 1390 to 1290 cm^{-1} (7.19 to 7.75 μm), using 0.2 mm NaCl cells. Proceed as as in (a), using min. at 1370 and 1310 cm^{-1} (7.30 and 7.63 μm), and det. A at max. 1320 cm^{-1} (7.58 μm).

6.C30 *Calculations*

$$\text{Wt \% } \alpha(\alpha\text{-chlordane}) \text{ or } \gamma(\gamma\text{-chlordane}) = (A_s \times F \times 100)/W_s$$

$F = [W(\alpha \text{ or } \gamma \text{ in std}) \times \%\text{ purity in std}]/A(\alpha \text{ or } \gamma \text{ of std})$, where W = wt (g), F = factor, and subscript s refers to sample.

(Wts given are for cell thicknesses specified. For other cells, adjust wts to yield peak A between 0.2 and 0.5 (30–65% T).)

AG-Chlordane in Granular Formulations—Infrared Method

(*Caution: See* 46.011, 46.041, 46.046, and 46.048.)

6.C31 *Apparatus*

(a) *Infrared spectrophotometer.*—*See* 6.C27.

(b) *Soxhlet extraction apparatus.*—With 25 × 80 mm Whatman cellulose thimble.

(c) *Vigreux distilling tube.*—15 mm long.

(d) *Vials.*—5 dram, with plastic-lined screw caps.

6.C32 *Reagents*

(a) *Acetone.*—Spectral grade.

(b) *Std soln.*—Into tared 5 dram vial, weigh

1.00±0.05 g ref. std α-chlordane and 0.38±0.02 g ref. std γ-chlordane (Velsicol Chemical Corp.) and pipet in 10 ml CS_2.

6.C33 *Preparation of Sample*

Into tared thimble, weigh sample equiv. to wt of std and cover with glass wool. Insert into extn app. and attach to 250 ml flat-bottom ⅎ flask contg boiling chips. Add 125 ml acetone, attach extractor to condenser, and reflux 1 hr.

Rinse extn app. with acetone. Sep. flask from extractor and condenser, attach distg tube to flask, and evap. acetone on steam bath. Remove tube, add 5 ml CS_2, and evap. carefully. Repeat addn and evapn of CS_2 4 more times. (All residual acetone must be removed because acetone interferes with IR measurement.) Dry residue further, using forced air, until crystalline solid appears. Pipet 10 ml CS_2 into flask, and swirl carefully to dissolve solid. Release stopper pressure.

6.C34 *Determination*

Proceed as in 6.C29.

6.C35 *Calculation*

Proceed as in 6.C30 for calcn of wt %.

% Total AG-chlordane
= % total $(\alpha + \gamma) \times 100/\%$ assay

Generally, factor representing specification grade of 95% AG-chlordane may be used. Then, % AG-chlordane = % total $(\alpha + \gamma) \times 1.053$.

Heptachlor in AC Chlordane—Gas Chromatographic Method

6.C36 *Apparatus and Reagents*

(a) *Gas chromatograph.*—Equipped with flame ionization detector and 5′ × ⅛″ id glass column packed with 5% DC-200 (Analabs, Inc.) on 130–140 mesh Anakrom ABS. Operating conditions: temps (°)—column 165, injector 215, detector 220; N carrier gas 30 ml/min (ca 80 psig at inlet); and chart speed 0.5″/min.

(b) *Stds.*—Ref. std α-chlordane, γ-chlordane, and heptachlor (Velsicol Chemical Corp.) and hexachlorobenzene (C_6Cl_6) internal std (Eastman Kodak Co.), recrystd from benzene.

(c) *Std soln.*—Accurately weigh following components into 10 ml vol. flask, dissolve in CS_2, and dil. to vol.: 0.48 g α-chlordane, 0.18 g γ-chlordane, 0.010 g C_6Cl_6, and 0.010 g heptachlor.

6.C37 *Preparation of Sample*

Melt entire sample in 100° oven and mix. Accurately weigh 0.73 g sample and 0.010 g C_6Cl_6 into 10 ml vol. flask, and dil. to vol. with CS_2.

6.C38 *Determination*

Inject 1.5 μl sample soln into gas chromatograph; retention times for C_6Cl_6 and heptachlor are ca 2 and 4 min, resp. Also inject 1.5 μl std soln to det. response factor (*RF*).

Use attenuation (ca 2×) to keep internal std peak on scale and include in calcns.

6.C39 *Calculations*

Calc. each peak area by any convenient means. Built-in integrators or planimeters provide most accurate method of detg areas where peaks are not perfectly symmetrical.

% Heptachlor
$$= (PH \times RF \times WI \times 100)/(PI \times W')$$
$$RF = (PI \times W')/(PH' \times WI)$$

where PH, PH', and PI = peak areas of sample and std heptachlor and internal std, resp.; W, W', and WI = g sample and std heptachlor and internal std, resp.

Collaborative Study

The 6 samples tested were considered typical technical and formulated products. The formulations were prepared analytically by using the methods and materials recommended by the Velsicol Chemical Corp. All samples were carefully blended to insure homogeneity. The samples are described in Table 1.

Fifteen collaborators were supplied with representative portions of each of the samples, analytical reference grade α- and γ-chlordane, heptachlor, and hexachlorobenzene, and a copy of each of the methods. They were instructed to analyze each sample in duplicate, using the methods exactly as written.

Results and Recommendations

Fifteen collaborators, including 5 CIPAC collaborators, participated in this study. The results from the collaborative laboratories are given in Tables 2–5.

Table 1. Description of collaborative samples

Sample	Description	True assay
1	Technical	—
2	Technical	—
3	25% Granular	24%
4	25% Granular	26%
5	4 lb/gal. emulsifiable concentrate	3.8 lb/gal.
6	4 lb/gal. emulsifiable concentrate	4.2 lb/gal.

Table 2. Collaborative results (%) for the infrared analysis of AG-chlordane

Coll.	Sample 1			Sample 2			Av.
	α-Isomer	γ-Isomer	Total	α-Isomer	γ-Isomer	Total	
A	71.6,69.9	25.9,24.9	96.1	70.9,70.2	26.3,25.7	96.6	
B	67.9,72.2	24.3,26.6	95.5	65.1,64.7[a]	23.4,25.2	—	
C	69.6,69.0	20.8,20.1[a]	—	70.8,69.4	25.3,26.1	95.8	
D	72.0,71.2	24.0,23.8	95.5	71.3,71.5	23.7,23.8	95.2	
E	69.2,69.8	24.6,24.4	94.0	69.4,69.8	24.4,24.6	95.1	
F	69.6,69.2	24.8,24.9	94.3	68.2,68.7	25.3,24.6	93.5	
G	67.3,67.6[a]	27.1,27.0	—	62.6,62.0[a]	26.9,27.0	—	
H	69.5,70.0	25.3,25.3	95.1	69.3,68.6	25.1,25.0	94.1	
I	70.4,70.3	—	—	68.9,68.9	—	—	
J	69.7,70.6	25.4,24.4	95.0	68.0,71.1	25.3,25.1	94.8	
K	71.2,70.3	26.6,29.6	98.8	72.7,70.0	27.8,27.1	98.9	
L	70.7,69.6	25.4,24.7	95.2	69.5,68.7	25.4,24.7	94.2	
M	69.7,69.8	26.5,27.3	96.7	68.3,68.2	27.4,26.9	95.5	
N	73.6,77.5[a]	22.4,23.2	—	72.6,77.3[a]	26.4,25.8	—	
O	71.8,72.4	24.1,24.0	96.2	71.2,71.8	23.7,23.7	95.2	
Av.	70.3	25.2	95.7	69.9	25.5	95.4	
Std dev.	0.84	1.42	1.31	1.15	1.21	1.45	1.38
Coeff. of var.	0.012	0.056	0.014	0.016	0.047	0.015	0.015

[a] These results were rejected from the statistical evaluation of the data on the basis of the Dixon test (Dixon, W. J. (1953) *Biometrics* 9, 74–89).

Table 3. Collaborative results (%) for the gas chromatographic analyses of the heptachlor content in AG-chlordane

Coll.	Sample 1	Sample 2	Av.
A	—	—	
B	0.40,0.41	0.27,0.28	
C	—	—	
D	0.40,0.30	0.40,0.28	
E	0.51,0.48	0.36,0.34	
F	0.42,0.41	0.32,0.37	
G	—	—	
H	2.23,2.25[a]	2.15,2.19[a]	
I	0.23,0.27	0.26,0.24	
J	0.36,0.43	0.23,0.31	
K	0.38	0.36	
L	—	—	
M	0.45,0.47	0.36,0.32	
N	0.40,0.40	0.32,0.39	
Av.	0.39	0.32	
Std dev.	0.075	0.040	0.058
Coeff. of var.	0.19	0.13	0.16

[a] See footnote, Table 2.

The infrared method for the determination of α- and γ-chlordane isomer content (Table 2) exhibited acceptable precision, with coefficients of variation of 0.014, 0.051, and 0.015 for α, γ, and total chlordane, respectively. The gas chromatographic determination of heptachlor content (Table 3) also exhibited good precision, with a standard deviation of 0.058. Several collaborators used conditions which were different from the ones prescribed: 4′ × ¼″, 5% UC-W98 at 200°C; 5′ × ¼″, 10% DC-200 at 175°C; 3% SE-30 at 180°C; 6′ × ⅛″, 10% SE-30 at 170°C; 6′ × ¼″, 10% QF-1 at 200°C. These modifications were found acceptable.

The infrared assay method for granular formulations (Table 4) exhibited unbiased results with acceptable, although disappointing, precision. The coefficients of variation were 0.051, 0.14, and 0.067 for α, γ, and total chlordane, respectively. The error was found to be only +0.25% relative.

The gas chromatographic assay method for emulsifiable concentrate formulations (Table 5) exhibited unacceptable results. Both precision and accuracy were poor, with a coefficient of variation of 0.096 and an error of +9.1% relative. Much of this problem can probably be attributed to the fact that half of the collaborators used gas chromatographic conditions other than those prescribed. Collaborators F, H, J, and K are known to have used modified procedures. While these modifications did not adversely affect the determination of heptachlor in AG-chlordane, they did result in unacceptable assays for this study.

It is recommended that—

(1) The infrared method for the determination of the α- and γ-chlordane isomer content in AG-chlordane be adopted as official first action.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were adopted by the Association; see (1973) *JAOAC* 56, 393–394.

Table 4. Collaborative results (%) for the infrared analysis of AG-chlordane granular formulations

Coll.	Sample 1			Sample 2			Av.
	α-Isomer	γ-Isomer	Total	α-Isomer	γ-Isomer	Total	
A	18.5,18.3	6.4,6.3	26.1	18.6,18.5	6.6,6.5	26.5	
B	18.1,16.9	7.0,6.2	25.4	19.7,18.3	7.1,6.8	27.4	
C	17.3,18.6	5.7,5.9	25.0	17.2,—	4.8,—	23.2	
D	17.0,17.0	5.0,5.2	23.3	17.0,17.1	5.3,5.2	23.6	
E	16.6,16.3	6.3,6.2	24.0	18.2,17.6	6.9,6.8	26.1	
F	—,15.2	—,5.3	21.6	—,14.9[a]	—,5.7	—	
G	16.5,18.1	3.9,3.8	22.3	18.1,17.7	7.1,7.0	26.3	
H	18.1,18.6	5.0,5.1	24.8	17.9,17.2	6.7,6.7	25.6	
I	17.3,16.8	—	—	17.9,17.8	—	—	
J	15.4,16.0	6.0,6.0	22.9	17.0,18.0	6.5,6.5	25.3	
K	20.1,—	6.8,—	28.3	19.9,—	7.5,—	28.9	
L	—	—	23.1	—	—	24.1	
M	17.5,17.1	6.9,6.8	25.5	17.8,18.5	6.7,6.9	26.3	
N	16.0,18.4	6.0,5.3	24.0	21.1,21.7[a]	4.7,4.2	—	
O	16.6,16.3	6.2,6.1	23.8	18.0,17.3	6.4,6.4	25.3	
Av.	17.2	5.82	24.3	18.1	6.26	25.8	
Theoretical	—	—	24.0	—	—	26.0	
Std dev.	1.22	0.84	1.76	0.56	0.87	1.60	1.68
Coeff. of var.	0.071	0.14	0.072	0.031	0.14	0.062	0.067
Error, % relative			+1.25			−0.76	+0.25

[a] See footnote, Table 2.

Table 5. Collaborative results (%) for the gas chromatographic analysis of AG-chlordane emulsifiable concentrates

Coll.	Sample 1			Sample 2			Av.
	α-Isomer	γ-Isomer	Total	α-Isomer	γ-Isomer	Total	
A	—	—	—	—	—	—	
B	3.01,2.99	1.01,1.00	4.22	3.21,3.17	1.08,1.07	4.50	
C	—	—	—	—	—	—	
D	4.10,3.90	1.40,1.50[a]	—	3.90,4.50	1.30,1.40	5.90	
E	2.82,2.72	0.99,0.96	3.94	3.20,3.20	1.10,1.10	4.53	
F	3.97,3.72	1.21,1.09	5.25[a, b]	4.15,3.98	1.32,1.30	5.65[b]	
G	—	—	—	—	—	—	
H	2.76,2.75	0.98,0.97	3.94	2.97,3.01	1.03,1.05	4.24	
I	3.04,3.02	1.15,1.18	4.41	3.23,3.16	1.25,1.27	4.69	
J	2.75,2.96	0.96,1.13	4.10	3.22,3.43	1.17,1.20	4.74	
K	2.68,2.12	0.92,0.93	3.51	2.73,2.44	0.84,0.92	3.64	
L	—	—	—	—	—	—	
M	3.09,2.82	1.06,1.01	4.19	3.11,3.10	1.07,1.08	4.41	
N	—	—	—	—	—	—	
Av.	3.17	1.03	4.04	3.41	1.15	4.70	
Theoretical	—	—	3.80	—	—	4.20	
Std dev.	0.19	0.082	0.29	0.56	0.16	0.58	0.43
Coeff. of var.	0.06	0.08	0.072	0.16	0.14	0.12	0.096
Error, % relative	—	—	6.3	—	—	11.9	9.1

[a] See footnote, Table 2.
[b] Column temperature of 210°C used for analysis.

(2) The gas chromatographic method for the determination of heptachlor content in AG-chlordane be adopted as official first action.

(3) The infrared assay method for AG-chlordane granular formulations be adopted as official first action.

(4) The gas chromatographic assay method for AG-chlordane emulsifiable concentrate formulations be further studied.

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Free Acid in Esters of 2,4-Dichlorophenoxyacetic Acid and 2,4,5-Trichlorophenoxyacetic Acid and Their Formulations

HOWARD HAMMOND
State Laboratories Department, Bismarck, N.Dak. 58501

The free acid content of 2,4-D and 2,4,5-T esters and formulations is determined by titration to pH 7.0, using a pH meter. Average recoveries and standard deviations for 9 collaborators, analyzing 4 samples containing different amounts of the esters, were 0.2067±0.0201 and 0.3778±0.0245 for 2,4-D ester and 0.6208±0.0555 and 1.4622±0.1356 for 2,4,5-T ester. The method has been adopted as official first action.

The Associate Referee received a report that CIPAC Method MT 66 Free Phenoxyalkanoic Acidity (*CIPAC Handbook* (1970) Vol. 1, p. 966) gave high results, and evidence was submitted in support of this claim (M. F. Loucks (1971) private communication). The high results are apparently due to saponification of the sample at a higher pH and the elevated pH of the thymol blue color change in a nonaqueous system. 2,4-Dichlorophenoxyacetic acid (2,4-D) crystals (0.5 g) of questionable purity were added to a sample of 2,4-D ester that had been titrated to a pH of 7.0. Titration to a pH 7.0 end point calculated to a 96.9% purity. Titration to a pH of 11.0 (equivalent to thymol blue in ethanol solution) calculated to a 105.9% purity. For comparison, a similar 2,4-D acid sample was titrated in 100 ml neutralized alcohol (85% strength). Titration to a pH 7.0 end point was calculated as 97.0% purity. Titration to pH 11.0 calculated to a purity of 99.1%. These results suggest that saponification of the ester at pH levels above 7.0 would lead to high values for the esters.

A method developed and used by Dow Chemical Co. was selected as a basis for the proposed method and subjected to a collaborative study. Four samples, consisting of commercial herbicide formulations, were sent to 5 collaborators by the Associate Referee: sample 1, mixture of butyl and isopropyl esters of 2,4-D; sample 2, propylene glycol butyl ester of 2,4-D; sample 3, propylene glycol butyl ester of 2,4,5-T (2,4,5-trichlorophenoxyacetic acid); and sample 4, propylene glycol ether ester of 2,4,5-T. M. F. Loucks, Dow Chemical Co., also participated in the study and submitted samples to 3 additional analysts. The collaborators were requested to make single determinations of the free acids on 2 different days, using the proposed method. The Dow Chemical Co. analysts and the Associate Referee used the CIPAC method for analyzing the esters, for comparison.

METHOD

Free Acid in Esters of 2,4-D and 2,4,5-T in Liquid Herbicides—Official First Action

6.C04 *Determination*

(*Caution: See* 46.041.)

Stdze electrode system against pH 6.87 buffer, 45.007(d). Accurately weigh ca 10 g sample into 250 ml beaker, add 100 ml 80% alcohol, and titr. with 0.1*N* NaOH to pH 7.0. 1 ml 0.1*N* NaOH = 0.02211 g 2,4-D or 0.02555 g 2,4,5-T.

Do not exceed pH 7, since esters saponify rapidly at pH >7. Me red may be used as indicator if pH meter is not available and sample is not excessively colored.

Results and Recommendation

Results were received from 9 analysts in 7 laboratories; *see* Table 1. Although the collaborators were requested to make single determinations, some of them reported several values. The average value of each collaborator was used for the calculation of the overall average and standard deviation.

The results show good reproducibility between laboratories, indicating that the proposed method has good precision. The method is biased to a low result, since the end point at pH 7.0 is a compromise end point rather than the equivalence point. This is necessary because saponification above pH 7.0 proceeds rapidly and yields high results. There are a limited number of results by CIPAC method MT 66 Free Phenoxyalkanoic Acidity for comparison. The problem presented was discussed at the CIPAC Meeting, October 1971, and CIPAC was invited to participate in the study.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was adopted by the Association; *see* (1973) *JAOAC* 56, 393.

Table 1. Collaborative results (%) of the determination of free acid content of 2,4-D and 2,4,5-T esters

	Sample 1: 2,4-D ester		Sample 2: 2,4-D ester		Sample 3: 2,4,5-T ester		Sample 4: 2,4,5-T ester	
Coll.	% Free acid	CIPAC	% Free acid	CIPAC	% Free acid	CIPAC	% Free acid	CIPAC
1	0.20		0.32		0.67		1.34	
2	0.19	0.29	0.38	1.25	0.67	1.97	1.41	3.13
	0.20	0.28	0.38	1.24	0.69	1.98	1.42	3.06
	0.20	0.28	0.40	1.14	0.68	1.98	1.42	3.21
	0.21	0.27	0.40	1.18	0.68	1.97	1.43	3.26
Av.	0.20	0.28	0.39	1.20	0.68	1.975	1.42	3.165
3	0.17	0.47	0.35	1.28	0.60	2.06	1.30	3.31
	0.18	0.45	0.35	1.28	0.58	2.05	1.31	3.33
	0.17	0.46	0.36	1.22	0.62	2.00	1.35	3.23
	0.16	0.46	0.36	1.28	0.61	2.04	1.35	3.28
Av.	0.17	0.46	0.355	1.265	0.602	2.038	1.33	3.288
4	0.19	0.43	0.37	1.35	0.62	2.23	1.52	3.25
	0.19		0.37		0.63		1.53	
Av.	0.19		0.37		0.625		1.525	
5	0.23		0.40		0.68		1.73	
	0.22		0.40		0.68		1.74	
Av.	0.225		0.40		0.68		1.735	
6	0.18		0.44		0.54		1.41	
	0.20		0.35		0.55		1.28	
Av.	0.19		0.395		0.545		1.345	
7	0.22		0.40		0.67		1.64	
	0.23		0.40		0.68		1.65	
Av.	0.225		0.40		0.675		1.645	
8	0.22		0.39		0.56		1.41	
	0.22		0.41		0.58		1.43	
Av.	0.22		0.40		0.57		1.42	
9	0.24		0.37		0.54		1.40	
Overall av.	0.2067		0.3778		0.6208		1.4622	
Std dev.	0.0201		0.0245		0.0555		0.1356	

Based on the results reported it is recommended that this method be adopted as official first action.

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Boyd L. Samuel, Department of Agriculture and Commerce, Richmond, Va.

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DECOMPOSITION IN FOODS (CHEMICAL METHODS)

Collaborative Study of the Determination of Ammonia as an Index of Decomposition in Crabmeat

KURT STEINBRECHER
Food and Drug Administration, 909 First Ave., Seattle, Wash. 98104

A colorimetric method for determining the ammonia content in crabmeat in various stages of decomposition was studied collaboratively. The method involves the color reaction between ammonia, thymol, and alkaline bromine water. The mean values for organoleptic Class 1, Class 2, and Class 3 of decomposition were found to be 281, 568, and 1376 μg NH_3/g crabmeat, respectively. By statistical analysis these means were found to be significantly different from each other, and were found to arise from different populations. Useful cut-off points can be selected to separate the classes. The method has been adopted as official first action.

The determination of a chemical byproduct of decomposition subject to objective analysis has been a sought-after goal to confirm organoleptic classification. In his study, Burnett (1) showed a general correlation between the concentration of ammonia in crabmeat and its degree of decomposition by organoleptic standards. The method is based on the interaction of ammonia, thymol, and alkaline bromine water to give a color dependent on the ammonia concentration. The reaction scheme (Fig. 1) may be analogous to the indophenol mechanism proposed by Bolleter *et al.* (2).

A modification of the method of Fernandez-Flores and Salwin (3) resulted in an 8-fold increase in sensitivity when applied to codfish. A collaborative study was performed with crabmeat and reported at the 83rd Annual Meeting of the AOAC (4). The results indicated that differentiation between Class 1 and Class 2 of decomposition on the basis of ammonia content was feasible. However, revision of the method was required to improve precision and reproducibility.

The method of this collaborative study incorporated several modifications suggested by previous collaborators as well as additional experimentation by the Associate Referee and intralaboratory collaboration.

Collaborative Study

Nine collaborators representing 6 Food and Drug laboratories, 2 State laboratories, and 1 trade association laboratory participated in the study. The samples were prepared in the following manner: Fresh frozen Dungeness crab was allowed to thaw in the shell at room temperature. It was periodically examined by organoleptic analysis, and meat at a particular stage of decomposition was removed from the shell, ground 3 times through a meat grinder with thorough mixing, and frozen for subsequent distribution to collaborators. Collaborators were requested to analyze 6 samples: I. Class 1; II. late Class 1; III. early Class 2; IV. early Class 2; V. early Class 3; VI. early Class 3. The definition of classes conforms to the usage of Hillig *et al.* (5).

METHOD

18.C01 ***Reagents***

(Use NH_3-free H_2O thruout; ordinary distd H_2O is suitable.)

(a) *Bromine soln.*—Dil. 10 ml NaOH soln (1+1), 45.034(b), to ca 100 ml with H_2O, add 1.0 ml Br, shake, and dil. to 200 ml with H_2O. Prep. fresh daily.

(b) *Thymol soln.*—10% in alcohol. Prep. fresh daily.

(c) *Dilute sodium hydroxide soln.*—Dil. 25 ml NaOH soln (1+1), 45.034(b), to 100 ml with H_2O.

(d) *Ammonia std soln.*—40 μg/ml. Dissolve 0.314 g NH_4Cl, previously dried 1 hr at 100°, in H_2O, and

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

dil. to 100 ml. Transfer 4.0 ml to 100 ml vol. flask, and dil. to vol. with H_2O.

18.C02 *Preparation of Samples*

Remove meat from shell, if necessary, and grind 3 times thru food chopper, mixing after each grinding.

18.C03 *Determination*

Place 20 g prepd sample in 500 ml g-s erlenmeyer. Add 180 ml 2.5% *phosphotungstic acid soln*, shake vigorously 2 min, and filter thru Whatman No. 1, or equiv., paper into 250 ml g-s erlenmeyer. Pipet 2 ml filtrate (equiv. to 0.2 g sample) into 125 ml separator. Save remainder of filtrate. To another separator, add 2.0 ml 2.5% phosphotungstic acid soln as blank.

To each separator add 8.0 ml H_2O. Then, in immediate succession, add 1.0 ml dil. NaOH soln, (c), swirl to mix, 2.0 ml thymol soln, (b), swirl to mix, and 5.0 ml Br soln, (a), in ca 30 small addns, swirling vigorously after each addn. Shake vigorously 1 min. With series of samples or stds, complete reagent addns in sequence on each separator before proceeding to next. Let stand $\geq$20 min.

To each separator add 20.0 ml *n*-butyl alcohol and shake vigorously 1 min. Let stand 20 min. Drain aq. layer and pass *n*-butyl alcohol thru ca 30 g anhyd. Na_2SO_4 in glass funnel plugged with glass wool into g-s erlenmeyer. Measure *A* of soln at max. ca 680 nm in 1 cm cell against blank as ref.

If *A* is greater than that of highest NH_3 std, quant. dil. reserved filtrate with 2.5% phosphotungstic acid soln so that 2.0 ml dild soln will produce *A* below this level.

18.C04 *Preparation of Standard Curve*

Pipet 0.0, 1, 2, 3, 4, and 5 ml std NH_3 soln into 125 ml separators. Add 2.0 ml 2.5% phosphotungstic acid soln to each and dil. to 10.0 ml with H_2O. Proceed as in 18.C03, beginning "Then, in immediate succession, add 1.0 ml dil. NaOH soln, . . ." Using 0.0 soln as ref., measure *A* of each std at max. as above. Prep. std curve.

Results and Discussion

As in the previous collaborative study (4), collaborators were asked to report absorbance values only for the standards, and absorbance values and dilutions performed for the samples. The Associate Referee calculated the ammonia content, expressed in μg NH_3/g crabmeat, by deriving the equation of a line from the standard absorbance values by the least squares method and by substituting sample absorbances in the equation derived. Absorbances were essentially

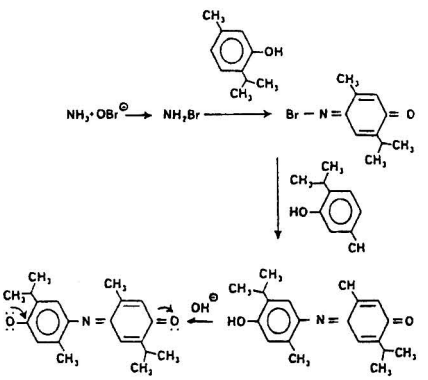

FIG. 1—Reaction scheme for colorimetric determination of ammonia.

linear over the concentration range of the standards.

The results confirm the findings of the previous collaborative study as to the range of ammonia content found in the various classes of decomposition. The data submitted also indicate that the modifications of the method have improved precision and reproducibility.

The 6 samples sent to collaborators, in effect, represented 4 classes of decomposition: one sample Class 1, one sample late Class 1, duplicate samples early Class 2, and duplicate samples early Class 3.

Table 1 lists the amount of ammonia reported in the crabmeat samples in μg NH_3/g crabmeat. On the basis of the 2-tailed ranking test for 9 laboratories and 6 materials (6), results from Collaborators 3 and 6 may be eliminated. The means of ammonia content for the various classes of decomposition with their standard deviations, calculated from the data of the remaining 7 collaborators, are shown in Table 2.

The mean values of ammonia content for the different classes of decomposition may be compared with respect to the significance of their differences from each other by a substitute *t*-test (7).

Table 3 depicts calculated M values for pairs of decomposition classes which are compared, and the values for each pair that must be exceeded to show a significant difference. It may be seen that the M value for the pair Class 1 and late Class 1 (0.192) exceeds the critical value for the 1-tailed test (0.174), indicating that the means for these 2 classes are significantly different from each other. The M value for the 2-tailed test (0.213) is

Table 1. Collaborative results of ammonia content in 6 samples, μg NH_3/g crabmeat

Coll.	Class 1	Late Class 1	Early Class 2	Early Class 2	Early Class 3	Early Class 3
1	248	326	455	428	1323	961
2	276	366	720	679	694	1729
3	108	236	226	236	1007	815
4	188	222	351	452	795	956
5	342	296	503	645	1778	2292
6	143	143	258	182	1014	770
7	264	311	727	570	1578	1628
8	275	314	593	578	1352	1279
9	137	366	619	628	1572	1326

Table 2. Ammonia content of crabmeat, according to its stage of decomposition

Class	Mean, μg NH_3/g	Std dev.
Class 1[a]	247	67
Late Class 1[a]	314	49
Total Class 1[b]	281	66
Early Class 2[b]	568	114
Early Class 3[b]	1376	433

[a] Mean of 7 determinations.
[b] Mean of 14 determinations.

Table 3. Significance of difference between means of classes of decomposition, according to 1-tailed test and 2-tailed test[a]

Classes compared	M values calcd[b]	Critical value 1-tailed test	Critical value 2-tailed test
1 and late 1	0.192	0.174	0.213
Late 1 and early 2	0.489	0.128–0.148	0.155–0.181
1 and early 2	0.474	0.094	0.114
Early 2 and early 3	0.409	0.094	0.114

[a] Critical values obtained from Bauer (7): 1-tailed test, $P_1 = 0.05$; 2-tailed test, $P_2 = 0.05$.
[b] $M = \bar{x} - \bar{y}/R_x + R_y$, where $\bar{x}$ and $\bar{y}$ = means and R_x and R_y = ranges of classes being compared.

Table 4. Range of ammonia content of crabmeat, according to its stage of decomposition

Class	μg NH_3/g Mean ±1 std dev.	Mean ±2 std dev.
Class 1	180–314	113–381
Late Class 1	265–363	216–412
Total Class 1	215–347	149–413
Early Class 2	454–682	340–796
Early Class 3	943–1809	510–2242

not exceeded, however, indicating that both means come from the same population.

For the 3 remaining pair comparisons, both the critical values for the 1-tailed test and for the 2-tailed test are exceeded, indicating both a significant difference between their respective means, and that the means arise from different populations.

Of particular interest is the difference in the means between all of Class 1 (Class 1 and late Class 1) and early Class 2, because here the decision whether crabmeat is actually decomposed must be made. Comparison of these 2 classes shows both a significant difference in means and means arising from different populations.

Table 4 lists the ranges of ammonia content values obtained between ±1 standard deviation (68% of the population) and ±2 standard deviations (95% of the population). The cut-off point between Classes 1 and 2 may be selected, depending on the importance of rejecting Class 2 samples or passing Class 1 samples. For example, if a value of ammonia content of 450 μg/g is selected as a cut-off point, since 413 μg/g is the upper limit of 2 standard deviations for Class 1, more than 97.5% of the population of Class 1 would be passed as undecomposed. Since 454 μg/g is the lower limit of 1 standard deviation for Class 2, about 84% of the population of Class 2 would be rejected as decomposed. If the cut-off point is lowered, rejections of both Class 1 and Class 2 are increased.

When the data of all 9 collaborators are used in computing means and standard deviations, it is found that the means are lowered about 10% and the standard deviations are increased. Nevertheless, application of the substitute *t*-test still shows significant differences between the mean values of ammonia content for the various classes of decomposition and that the means do not come from the same population.

Recommendations

Since the modified methodology of this collaborative study has given results that show statistically significant differences in the ammonia content of crabmeat at various stages of decomposition, it is recommended that this method be adopted as official first action for the determination of ammonia in crabmeat. It is also recommended that the study be continued to further improve precision and reproducibility.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee C and were adopted by the Association; *see* (1973) *JAOAC* 56, 398.

Acknowledgments

The Associate Referee wishes to thank the following collaborators:

D. M. Cosgrove, National Canners Association, Seattle, Wash.

R. Coduri, Food Chemistry Laboratory, Rhode Island Department of Health, Providence, R.I.

W. C. Funderburk, Florida Department of Agriculture and Consumer Services, Tallahassee, Fla.

R. C. L. Chu, L. Feldman, R. H. Johnson, R. C. Mipro, E. C. Netz, and E. Young, Food and Drug Administration, San Francisco, New York, Seattle, New Orleans, Minneapolis, and Atlanta, respectively.

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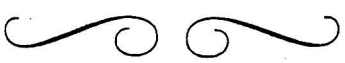

ASSOCIATE REFEREES—Have you conducted a successful collaborative study, and do you want your method adopted by AOAC at the next annual meeting? Please be sure to observe the following points:

1. Submit five copies of your report to the AOAC Business Office for evaluation by the Subcommittee members.
2. IN ADDITION, send one copy of the report direct to your General Referee so that he can prepare his own recommendations in time for consideration by the Subcommittee.
3. Be very specific in your recommendations. If the method you propose for adoption is to replace a method currently in effect, say so, and recommend that the older method be deleted. If your method is to be adopted for alternative use with another official method, state this specifically. If you are recommending that only certain portions of an official method be revised, write out the sections involved so that no misunderstanding can occur.
4. For more specific details, write or call the AOAC Editorial Office.
5. Deadline for 1973 AOAC Associate Referee reports to be received in the AOAC office: August 17, 1973.

FOOD ADDITIVES

Gas Chromatographic Determination of Brominated Sesame Oil in Orange Drinks: Collaborative Study

H. B. S. CONACHER
Research Laboratories, Health Protection Branch, Ottawa, Ontario, Canada K1A OL2

A gas-liquid chromatographic method for the determination of brominated vegetable oils in soft drinks was collaboratively studied, using a commercial orange drink spiked with known amounts of brominated sesame oil. Initial results based on calculations involving total peak area measurement were considered erroneously high, since drink components coeluted with the C_{16} and C_{18} methyl esters, especially at low levels of brominated oil. A modified calculation, based only on brominated ester content, showed recovery values (6 collaborators) of 94.6, 95.3, and 96.0% for drinks containing 3.10, 5.22, and 10.66 mg/10 fluid oz., respectively, with corresponding standard deviation values of 0.40, 0.46, and 0.64. The method with the modified calculation incorporated has been adopted as official first action.

In 1969, a gas-liquid chromatographic (GLC) method was reported for the determination of brominated vegetable oil (BVO) in soft drinks (1). Since then, permitted levels of BVO in Canadian drinks have been lowered from 150 ppm (ca 42 mg/10 fluid oz.) to 15 ppm (ca 4.2 mg/10 fluid oz.). The GLC procedure has been revised and studied collaboratively, using an orange-flavored beverage.

Collaborative Study

Ten fluid oz. bottles of a commercial orange drink containing all normal ingredients except BVO were spiked at 3 levels (A, 3.10; B, 5.22; C, 10.66 mg/10 fluid oz.) with known amounts of brominated sesame oil (BSO) in acetone solution.

Collaborators received 9 bottles of drink, comprising 3 bottles containing no BSO, and 3 test samples in duplicate—A_1, A_2, B_1, B_2, C_1, and C_2. They were also provided with a copy of the method and the following standards: methyl dibromostearate (DBS, about 200 mg), methyl tetrabromostearate (TBS, about 200 mg), methyl pentadecanoate (MPD, about 100 mg), and brominated sesame oil (BSO, about 2 g).

The collaborators were requested to prepare relevant standard solutions and to determine response factors for DBS and TBS, relative to MPD, and also the correction necessary for the overlapping peaks of DBS and TBS; to use these response factors to determine BSO directly at the 5 and 10 mg levels; to determine BSO at the same levels in the presence of drink extract, using 2 of the blanks provided; to determine BSO in 6 unknown samples (3 duplicates). Collaborators were advised to use the entire contents of each bottle for analysis, using the first 100 ml diethyl ether to rinse each bottle prior to extraction. They were also advised to use 1 mg MPD with samples A_1, A_2, B_1, and B_2, and 2 mg with samples C_1 and C_2.

METHOD

Brominated Vegetable Oils

Gas Chromatographic Method—Official First Action

(*Caution: See* 46.011, 46.018, 46.034, 46.039, 46.040, 46.045, 46.054, and 46.066.)

20.C07 *Principle*

Extd brominated oil is methylated and detd by GLC, using internal std.

20.C08 *Apparatus and Reagents*

(a) *Gas chromatograph.*—Varian Model 1740-10, or equiv., with flame ionization detector, strip chart recorder fitted with disk integrator, 3′ × ⅛″ od stainless steel column packed with 3% JXR or SE-30 (Supelco, Inc., Bellefonte, PA 16823) on 80–90 mesh Anakrom ABS. Operating conditions: temps (°)—injector 260, detector 270, column programmed from 150 to 270 at 10°/min; He carrier gas 40 ml/min.

(b) *Solvents.*—Ether, distill before use; anhyd. benzene, distill from Na wire; anhyd. MeOH, prep. as follows: Place 5 g clean, dry Mg turnings and 0.5 g resublimed I in 2 L r-b Pyrex flask fitted with double surface reflux condenser. Add 50–75 ml com. anhyd. MeOH thru condenser and warm mixt. on 100° H_2O bath until I disappears. As H is vigorously evolved, remove flask from H_2O bath. If vigorous evolution of H does not take place, add 0.5 g more I

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and heat mixt. until all Mg is converted to methoxide. Add 900 ml MeOH and boil 30 min under reflux. Distill mixt., with exclusion of moisture, discarding first 25 ml distillate.

(c) *Sodium in anhydrous methanol.*—Clean ca 1 g Na metal in hexane, dry with filter paper, and dissolve in 100 ml anhyd. MeOH in 250 ml conical flask fitted with silica gel drying tube.

(d) *Methyl 9,10-dibromostearate (DBS) std soln.*—2.0 mg/ml. Prep. DBS by dissolving 500 mg Me oleate (Applied Science Laboratories, Inc.) in 15 ml distd ether in 100 ml erlenmeyer held at 0°. Stir magnetically and add 0.15 ml Br dropwise over 20 min. Stir addnl 10 min at 0°. Transfer soln with addnl 30 ml ether to 250 ml separator contg 50 ml 10% aq. $Na_2S_2O_5$, shake, let layers sep., and discard aq. layer. Wash ether layer successively with two 10 ml portions 2% aq. $Na_2S_2O_3$ and two 10 ml portions H_2O. Dry ether layer over anhyd. Na_2SO_4 and evap. on rotary evaporator at 40°.

Dissolve 200 mg DBS in anhyd. benzene in 100 ml vol. flask and dil. to vol. with anhyd. benzene.

(e) *Methyl 9,10,12,13-tetrabromostearate (TBS) std soln.*—2.0 mg/ml. Prep. TBS as in (d), except use Me linoleate (Applied Science Laboratories, Inc.) and 0.30 ml Br. Prep. std soln as in (d).

(f) *Brominated vegetable oil (BVO) std soln.*—1.0 mg/ml. Dissolve 50 mg BVO (Abbott Laboratories, 14th St & Sheridan Rd, N. Chicago, IL 60064) in acetone in 50 ml vol. flask and dil. to vol. with acetone.

(g) *Methyl pentadecanoate (MPD) internal std soln.*—1.0 mg/ml. Dissolve 100 mg MPD (Applied Science Laboratories, Inc.) in anhyd. benzene in 100 ml vol. flask and dil. to vol. with anhyd. benzene.

20.C09 *Preparation of Standards*

(a) *Methyl 9,10-dibromostearate and methyl 9,10,12,13-tetrabromostearate std solns.*—Pipet 3, 5, and 10 ml (6, 10, and 20 mg) DBS std solns into 3 sep. dry conical flasks and add 3, 5, and 10 ml (3, 5, and 10 mg) MPD std soln to each. Similarly prep. TBS-MPD solns. Treat each soln as follows: Evap. solv. with N at 40°. Add 25 ml 1% Na in MeOH and 12 ml anhyd. benzene, and reflux 1 hr. Cool, and transfer to 125 ml separator contg 50 ml H_2O. Acidify with 2*N* H_2SO_4 and ext with three 30 ml portions ether, using first 30 ml to rinse flask. Combine ether exts in second separator, wash with two 10 ml portions H_2O, dry over anhyd. Na_2SO_4, filter, and evap. solv. on rotary evaporator at 40°. Dissolve residue in 3 ml ether.

(b) *Brominated vegetable oil.*—Pipet 5 and 10 ml BVO std soln (5 and 10 mg) into sep. dry conical flasks, add 1 and 2 ml MPD std soln (1 and 2 mg), resp., and evap. solv. with N at 40°. Proceed as in (a), beginning "Add 25 ml 1% Na in MeOH . . ."

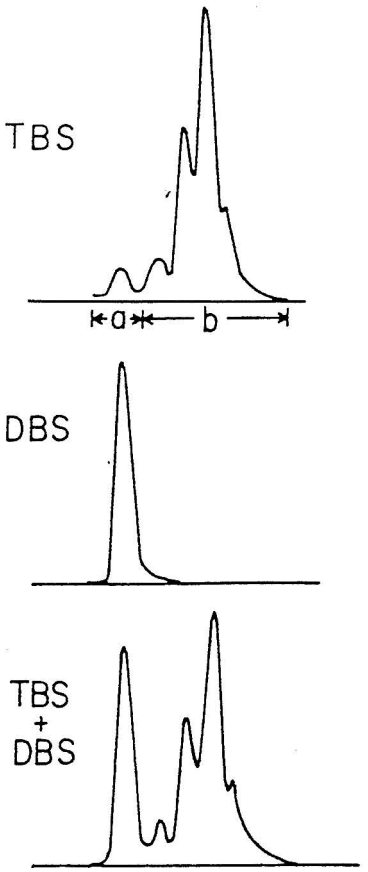

FIG. 20:C1—Gas chromatograms of TBS, DBS, and TBS + DBS.

20.C10 *Preparation of Sample*

Decarbonate and mix by transferring several times from 1 beaker to another. Transfer 280 ml sample to 500 ml separator, thoroly sat. with NaCl, and add 1 ml MPD std soln (1 mg). Ext with three 100 ml portions ether. Combine exts in second separator and wash successively with 50 ml 2*N* NaOH, 50 ml 2*N* HCl, and two 25 ml portions H_2O. Dry ether layer over anhyd. Na_2SO_4, filter, and evap. on rotary evaporator at 40°. Quant. transfer residue with ether to clean, dry conical flask, and evap. ether with N at 40°. Proceed as in 20.C09(a), beginning "Add 25 ml 1% Na in MeOH . . ."

20.C11 *Determination*

Inject duplicate portions methylated DBS, TBS, BVO std, and sample solns. Det. peak areas (*PA*)

with disk integrator. Correct apparent areas of both TBS and DBS for area of common peak *a* (Fig. 20:C1) as follows: Assume PA_a in TBS chromatogram is 5% of PA_b and apparent PA_{DBS} and PA_{TBS} are 100 and 200 units, resp.

True DBS PA = 100 − 1/20 (200) = 90 units

True TBS PA = 200 + 1/20 (200) = 210 units

Det. av. response factors (RF) for each soln.

$$RF_{DBS\ or\ TBS} = (\text{mg DBS or TBS} \times PA_{MPD})/(\text{mg MPD} \times \text{true } PA_{DBS\ or\ TBS})$$

(a) *Brominated vegetable oil (no drink) calculated from total peak areas.*—(Use for checking response factors, recoveries, and B, required for (b).)

$$\text{mg BVO} = (|PA_{16} + PA_{18} + (\text{true } PA \times RF)_{DBS} + (\text{true } PA \times RF)_{TBS}| \times \text{mg MPD})/PA_{MPD}$$

where PA_{16} and PA_{18} = areas of C_{16} and C_{18} Me esters, resp.

(b) *Brominated vegetable oil in drink calculated from brominated derivatives only.*—

$$\text{mg BVO} = |(\text{true } PA \times RF)_{DBS} + (\text{true } PA \times RF)_{TBS} \times \text{mg MPD}|/(PA_{MPD} \times B)$$

where B = % (DBS + TBS) in BVO found by direct analysis (a).

Results and Discussion

Of the 12 collaborators asked to participate in the study, 7 reported results. One set of results was eliminated because of the extreme variability, probably because this collaborator did not have a disk integrator and measured peak areas manually. Of the 5 collaborators who did not complete the study, 3 experienced instrumental problems and one had no disk integrator.

The response factors obtained by collaborators for the DBS and TBS derivatives are summarized in Table 1. Variations from laboratory to laboratory are probably due to different GLC instruments and slight differences in operating conditions. The Associate Referee has also experienced some variation when 2 different instruments were used (cf. (1) and (2)).

Table 1. Response factors[a] for methyl dibromostearate and tetrabromostearate

Coll.	RDBS	RTBS
1	1.72	2.25
2	1.56	1.99
3	1.51	2.30
6	1.82	2.08
9	1.51	1.95
12	1.57	2.16

[a] RDBS and RTBS are weight response factors for methyl dibromostearate and methyl tetrabromostearate, respectively, relative to methyl pentadecanoate.

Table 2. Recoveries of brominated sesame oil (BSO) from drinks spiked by collaborators

	Direct analysis[a]			From drinks		
Coll.	Added, mg	Rec., mg[b]	Rec., %	Added, mg	Rec., mg[b]	Rec., %
1	4.75	4.86	102.5	4.80	5.03	104.8
	9.50	9.91	104.3	9.50	9.04	95.2
2	4.99	5.15	103.2	4.99	5.42	108.6
	9.99	9.89	99.0	9.99	10.41	104.2
3	4.90	5.15	105.1	4.90	4.50	91.9
	9.80	9.66	98.6	9.80	9.21	94.0
6	5.15	5.09	98.7	5.15	5.54	107.5
	10.30	10.11	97.9	10.30	10.60	102.9
9	5.17	4.97	96.2	5.17	5.06	97.8
	10.34	10.23	98.9	10.34	9.78	94.6
12	5.22	5.14	98.5	5.22	5.32	101.8
	10.66	10.30	96.6	10.66	10.80	101.3

[a] BSO weighed directly by collaborators—no drink used.

[b] Each figure represents the average of duplicate GLC determinations.

Table 3. Collaborative results for determination of brominated sesame oil in test samples[a, b]

	Rec., mg		Rec. mg		Rec., mg	
Coll.	A_1	A_2	B_1	B_2	C_1	C_2
1	2.98	2.98	4.99	5.09	10.85	11.15
2	3.04	3.20	5.13	5.27	9.59	9.72
3	3.45	3.31	5.44	5.57	10.20	10.66
6	3.29	3.20	5.32	5.34	10.40	10.80
9	2.94	3.20	5.00	4.76	9.77	9.79
12	3.10	3.30	5.45	5.50	10.60	11.00
Mean	3.17		5.24		10.39	
Range:						
high	3.45		5.57		11.15	
low	2.94		4.76		9.59	
Std dev.[c]	0.14		0.25		0.55	
Coeff. of var., %	4.42		4.77		5.29	
Av. rec., %	102.2		100.4		97.5	

[a] Calculations based on total peak area measurement.

[b] Samples analyzed in duplicate A_1, A_2 (3.10 mg BSO); B_1, B_2 (5.22 mg); C_1, C_2 (10.66 mg).

[c] Average values used.

Satisfactory collaborative results were obtained for the determination of BSO without drink and also in the presence of drink extract (Table 2). In the direct determination, average recoveries were 100.7 and 99.2% at the 5 and 10 mg levels, respectively, and 102.1 and 98.7% at corresponding levels of BSO in drinks spiked by collaborators.

Table 4. Composition of brominated sesame oil found by collaborators in test samples

Coll.	Sample	Fatty acid composition, %[a] C_{16}	C_{18}	DBS	TBS
1	BSO	6.7	5.7	47.7	40.0
	A	12.6	16.8	38.6	32.0
	B	10.8	12.4	43.9	32.9
	C	7.6	8.9	48.3	35.2
2	BSO	6.4	5.0	44.7	43.9
	A	11.9	14.4	38.3	35.4
	B	8.4	9.6	40.0	42.0
	C	7.3	7.3	43.9	41.5
3	BSO	6.7	4.6	43.6	45.1
	A	4.7	3.1	44.4	47.8
	B	6.3	4.4	42.8	46.7
	C	5.0	1.7	46.3	47.0
6	BSO	6.6	3.9	49.8	39.7
	A	8.2	4.3	47.4	40.1
	B	7.2	4.1	48.6	40.1
	C	6.9	4.1	48.7	40.3
9	BSO	6.2	5.1	45.8	42.9
	A	6.1	9.7	42.7	41.5
	B	6.5	8.0	43.9	41.6
	C	6.8	6.2	44.9	42.1
12	BSO	5.4	4.2	47.7	42.7
	A	7.1	8.0	44.0	40.5
	B	6.4	7.0	45.1	41.5
	C	5.7	5.8	45.0	43.5

[a] Average compositions from direct determination of BSO and from duplicate determinations A_1, A_2, etc.

Table 5. Collaborative results for determination of brominated sesame oil in test samples[a, b]

Coll.	Rec., mg A_1	A_2	Rec., mg B_1	B_2	Rec., mg C_1	C_2
1	2.43	2.43	4.37	4.48	10.27	10.68
2	2.55	2.55	4.80	4.47	9.22	9.35
3	3.54	3.45	5.36	5.69	10.70	11.00
6	3.20	3.19	5.30	5.37	10.45	10.60
9	2.80	3.02	4.80	4.47	9.57	9.58
12	2.98	3.06	5.45	5.13	10.50	10.80
Mean	2.93		4.98		10.23	
Range: high	3.54		5.69		11.00	
low	2.43		4.37		9.22	
Std dev.[c]	0.40		0.46		0.64	
Coeff. of var., %	13.5		9.2		6.3	
Av. rec., %	94.6		95.3		96.0	

[a] Based on brominated ester determination only.
[b] Samples analyzed in duplicate A_1, A_2 (3.10 mg BSO); B_1, B_2 (5.22 mg); C_1, C_2 (10.66 mg).
[c] Average values used.

Results for BSO determination in test samples are presented in Table 3. On first inspection these results appear satisfactory at all 3 levels with average recoveries of 102.2, 100.4, and 97.5% at the 3, 5, and 10 mg levels, respectively. However, comparison of the true fatty acid composition of BSO with that found by some collaborators in test samples A, B, and C (Table 4) indicates that some results may be erroneously high (Table 3). For example, both Collaborators 1 and 2 reported total (C_{16}+C_{18}) values about 12% in the direct determination of BSO whereas in test sample A, total (C_{16}+C_{18}) contents were approximately 25–30%. Similarly, with test sample B, the total (C_{16}+C_{18}) values were approximately 18–23%, whereas in Sample C, the total amounts (15–17%) were closer to the true values. These results would appear due to an incomplete extraction of BSO accompanied by an enhancement in peak areas of C_{16} and C_{18} by some drink components—the latter effect being more pronounced at low BSO levels. Similar, although smaller, enhancements of C_{16} and C_{18} percentages were apparent in the A test samples of most other collaborators.

Subsequent analyses of several blank orange drinks in the Associate Referee's laboratory revealed components eluting with retention times similar to those of C_{16} and C_{18}. The content of these components was variable and ranged from approximately 0.1 to 0.4 mg/10 fluid oz.

Accordingly, the Associate Referee felt that more meaningful recovery data on the test samples would be obtained by a calculation based only on the total (DBS+TBS) content of BSO (20.C11(b)). This method of calculation has been used in the Associate Referee's laboratory for the analysis of several commercial samples of citrus-based drinks which appear to contain nonbrominated vegetable oils.

The collaborative results obtained by such calculations are given in Table 5. Recoveries of BSO were 94.6, 95.3, and 96.0% for 3.10, 5.22, and 10.66 mg/10 fluid oz. levels, respectively, and according to the ranking test (3), all collaborative results (average values) were within allowable score limits.

Components of variation for duplicate samples are presented in Table 6.

Recommendations

It is recommended that the GLC method for the determination of brominated vegetable oils in soft drinks, using calculations based on total

Table 6. Within- and between-laboratory components of variance (S^2) and coefficient of variation for test samples[a]

BSO added, mg	Mean found, mg	Total		Within lab.		Between lab.	
		S_d^2	Coeff. of var., %	S_r^2	Coeff. of var., %	S_b^2	Coeff. of var., %
3.10	2.93	0.158	13.5	0.004	2.1	0.154	13.4
5.22	4.98	0.212	9.2	0.037	3.9	0.175	8.4
10.66	10.23	0.406	6.3	0.035	1.6	0.371	3.6

[a] $S_r^2 = \Sigma(X - \bar{X})^2/2n$
$S_b^2 = S_d^2 - S_r^2$
$S_d^2 = S_b^2 + S_r^2$.

brominated ester content, be adopted as official first action.

It is also recommended that a further corroborative technique, possibly based on bromide determination, be developed and used in conjunction with the GLC technique.

Acknowledgments

The Associate Referee is indebted to R. K. Chadha for invaluable technical assistance, and to Coca-Cola (Canada) Ltd. for providing the orange drink used in the study. The participation of the directors and collaborating personnel at the following laboratories is gratefully acknowledged:

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R. Kallies, Health Protection Branch Research Laboratory, Ottawa, Ontario, Canada

D. Larry, Food and Drug Administration, Washington, D.C.

J. Lee, Health Protection Branch Regional Laboratory, Toronto, Ontario, Canada

R. K. Munns, Food and Drug Administration, Denver, Colo.

W. S. Schwien, Food and Drug Administration, Kansas City, Mo.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee C and were adopted by the Association; *see* (1973) *JAOAC* 56, 399.

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(2) Conacher, H. B. S., Meranger, J. C., & Leroux, J. (1970) *JAOAC* 53, 571–575

(3) Youden, W. J. (1967) *"Statistical Techniques for Collaborative Tests,"* AOAC, Washington, D.C.

Corrected Fluorescence Spectra of Polynuclear Materials and Their Principal Applications

THOMAS J. PORRO, ROBERT E. ANACREON, PAUL S. FLANDREAU, and IRVING S. FAGERSON*

The Perkin-Elmer Corp., Main Ave., Norwalk, Conn. 06852

* *Department of Food Technology, University of Massachusetts, Amherst, Mass. 01002*

Corrected fluorescence spectra are presented for 20 polynuclear aromatic compounds. The applications of corrected spectra, as well as instrumentation and techniques for providing these spectra, are described.

Luminescence spectroscopy has made great strides over the last number of years in the solution of chemical problems, primarily because of its fundamental properties of sensitivity and selectivity.

This progress has been aided by the availability of instrumentation capable of precision and stability approaching those of ultraviolet (UV) and visible absorption spectrophotometers. Much of the utility of fluorescence measurements has been in the quantitative analysis of trace components of mixtures in the 10^{-6}–10^{-9} g range, which is usually below the threshold level of sensitivity of UV absorption measurements. Fluorescence spectroscopy has also been an invaluable adjunct in the elucidation of biochemical reactions at the molecular level, particularly because of its greater sensitivity compared with UV and also because of the direct excited state information it provides.

The majority of fluorescence spectrophotometers in use do not provide accurate or "true" fluorescence spectra. Although the instruments are precise and stable, they still record spectra, for most part, which reflect the non-constant output of the excitation source vs. wavelength and the variation in spectral response of the photomultiplier.

The need for obtaining true fluorescence excitation and emission spectra and methods for correcting spectra were described in the literature as early as 1957 by Weber and Teale (1) and in 1958 by Parker (2). Several instruments have been constructed to record fluorescence spectra directly, either in energy units or in units of quanta/unit spectral bandwidth. The latter units are more useful for physical interpretation of molecular behavior and for determination of quantum efficiency.

The purpose of this paper is to (*1*) identify and illustrate a number of the principal uses of "corrected" or "true" fluorescence spectral data, (*2*) describe available equipment that provides these data conveniently and accurately, and (*3*) provide a compilation of corrected fluorescence spectra of 20 polynuclear aromatic compounds of direct interest to workers in the fields of air pollution and food and agricultural sciences.

Application of Corrected Fluorescence Spectra

The primary advantages of directly obtaining correct excitation and emission spectral data, in units of relative quanta, are the following:

(*1*) Apparent shifts in excitation and emission wavelength peaks, distortions in bands and shapes, and changes in relative band intensities introduced by the detector and xenon source are eliminated by correcting the spectra.

(*2*) Interfering narrow lines of the xenon source which occur in many uncorrected excitation spectra are cancelled in the corrected spectra.

(*3*) The "corrected" excitation spectrum can be used in place of the absorbance spectrum for identification of unknown materials at concentration levels far below minimum detectable levels of absorption.

(*4*) The "corrected" emission spectrum is required in the determination of fluorescence quantum efficiencies of pure materials. The quantum efficiency of a material is a measure of its ability to fluoresce and this is useful in predicting its minimum detectable concentration.

(*5*) The dissimilarity between an absorbance vs. an excitation spectrum, or the non-constancy of the quantum efficiency of a substance with wavelength, can be an indication of one or a number of the following: (*a*) the presence of an impurity or impurities, (*b*) molecular association

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(dimers, trimers, etc.), (*c*) tautomerism, and (*d*) the transfer of electronic energy among molecules.

(*6*) The routine availability of "correct," "true," or "accurate" fluorescence spectra data in relative quantum units is not only required to elucidate chemical structure and reactions but is important to good fluorescence analysis for the same reasons that accuracy is important to UV absorption analysis. In other words, "true" fluorescence spectral data are not only useful to solve an immediate problem but can be exchanged from instrument to instrument and laboratory to laboratory. The compilation of such information is extremely useful for many analytical problems, which will reduce the need to continually rerun spectra. In a recent review by Sawicki (3), the need for true excitation spectra for identification of polynuclear compounds in air pollution research was identified.

Experimental

Materials and Methods

Polycyclic aromatics were obtained commercially from sources indicated in Table 1. Although no attempts were made to purify the samples, an assessment of purity was made by thin layer chromatography as well as by correlation of the excitation and absorption spectra of each sample.

Two systems, as described by Halaby and Fagerson (4), were used for thin layer chromatography. The compounds were screened for impurities; those compounds were rejected that showed more than a faint fluorescence for spots other than the main compound.

Luminescence spectra were obtained for the compounds retained: Unless correlations were observed between the corrected excitation and absorption spectra, the compound was rejected.

Solutions of the compounds retained were prepared in redistilled ACS grade methanol (w/v) and serially diluted with redistilled methanol or with commercially available absolute ethanol to the appropriate concentration.

Luminescence and absorption spectra were obtained from solutions in quartz cells of 10 mm pathlength.

Instrumentation

Figure 1 is a block diagram of an excitation and emission correction system as an accessory to the Perkin-Elmer Models MPF-2A and MPF-3 fluorescence spectrophotometers. The excitation spectrum is corrected by recording the ratio of the sample detector output signal to that of a reference detector which monitors the emission of the quantum counter, Rhodamine B, which has the property of maintaining a constant ratio of quanta absorbed from 200 to 600 nm to quanta emitted at 630 nm (5). After the beam exits from the first monochromator it strikes a lens, and then a beam splitter which reflects a small percentage of the light to a triangular cell filled with the quantum counter. The detected signal is fed to the reference amplifier. The sample fluorescence is directed through the emission monochromator and then to the sample photomultiplier. The amplified sample signal is electronically ratioed to the reference signal and recorded. A 25-tap preprogrammed potentiometer is coupled to the wavelength drive of the excitation monochromator to alter the sample amplifier gain to compensate for the non-constant reflectance of the beam splitter.

The emission correction produces a flat response curve for the detector output by preadjustment of another 25-tap potentiometer coupled to the emission monochromator between 300 and 600 nm.

Figure 2 shows the spectral output of the xenon

Table 1. Source of polycyclic aromatic compounds

Compound	Fig. No.	Source
Anthanthrene	10	K&K Laboratories, Plainview, N.Y.
Anthracene	11	Aldrich Chemical Co., Milwaukee, Wis.
3,4:9,10-Dibenzprene	12	K&K
3,4:8,9-Dibenzprene	13	K&K
Benz(a)pyrene	14	K&K
1,2:3,4-Dibenzprene	15	K&K
1,2:5,6-Dibenzacridine	16	Aldrich
m-Quaterphenyl	17	Aldrich
Iminodibenzyl	18	Aldrich
5,6-Benzoquinoline	19	Aldrich
p,p-Bitolyl	20	K&K
Coronene	21	Aldrich
1,2:5,6-Dibenzanthracene	22	Pfaltz & Bauer, Flushing, N.Y.
9-Vinyl anthracene	23	Aldrich
Azulene	24	K&K
2,3-Benzofluorene	25	K&K
1 12-Benzoperylene	26	Aldrich
Pyrene	27	Koch-Light, Colnbrook, Bucks, England
Chrysene	28	Aldrich
Fluoranthene	29	Koch-Light

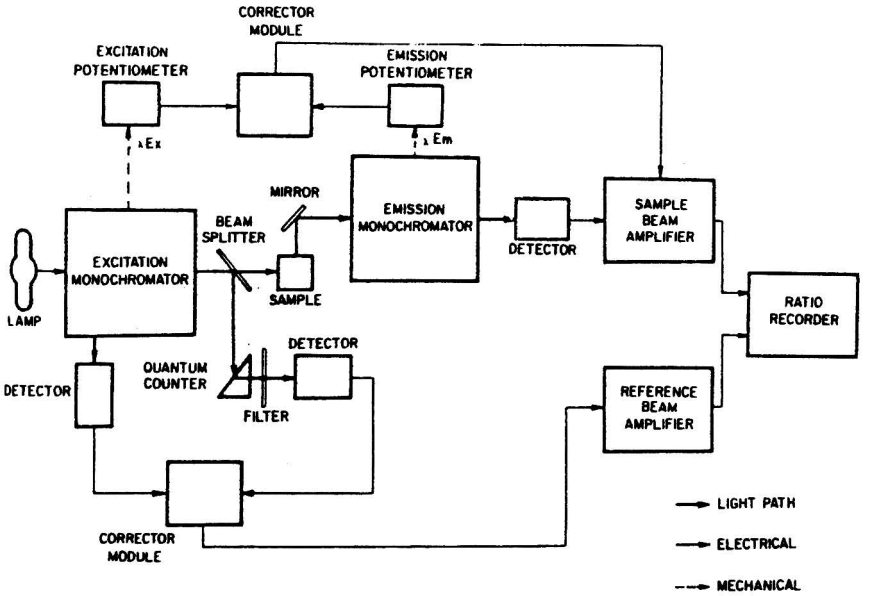


FIG. 1—Block diagram: corrected excitation and emission system.

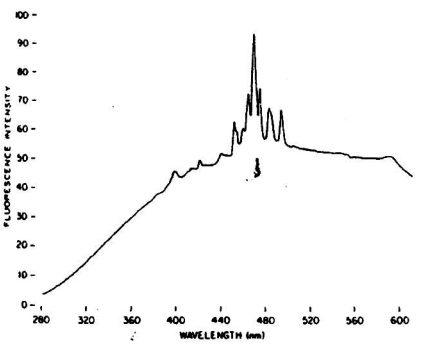


FIG. 2—Spectral energy output—xenon source.

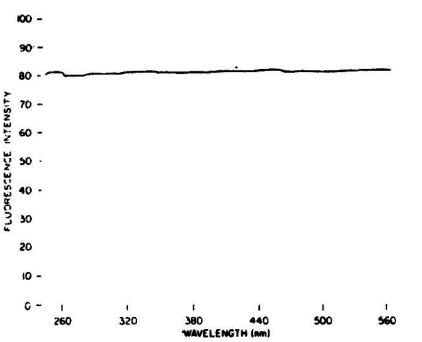


FIG. 3—Compensation for source, Rhodamine B vs. Rhodamine B.

source which is the main parameter that the correction system compensates for in obtaining corrected excitation spectra. Note the sharp lines around the peak emission.

When the excitation system is properly readjusted, the excitation spectrum of a sample of Rhodamine B is run against the Rhodamine B reference cell. A plot, as shown in Fig. 3, is flat to within ±5%.

The main parameter to be compensated for in obtaining corrected emission spectra is the nonflat detector response, shown for a R-136 type cathode in Fig. 4.

One method of preadjustment of the emission system utilizes an externally mounted NBS-calibrated tungsten lamp whose output is measured directly through the emission monochromator. Figure 5 shows the uncorrected output of the

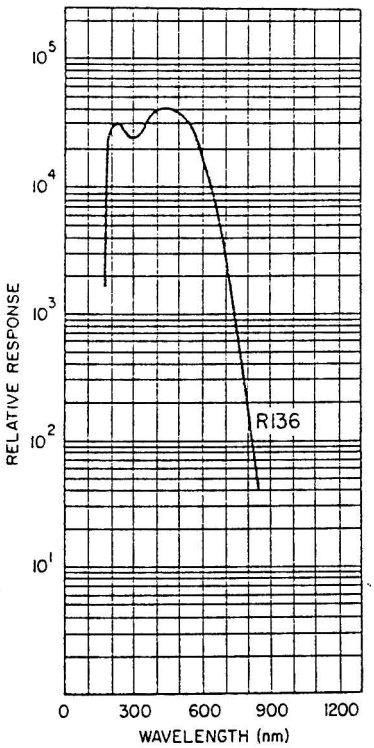


FIG. 4—Detector response, R-136 photocathode.

lamp, which is adjusted to match the calibrated values by using the 25-tap compensator. When the instrument is adjusted one can routinely obtain corrected or uncorrected emission and excitation spectra data.

Figure 6 shows the corrected and uncorrected excitation spectra of an ethanol solution of anthracene (6). Note the large change in relative band intensities and the shift of the wavelength maximum of the 250 nm band. Figure 7, showing the corrected vs. uncorrected excitation spectrum of a fluorescence dye used in the antibody-antigen analytical technique, illustrates quite well the ability of the correction system to eliminate the sharp xenon lines (labeled with asterisks) which would otherwise be impossible to remove by filtering alone.

Figure 8 shows the corrected and uncorrected emission spectra of a phosphor powder. It illustrates very dramatically the error of about 13 nm in the wavelength of the emission maximum that

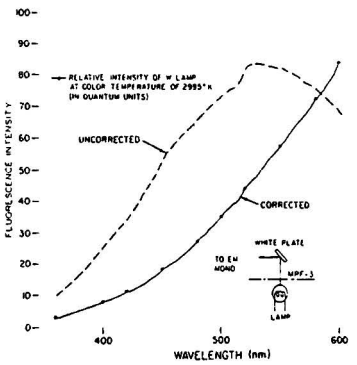


FIG. 5—Uncorrected spectral output of tungsten lamp.

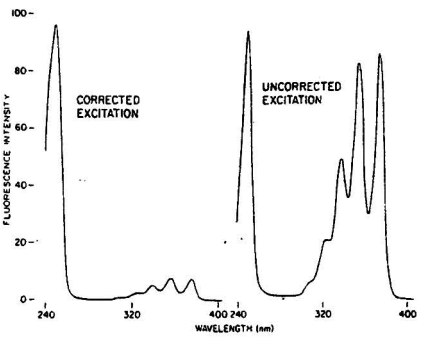


FIG. 6—Anthracene in ethanol, corrected vs. uncorrected excitation spectra.

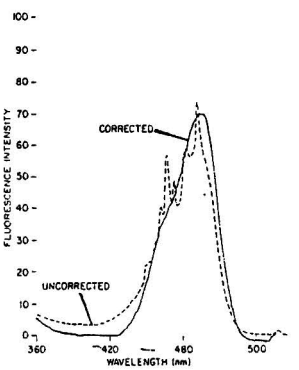


FIG. 7—Antibody dye, corrected vs. uncorrected excitation spectra.

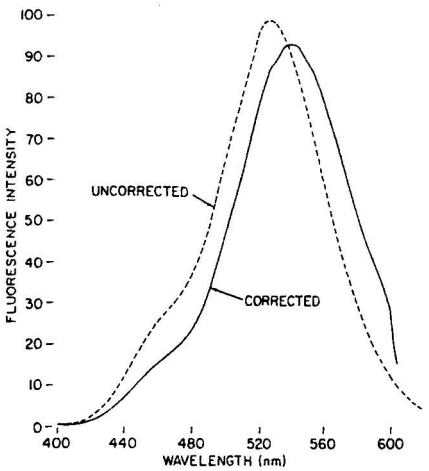


FIG. 8—Phosphor powder, corrected vs. uncorrected emission spectra.

is incurred in this portion of the spectrum if the spectrum is not corrected for the photomultiplier response.

The ratio of light emitted to that absorbed by a molecule is called its quantum efficiency, a measure of its ability to fluoresce. The determination of quantum efficiencies is important because they are measures of a fundamental constant of a fluorescing compound. For the most part these measurements have been very tedious and require special equipment. If the integrated areas of the corrected emission spectra of an unknown and also of a standard material (we used an electronic integrator) are measured under the same instrument conditions and if the absorbance of each is determined at the wavelengths of excitation, the efficiencies of materials can be determined efficiently and accurately (7).

$$\phi_2 = \phi_1 \times (\text{area}_2/\text{area}_1) \times (1 - c - 2.3A_1)/(1 - Xe - 2.3A_2) \times (E_1/E_2) \quad (1)$$

Equation (*1*) was used to calculate the quantum efficiency of a substance (ϕ_2) relative to a given standard (ϕ_1); the integrated areas under the corrected emission curves are area_2 and area_1, respectively. The absorbances of the unknown and standard solutions measured at the corresponding wavelength of excitation are A_2 and A_1, while the relative energies of excitation of the standard and unknown are given by E_1 and E_2. The E values cancel out if both the standard and the unknown solutions are excited at the same wavelength.

Table 2 summarizes the relative quantum efficiencies determined in this way, using a solution of quinine sulfate as a standard ($\phi_1 = 0.55$) and calculating the relative quantum efficiency for anthracene in ethanol. Both solutions were excited at 250 nm so that $E_1/E_2 = 1$ in the formula. The calculated value of 0.31 agrees closely with the value of 0.30 reported by Parker (8). A minor correction factor should be applied for the difference in refractive index between ethanol and 0.1*N* H_2SO_4.

Similarly the relative quantum efficiency of perylene in ethanol was calculated, using anthracene in ethanol as a reference. These data are summarized in Table 3. The calculated value of 0.82 agrees within 6% with the published value of 0.87.

In the measurement of relative quantum efficiencies, the bandwidth of the excitation mono-

Table 2. Relative quantum efficiency of anthracene vs. quinine sulfate solutions

Compd	Solv.	λ_{ex}, nm	Absorbance	Area units	ϕ^a
Anthracene, 0.25 μg/ml	Ethanol	350	0.015	3620	0.31 (calcd)
Quinine sulfate, 1 μg/ml	0.1*N* H_2SO_4	350	0.010	4946	0.55 (known)

[a] See equation (*1*) in text.

Table 3. Relative quantum efficiency of perylene vs. anthracene solutions

Compd	Solv.	λ_{ex}, nm	Absorbance	Area units	ϕ^a
Perylene, 0.10 μg/ml	Ethanol	250	0.031	22,466	0.82
Perylene (8)	Ethanol	360			0.87
Anthracene, 0.08 μg/ml	Ethanol	250	0.090	23,283	0.30 (8) (known)

[a] See equation (*1*) in text.

chromator must be the same as that for the absorption spectrophotometer which was used for the absorbance measurement.

Another example of the utility of corrected excitation spectral data is the ability to detect chemical effects, such as the presence of impurities, molecular associations, and energy transfer. In Fig. 9 the excitation and absorbance spectra of a naphthalene solution in cyclohexane are compared. The lack of correspondence of the 2 spectra, particularly at 288 nm, is a direct indication of an impurity which, in this case, we know to be the presence of a small amount of naphthacene. Since the impurity level is low, it is not reflected in the absorbance spectrum.

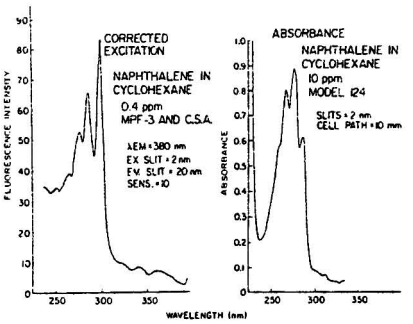


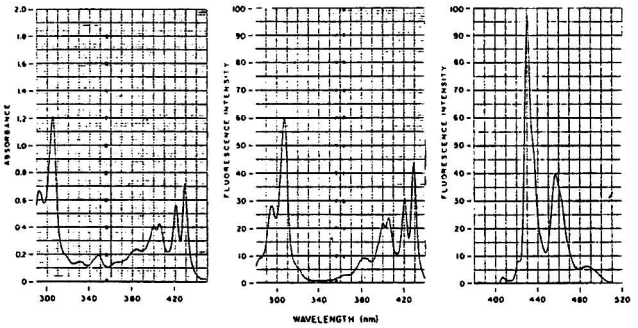
FIG. 9—Naphthalene in cyclohexane, excitation and absorbance spectra.

Probably the single most important use of corrected excitation data is in place of an absorbance spectrum when solution concentrations are below absorption thresholds. For a pure compound having a single fluorescing group, the corrected fluorescence excitation spectrum of a dilute solution should be identical to its absorbance spectrum. Since many compounds fulfill this condition, the ability to generate easily and accurately corrected excitation spectra will be extremely useful to those requiring an accurate UV absorption spectrum for comparison with the large compilation of reference spectra for qualitative identification of an unknown substance. In addition, it is always important to be able to compare spectral data in a quantitatively accurate way, not only from instrument to instrument, but also within the same instrument over a period of time. This capability has been generally available with UV absorption spectrophotometers for many years, but only recently has standard equipment been introduced that produces corrected spectral data on a routine and convenient basis.

Since we had on hand a number of aromatic polynuclear compounds relating to the fields of food and agricultural science, as well as air pollution, we felt that it would be useful to compile their corrected fluorescence spectra to be used as reference data and to correlate the corrected excitation spectrum of each compound with its absorbance spectrum. From a choice of over 50 compounds, we were able to meet the above conditions on 20 whose spectral data are shown in Figs. 10 through 29. The data from the remaining compounds were not included, primarily because a good correlation between excitation and absorption could not be made, thereby causing their purity to be suspect and the quality of the fluorescence data to be questionable. Although the purity of the compounds studied was known to be high, we are including the supplier (Table 1), since the materials were not purified further. Also shown in each spectral illustration is the linear absorbance spectrum of each compound, using the same spectral band-pass as for the corrected excitation spectrum. (The fluorescence spectra were obtained on a Perkin-Elmer Model MPF-3 fluorescence spectrophotometer with a corrected spectra accessory. The absorbance spectra were obtained on a Perkin-Elmer Model 124 absorption spectrophotometer.)

To compare the corrected excitation spectra with the linear absorbance spectra, the ratio of peak intensity of one band in the corrected excitation spectrum is compared to the intensity of the corresponding peak at the same wavelength in the linear absorbance spectrum. Several ratios were taken for different bands in each compound. If the correspondence to absorbance is true, then the ratio should be the same for each set of peaks. The average ratio for all the corresponding peaks was calculated and the standard deviation of these averages was then determined. The error in the measurement of true excitation spectra compared to the absorbance spectra was 5% or less. The coefficient of variation, (standard deviation/average value) × 100, was used as the measure of the accuracy of the correlation.

Comparison of spectra of 1,2-benzanthracene in ethanol shows good correlation (Fig. 30). In Fig. 31, the excitation spectrum of 1,2-benzanthracene (2) is compared with one obtained on our equipment; the corresponding ratios of the 5 bands at 255, 268, 278, 288, and 342 correlate to ±4%.



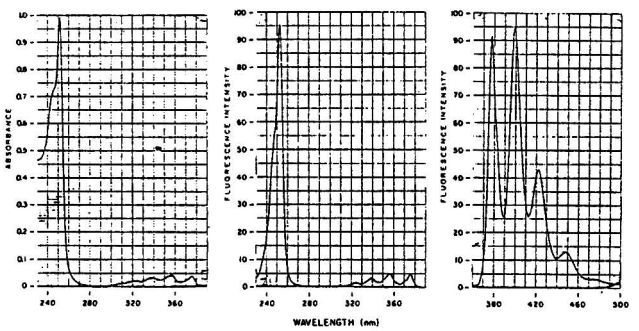
ANTHANTHRENE

ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 458 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.02 μg/ml

CORR, EMISSION
λ Ex 306 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.02 μg/ml

FIG. 10



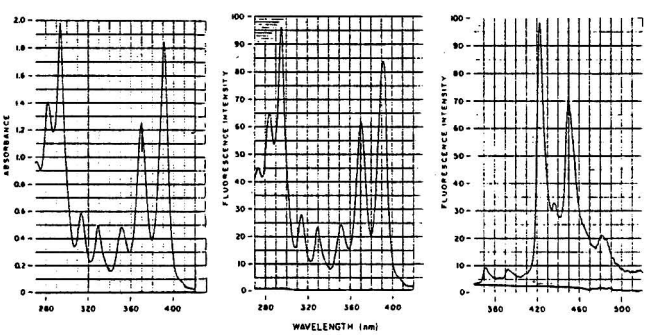
ANTHRACENE

ABSORBANCE
Slit 2 nm
16 μg/ml
Ethanol

CORR. EXCITATION
λ Em 400 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.08 μg/ml
Ethanol

CORR. EMISSION
λ Ex 250 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.08 μg/ml
Ethanol

FIG. 11



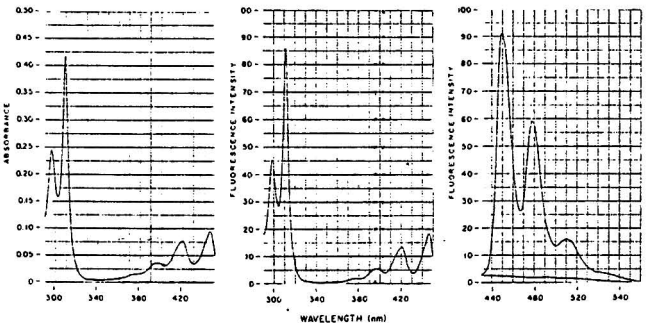
3,4-9,10 DIBENZPYRENE

ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 430 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.02 μg/ml

CORR. EMISSION
λ Ex 294 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.02 μg/ml

FIG. 12



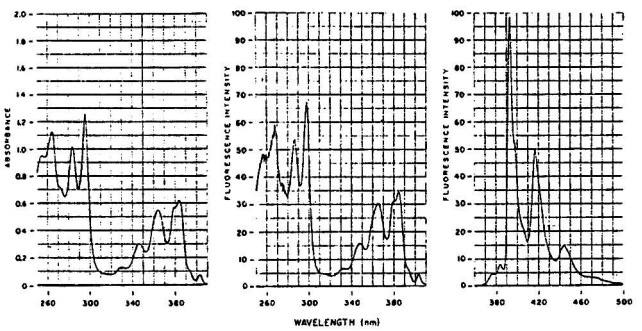
3,4:8,9 DIBENZPYRENE

ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 480 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.02 μg/ml

CORR. EMISSION
λ Ex 309.5 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.02 μg/ml

FIG. 13



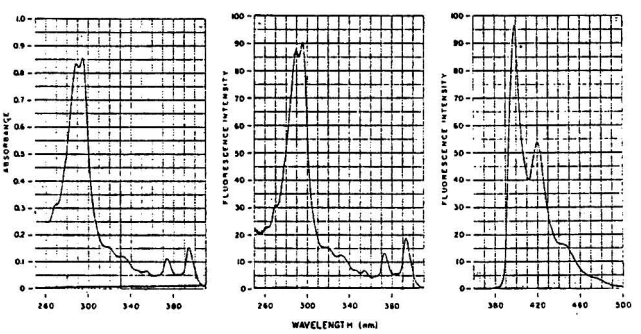
BENZ[a]PYRENE

ABSORBANCE
Slit 2 nm
Conc 9 μg/ml
Ethanol

CORR. EXCITATION
λ Em 420 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.36 μg/ml
Ethanol

CORR. EMISSION
λ Ex 296 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.36 μg/ml
Ethanol

FIG. 14



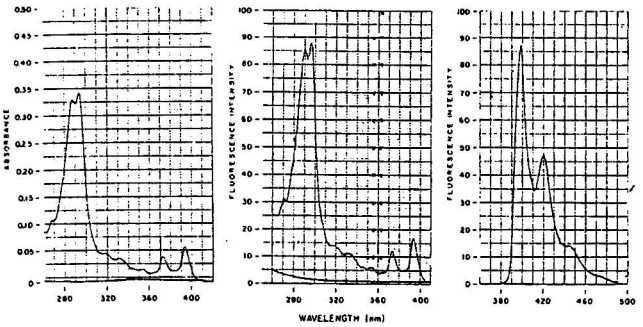
1,2:3,4 DIBENZPYRENE

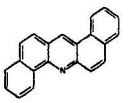
ABSORBANCE
Slit 2 nm
Conc 0.25 μg/ml

CORR. EXCITATION
λ Em 420 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.025 μg/ml

CORR. EMISSION
λ Ex 297 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.025 μg/ml

FIG. 15





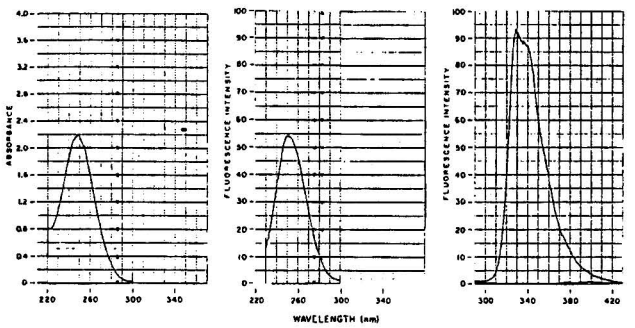
1,2-5,6 DIBENZACRIDINE

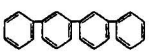
ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 420 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.01 μg/ml

CORR. EMISSION
λ Ex 293 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.01 μg/ml

FIG. 16





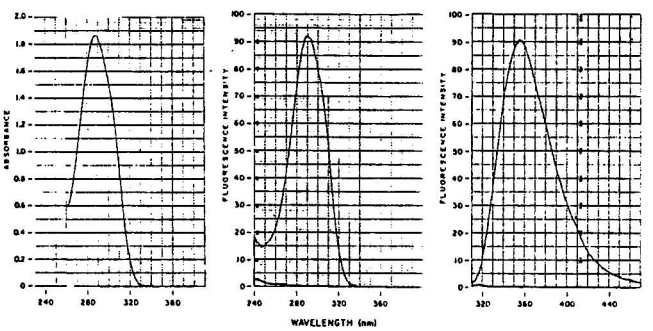
m-QUATERPHENYL

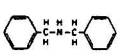
ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 330 nm
Ex slit 2 nm
Em slit 20 nm
Conc 0.04 μg/ml

CORR. EMISSION
λ Ex 250 nm
Ex slit 10 nm
Em slit 3 nm
Conc 0.04 μg/ml

FIG. 17





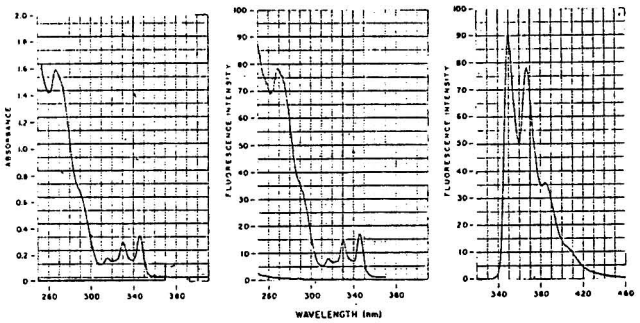
IMINODIBENZYL

ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 350 nm
Ex slit 2 nm
Em slit 15 nm
Conc 0.04 μg/ml

CORR. EMISSION
λ Ex 290 nm
Ex slit 10 nm
Em slit 3 nm
Conc 0.04 μg/ml

FIG. 18



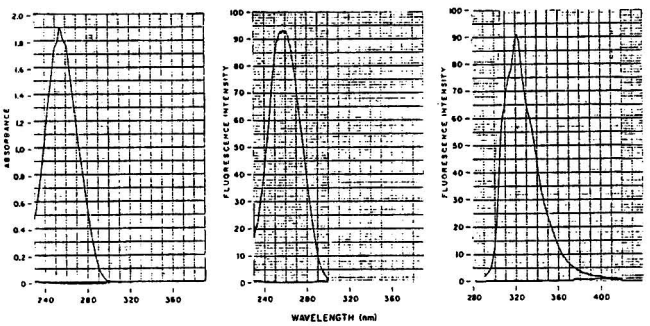
ABSORBANCE
Slit 2 nm
Conc 0.04 µg/ml

CORR. EXCITATION
λ Em 360 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.0016 µg/ml

CORR. EMISSION
λ Ex 276 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.0016 µg/ml

5,6 BENZOQUINOLINE

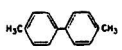
FIG. 19



ABSORBANCE
Slit 2 nm
Conc 1 µg/ml

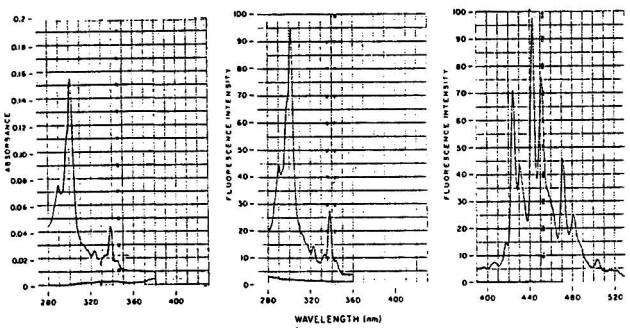
CORR. EXCITATION
λ Em 320 nm
Ex slit 2 nm
Em slit 15 nm
Conc 0.04 µg/ml

CORR. EMISSION
λ Em 256 nm
Ex slit 10 nm
Em slit 3 nm
Conc 0.04 µg/ml



p,p-BITOLYL

FIG. 20



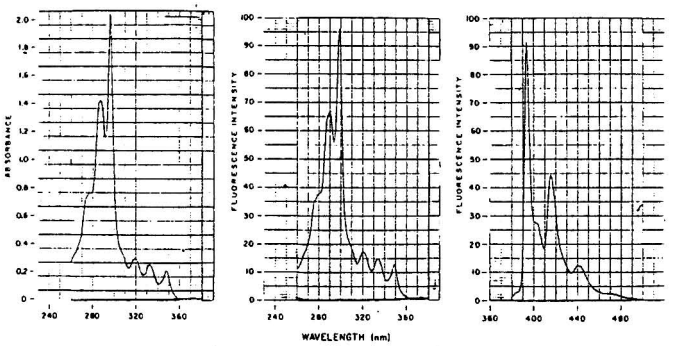
ABSORBANCE
Slit 2 nm
Conc 1 µg/ml

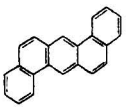
CORR. EXCITATION
λ Em 446 nm
Ex slit 2 nm
Em slit 10 nm
Conc 1 µg/ml

CORR. EMISSION
λ Ex 299 nm
Ex slit 10 nm
Em slit 3 nm
Conc 1 µg/ml

CORONENE

FIG. 21





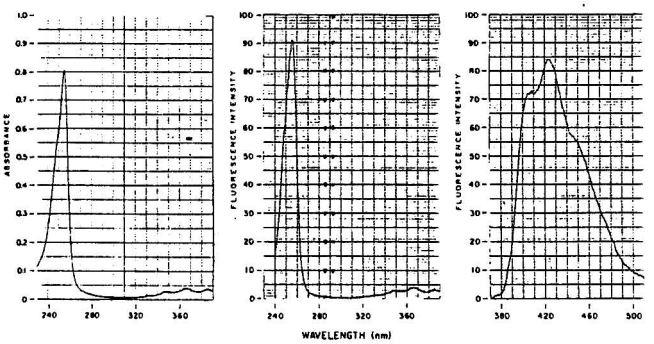
1,2 · 5,6
DIBENZANTHRACENE

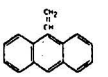
ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ EM 372 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.02 μg/ml

CORR. EMISSION
λ Ex 280 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.02 μg/ml

FIG. 22





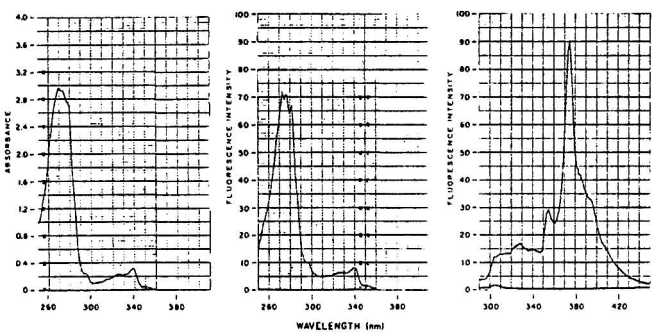
9 VINYL ANTHRACENE

ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 360 nm
Ex slit 2 nm
Em slit 10 nm
Conc 0.022 μg/ml

CORR. EMISSION
λ Ex 264 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.022 μg/ml

FIG. 23



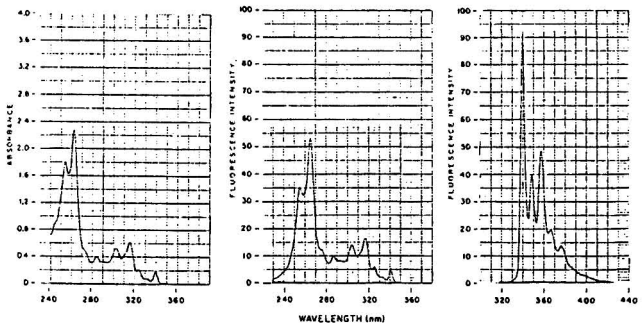
AZULENE

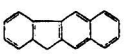
ABSORBANCE
Slit 2 nm
Conc 0.25 μg/ml

CORR. EXCITATION
λ Em 396 nm
Ex slit 2 nm
Em slit 16 nm
Conc 0.01 μg/ml

CORR. EMISSION
λ Ex 296 nm
Ex slit 2 nm
Em slit 16 nm
Conc 0.01 μg/ml

FIG. 24





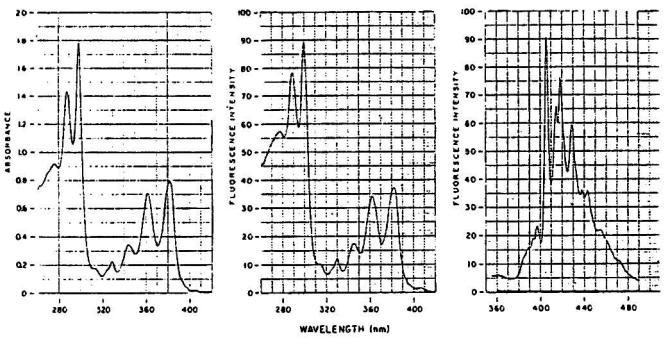
2,3 BENZOFLUORENE

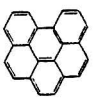
ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 420 nm
Ex slit 2 nm
Em slit 12 nm
Conc 0.02 μg/ml

CORR. EMISSION
λ Ex 255 nm
Ex slit 12 nm
Em slit 2 nm
Conc 0.02 μg/ml

FIG. 25





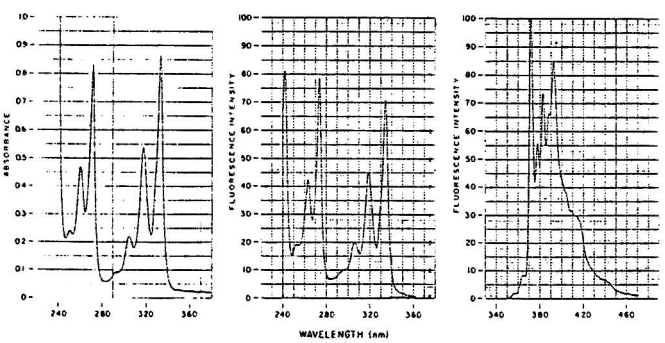
1,12 BENZOPERYLENE

ABSORBANCE
Slit 2 nm
Conc 1 μg/ml

CORR. EXCITATION
λ Em 410 nm
Ex slit 2 nm
Em slit 15 nm
Conc 0.02 μg/ml

CORR. EMISSION
λ Ex 299 nm
Ex slit 10 nm
Em slit 2 nm
Conc 0.02 μg/ml

FIG. 26



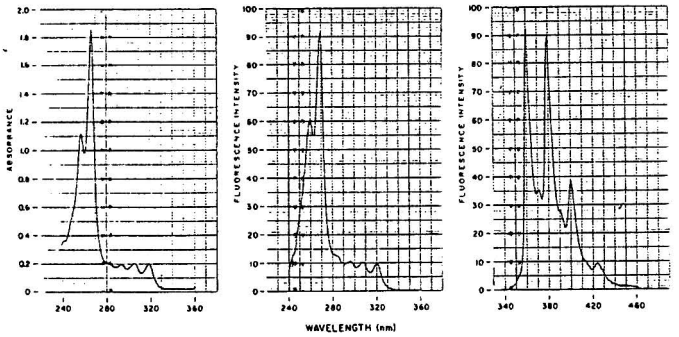
PYRENE

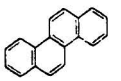
ABSORBANCE
Slit 2 nm
Conc 4 μg/ml

CORR. EXCITATION
λ Em 380 nm
Ex slit 2 nm
Em slit 8 nm
Conc 0.1 μg/ml

CORR. EMISSION
λ Ex 320 nm
Ex slit 8 nm
Em slit 2 nm
Conc 0.1 μg/ml

FIG. 27





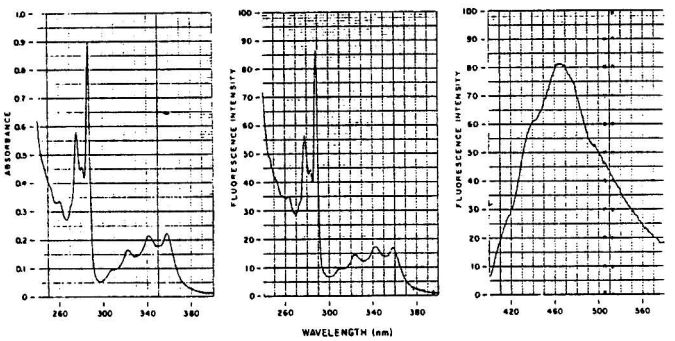
CHRYSENE

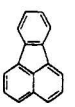
ABSORBANCE
Slit 2 nm
Conc 5 μg/ml

CORR. EXCITATION
λ Em 400 nm
Ex slit 2 nm
Em slit 14 nm
Conc 0.1 μg/ml

CORR. EMISSION
λ Ex 260 nm
Ex slit 14 nm
Em slit 2 nm
Conc 0.1 μg/ml

FIG. 28





FLUORANTHENE

ABSORBANCE
Slit 2 nm
Conc 10 μg/ml

CORR. EXCITATION
λ Em 460 nm
Ex slit 2 nm
Em slit 14 nm
Conc 0.6 μg/ml

CORR. EMISSION
λ Ex 290 nm
Ex slit 14 nm
Em slit 2 nm
Conc 0.6 μg/ml

FIG. 29

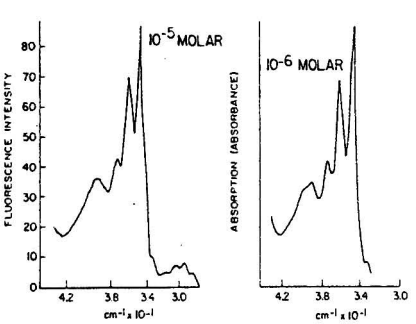


FIG. 30—1,2-Benzanthracene in ethanol, excitation and absorbance spectra—comparison of literature data (2).

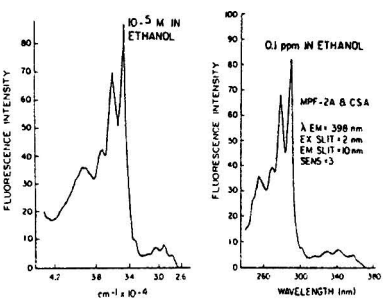


FIG. 31—1,2-Benzanthracene in ethanol, excitation and absorbance spectra—comparison of Parker's data (2) and MPF-3 data.

Acknowledgments

We would like to acknowledge the contribution of Mercedes R. Huang, University of Massachusetts, for obtaining a number of the corrected emission and excitation spectra that we have shown. We would also like to thank J. L. Monkman and R. S. Thomas of the Environmental Health Center, Ottawa, Ontario, Canada, for quantities of zone-refined samples of fluoranthene and pyrene.

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Nitrate, Nitrite, and Dimethylnitrosamine in Cured Meat Products

THAVIL PANALAKS, JAGANNATH R. IYENGAR, and NRISINHA P. SEN
Research Laboratories (Foods), Health Protection Branch, Department of National Health and Welfare, Ottawa, Ontario, Canada K1A OL2

Nitrate, nitrite, and dimethylnitrosamine (DMN) were determined in 197 samples of various kinds of meat products. Nitrate and nitrite were determined by the method of Kamm *et al.* (1965), and DMN was estimated semiquantitatively by a GLC method using a Coulson electrolytic conductivity detector (pyrolytic mode). The average levels of nitrate and nitrite were 181 ppm (range, 0–3467 ppm) and 28 ppm (range, 0–252 ppm), respectively. Trace amounts (2–12 ppb) of DMN were present in 57 samples; others were negative. As no mass spectrometric confirmation of the identity of DMN was carried out, the results should be considered as only tentative and not as an absolute proof of the presence of DMN. Except in a few types of meat products, the concentrations of nitrate or nitrite did not correlate with that of DMN detected in the samples.

Since the discovery of the carcinogenicity of dimethylnitrosamine (DMN) in rats by Magee and Barnes (1) in 1956, various reports (2–5) have been published regarding the carcinogenicity of DMN and other *N*-nitrosamines, their metabolism and mode of action, and occurrence in foodstuffs. The first evidence that DMN could be present in the environment came from the work of Norwegian researchers (6, 7) investigating a liver disease in farm animals that were fed nitrite-preserved herring meal. Subsequent studies (8, 9) established that the toxicity of the meal was due to the formation of fairly large amounts (30–100 ppm) of DMN from the added nitrite and the amines which occur naturally in the fish. Recent work from various laboratories has established that trace amounts of DMN are present in certain types of fish and meat products used for human consumption (10–12; Wasserman, A. E., Fiddler, W., Doerr, R. C., Osman, S. F., and Dooley, C. J., USDA, Philadelphia, Pa., 1972, personal communication).

Both sodium nitrate (500 ppm or more) and sodium nitrite (up to 200 ppm) are permitted as food additives in meat products in many countries of the world (13–15). It was thought, therefore, that a comparison of the levels of these additives with that of DMN in various commodities of meat might provide us with valuable data on the nature and extent of the distribution of these chemicals in this important source of human food. Here we wish to report the result of such a survey carried out at the Health Protection Branch Laboratory at Ottawa.

Experimental

Collection of samples.—Meat samples were purchased from commercial outlets located at 5 main centers (Vancouver, Winnipeg, Montreal, Toronto, and Halifax) and sent by air freight to this laboratory. Upon arrival the samples were comminuted in a meat mincer, and stored in polyethylene bags at −10°C until further use.

Determination of nitrate and nitrite.—These substances were determined by the method of Kamm *et al.* (16).

Determination of DMN.—The method used is very similar to that reported earlier (17). The only difference was that the amount of DMN in the final extract was semiquantitatively estimated by a gas-liquid chromatographic (GLC) method using a Coulson electrolytic conductivity detector (pyrolytic mode). The method can be described briefly as follows: 100 g sample was extracted with methylene chloride at an alkaline pH, and the extract was distilled over 3*N* KOH until all the methylene chloride was removed. The aqueous residue was distilled, and the distillate was made alkaline with 20% K_2CO_3 and re-extracted with methylene chloride. Interfering amines were removed by washing the extract with glycine-hydrochloric acid buffer (pH 2.1). After further purification of the extract by chromatographic cleanup on a basic alumina column the solution was concentrated to 1.0 ml. A 15–20 µl aliquot of the final methylene chloride extract was analyzed by GLC as described earlier (18).

Results and Discussion

Table 1 summarizes the results of the study. The average values for nitrate and nitrite were 181 ppm and 28 ppm, respectively. The concen-

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Table 1. Nitrate, nitrite, and DMN contents of various meat products

Sample No.	Variety	Nitrate, ppm[a]	Nitrite, ppm[a]	DMN, ppb
	Shelf-stable, canned cured meat			
1	Beef-ham meat spread	80	25	N[b]
2	Beef-pork chicken spread	N	4	N
3	Ham spread	69	4	N
4	Ham spread	88	14	3
5	Luncheon meat	124	4	N
6	Luncheon meat	122	14	N
7	Luncheon meat	124	4	2
8	Pork luncheon meat	9	4	N
9	Meat loaf	97	7	N
10	Meat loaf	159	7	N
11	Meat loaf	48	N	N
12	Corned beef loaf	67	18	N
13	Ham	1148	11	N
14	Ham	31	7	N
15	Ham	49	N	N
16	Ox tongue	27	7	3
	Pasteurized, canned cured meat			
17	Ham	185	148	3
	Sausages, smoked and unsmoked			
18	Wieners	309	21	2
19	Wieners	151	14	3
20	Wieners	223	11	3
21	Wieners	138	14	2
22	Wieners	255	18	2
23	Wieners	154	7	N
24	Wieners	83	18	N
25	Wieners	23	14	N
26	Wieners	26	N	2
27	Wieners	56	11	3
28	Wieners	93	14	N
29	Wieners	50	7	N
30	Beef wieners	151	14	N
31	Beef wieners	69	N	N
32	Smoked sausage	202	N	N
33	Smoked sausage	269	14	N
34	Smoked sausage	326	77	N
35	Smoked beef sausage	156	N	N
	Bologna			
36	Bologna	75	7	5
37	Bologna	201	11	3
38	Bologna	154	44	3
39	Bologna	98	14	N
40	Bologna	17	4	N
41	Bologna	154	7	3
42	Bologna	278	11	2
43	Bologna	336	108	8
44	Bologna	88	14	N
45	Bologna	15	44	7
46	Bologna	52	18	2
47	Bologna	112	4	5
48	Bologna	125	84	3
49	Bologna	88	77	4
50	Bologna	63	58	N
51	Bologna	13	4	N
52	Bologna	85	4	N
53	Garlic bologna	70	56	N
54	Garlic bologna	265	136	5
55	German bologna	178	216	N
56	Italian sausage	49	N	N

(Continued)

Table 1. (Continued)

Sample No.	Variety	Nitrate, ppm[a]	Nitrite, ppm[a]	DMN, ppb
	Meat loaves[c]			
57	Pickle & pimento loaf	76	11	N
58	Meat, pickle, & pimento loaf	48	N	N
59	Meat, macaroni, & cheese loaf	70	7	N
60	Smoked beef luncheon meat	112	7	2
61	Beef luncheon meat	13	N	N
62	Luncheon meat	124	7	N
63	Luncheon meat	84	N	N
64	Luncheon meat	4	4	N
65	Luncheon meat	13	N	N
66	Mock chicken loaf	222	25	10
67	Mock chicken loaf	185	14	2
68	Mock chicken loaf	108	7	2
69	Mock chicken loaf	147	4	3
70	Mock chicken loaf	175	39	N
71	Meat loaf	48	4	2
72	Meat loaf	203	118	N
73	Pork turkey loaf	289	11	N
74	Barbequed meat loaf	90	4	3
75	Ham	69	4	N
76	Cooked ham	17	N	N
	Salami, European-type sausages, etc.			
77	Belitalia	339	7	N
78	Pepperoni sausage	374	29	N
79	Ukranian sausage	251	35	10
80	Skinless sausage	83	N	N
81	Tyrola sausage	21	4	N
82	German-style sausage	97	25	N
83	Beer sausage	35	40	N
84	Summer sausage	185	14	N
85	Tradition ham sausage	17	99	N
86	Garlic ring sausage	42	18	N
87	Polish sausage	87	56	3
88	Salami	32	21	3
89	Salami	163	11	N
90	Salami	203	51	N
91	Salami	46	14	5
92	Salami	54	4	N
93	Salami	75	7	4
94	Salami	147	7	N
95	Beef salami	84	40	N
96	Beef salami	233	39	3
97	Beef salami	74	72	2
98	Beef salami	50	7	N
99	Polish cooked salami	123	4	N
100	Dry cured salami	5	18	N
101	Beef frankfurters	227	21	4
	Unpackaged cured meat products[d]			
102	Pastrami	154	40	N
103	Pastrami	106	68	N
104	Pastrami	189	10	2
105	Onion (pork & beef) loaf	65	58	N
106	Spiced beef	359	7	N
107	Aged & cured Hungarian salami sausage	384	N	N
108	Cured beef (uncooked and unsmoked)	22	N	N

(Continued)

Table 1. *(Continued)*

Sample No.	Variety	Nitrate, ppm[a]	Nitrite, ppm[a]	DMN, ppb
	Unpackaged cured meat products[d] (cont'd)			
109	Cured beef (uncooked and unsmoked)	17	N	N
110	Smoked meat	155	10	2
111	Westphalian ham	407	47	N
112	Westphalian ham	156	N	N
113	Cooked ham	122	4	N
114	Smoked ham	192	44	N
115	Smoked pork loins	22	90	2
116	Smoked European bacon	310	4	N
	Bacon			
117	Bacon	9	252	2
118	Bacon	32	18	N
119	Bacon	17	7	3
120	Bacon	37	64	N
121	Bacon	45	123	2
122	Bacon	216	21	N
123	Back bacon	223	10	N
124	Back bacon	99	14	N
125	Side bacon	296	67	N
126	Side bacon	168	7	N
127	Side bacon	87	14	N
128	Side bacon	108	29	N
129	Side bacon	290	10	N
130	Side bacon	67	21	3
131	Side bacon	111	4	N
132	Side bacon	440	4	N
133	Side bacon	86	7	3
134	Bacon	199	14	N
135	Peameal bacon end cut	37	56	2
136	Smoked bacon	64	14	N
	Hams			
137	Ham	88	127	N
138	Ham	65	60	N
139	Ham	223	N	N
140	Ham	N	56	3
141	Ham	26	N	N
142	Ham	N	21	N
143	Chopped ham loaf	36	14	N
144	Smoked ham	500	205	N
145	Smoked ham	106	90	N
146	Smoked ham	250	29	N
147	Semi-cured ham	104	52	2
148	Semi-cured ham	17	4	N
149	Semi-cured ham	18	7	N
150	Semi-cured ham	36	104	N
151	Uncooked cured ham	1946	7	3
152	Cooked ham	371	72	N
153	Cooked ham	74	25	N
154	Cooked ham	272	4	N
155	Cooked ham	9	32	N
156	Cooked ham	25	45	N
157	Canned cooked ham	60	7	N
158	Cooked boneless ham	31	45	N
159	Cooked boneless ham	444	N	N
	Picnics (shoulders)			
160	Corned pork shoulder	108	7	N
161	Picnic pork shoulder	89	21	N
162	Picnic pork shoulder	96	7	N

(Continued)

Table 1. *(Continued)*

Sample No.	Variety	Nitrate, ppm[a]	Nitrite, ppm[a]	DMN, ppb
	Picnics (shoulders) (cont'd)			
163	Picnic pork shoulder	311	108	2
164	Picnic pork shoulder	N	25	N
165	Picnic pork shoulder	379	7	N
166	Cottage roll	86	58	N
167	Cottage roll	70	85	N
168	Smoked picnic pork shoulder	191	90	N
169	Smoked picnic pork shoulder	167	7	N
170	Smoked picnic pork shoulder	60	7	N
171	Smoked picnic pork shoulder	22	21	N
172	Smoked cottage roll	142	7	2
173	Semi-cured picnic ham	172	4	N
174	Uncooked cured cottage roll	2831	14	N
	Corned beef products			
175	Corned beef	165	14	N
176	Corned beef	60	7	N
177	Corned beef	97	7	N
178	Corned beef	264	10	N
179	Corned beef	75	7	N
180	Corned beef	191	4	N
181	Corned beef	668	7	N
182	Corned beef	376	231	N
183	Corned beef	156	N	N
184	Corned beef	18	10	N
185	Cured corned beef	3467	39	12
186	Corned beef round	22	4	N
187	Corned beef loaf	17	4	N
188	Corned beef brisket	373	4	N
189	Corned beef brisket	159	7	7
190	Corned beef brisket	44	4	N
191	Corned beef brisket	79	N	N
192	Corned beef brisket	139	N	N
193	Corned beef brisket	45	4	N
194	Pickled corned beef brisket	111	N	3
195	Pickled cured corned beef	82	32	3
196	Pickled cured corned beef	168	21	3
197	Pickled beef	21	84	N

[a] Results expressed as the sodium salt.

[b] N = none detected (detection limit for DMN is 2 ppb and for nitrate and nitrite, 1 ppm).

[c] Products such as packaged luncheon meat slices, sliced ham, mock chicken, and macaroni and cheese luncheon meat slices.

[d] Pastrami, smoked meat, dried uncooked ham, etc.

trations of nitrate in 5 samples were over 500 ppm and that of nitrite in 4 samples exceeded the officially permitted levels. It is interesting to note that although some of these samples contained over 1000 ppm nitrate the corresponding values for nitrite were quite low. This may indicate that

only slow conversions, if at all, of nitrate to nitrite took place during storage or processing.

Of 197 samples analyzed, 57 contained detectable amounts (2–12 ppb) of DMN; the rest were negative (detection limit ≈ 2 ppb). The recoveries of added DMN at 10 ppb and 20 ppb levels ranged between 70 and 80%. Figure 1 shows a few typical gas chromatograms obtained in this study.

The semiquantitative estimation and identification of DMN was based solely on GLC analysis using the Coulson detector (pyrolytic mode). Under such conditions (Fig. 1) the detector is highly specific for nitrosamines (18), and it is therefore highly likely that these data represent a true measure of DMN and not of the interfering materials. It would probably be extremely laborious and time-consuming to confirm each of the low positive results by GLC-mass spectrometry. It should be pointed out that in a previous study (11) we have unequivocally confirmed the presence of DMN in a few meat samples by GLC-mass spectrometry.

The results reported here are in general agreement with our previous data and those reported by others (10–12; Wasserman *et al.*) that the concentrations of DMN, if present, in cured meat products are normally very low. Only in 3 instances have relatively high levels of DMN been confirmed; one salami examined by us contained about 60–80 ppb DMN (11) and two frankfurters analyzed by the USDA Laboratories were found to contain 48 and 84 ppb DMN, respectively (Wasserman *et al.*).

By comparing the data in Table 1 it would appear that there is no clear-cut correlation between the levels of DMN and that of nitrate or nitrite. Of the 9 samples (Nos. 13, 55, 117, 144, 151, 174, 181, 182, 185) that contained excess levels of nitrate or nitrite, only one (No. 185) contained significant amounts (12 ppb) of DMN; others contained either none or trace quantities (2–3 ppb). Similarly, of 9 samples (Nos. 36, 43, 45, 47, 54, 66, 79, 91, 185) that contained 5 ppb or more of DMN, only 2 (Nos. 43, 54) contained high levels of nitrite and one (No. 185) contained a high level of nitrate. These findings agree well with those of Wasserman *et al.* (personal communication) who found no correlation between the levels of DMN and those of nitrite in commercially processed samples of frankfurters.

The data were statistically analyzed by Kendall's rank correlation test (19), and the results are presented in Table 2. Significant positive correlations ($P \leq 0.1$) seem to exist between the following pairs of variables: nitrate-DMN and nitrite-DMN in corned beef; nitrate-DMN in meat loaves; nitrate-DMN in bologna. Negative correlations are observed between nitrate and DMN levels in bacons. Although no definite explanation of this negative relationship can be presented at the moment, it is possible that the manufacturers of these bacons used a combination of either high nitrate + low nitrite or low nitrate + high nitrite as the curing agent. This may explain why the concentrations of DMN in these samples were inversely proportional to those of

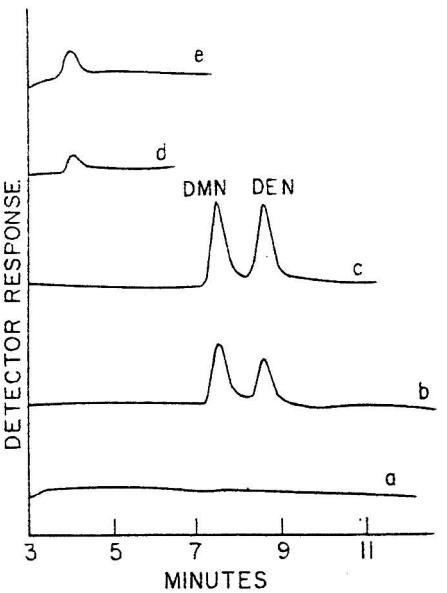

FIG 1—GLC analysis of DMN. (a) 20 μl aliquots of final extract from sample No. 175; (b) 20 μl aliquots of same sample spiked with DMN and DEN (diethylnitrosamine) at 10 ppb levels; (c) 20 ng each of DMN and DEN standard; (d) 5 ng DMN standard; and (e) 20 μl aliquots of extract from sample No. 185.

10% Carbowax 1540 on 80-100 Chromosorb W (HMDS/AW), 6′ × ¼″ stainless steel column, carrier gas (He) flow rate 30 ml/min. A Varian gas chromatograph, Model 2700, connected to a Coulson electrolytic conductivity detector (furnace 400–450°C, pyrolytic mode) was used. Detector voltage 30, attenuator at 2.

For (a) to (c) column temperature was 105°C for first 3 min, then programmed to 170°C at 20°C/min. For samples (d) and (e) the analysis was carried out under isothermal (150°C) conditions. The effluent from the column was allowed to vent in the air for first 3 min in each case.

Table 2. Kendall's rank correlation test for different variables in various groups of meat products[a]

Type of meat product	Sample size	Kendall's τ for	
		Nitrate-DMN	Nitrite-DMN
Shelf-stable meat	16	0.014 (0.93)	0.232 (0.20)
Sausages	18	0.026 (0.88)	0.196 (0.25)
Bologna	21	0.292 (0.06)	0.254 (0.11)
Meat loaves	20	0.320 (0.05)	0.252 (0.12)
Salami	25	0.100 (0.48)	0.196 (0.17)
Unpackaged cured meats	15	−0.098 (0.62)	0.271 (0.16)
Bacons	20	−0.445 (0.01)	0.148 (0.36)
Hams	23	0.048 (0.75)	0.049 (0.75)
Picnics	15	0.230 (0.23)	0.250 (0.19)
Corned beef	23	0.249 (0.10)	0.280 (0.07)

[a] Values in parentheses represent approximate significant probabilities for 2-tailed test.

nitrate. Since we do not know the exact input levels of nitrate and nitrite in these samples, it is difficult to say whether this was the case. Other equally good explanations may be possible.

The mechanism of formation of DMN in meat is a complex process. It depends not only on the concentration of nitrite but also on the availability of various amines such as dimethylamine, trimethylamine, and trimethylamine oxide, as well as some quaternary ammonium compounds (20). It is possible that some of the high nitrite samples did not contain enough of the necessary amines, and as a result were low in DMN. Other factors, such as the effect of various additives, bacterial flora in the meat, processing and storage conditions, etc., might influence the formation of DMN. If all the meat products were of the same type (same processing) and manufactured from the same batch of meat (same amine components), then it might have been possible to obtain a direct correlation between the levels of nitrite and of DMN. Recently, Fiddler *et al.* (Fiddler, W., Piotrowski, E. G., Pensabene, J. W., Doerr, R. C., and Wasserman, A. E., 1972, personal communication) have carried out such a study with frankfurters and noticed an increase in DMN formation with an increase in either the concentration of nitrite or the processing time.

The results of this study indicate that extremely low levels of DMN may be present in meat products. However, the biological effect of such low amounts of DMN in humans is not known. DMN has been shown to be carcinogenic to rats when fed in the diet at 2 ppm, and a no-effect level has not been established (21). Until such information is available it will not be possible to set tolerance guidelines for DMN in meat products or any other foods.

Acknowledgments

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SPICES AND OTHER CONDIMENTS

Detection of Turmeric in Foods by Rapid Fluorometric Method and by Improved Spot Test

ALBERT B. KARASZ, FRANK DE COCCO, and LINDA BOKUS
New York State Food Laboratory, 1220 Washington Ave., Albany, N.Y. 12226

The rapid test for turmeric is based on the fluorescence of curcumin, the principal pigment of turmeric. In a water-saturated *n*-butanol solution, its excitation maximum is 435 nm and the emission maximum is 520 nm. In the test described, which was applied to pickles, salad dressings, and baked goods, a sample is shaken with the butanol solvent and the filtered extract is scanned for a 520 nm peak on a spectrophotofluorometer that has been adjusted to give 100% scale deflection for a standard curcumin solution. Extracts of pickles and salad dressings are scanned directly. Extracts of enriched baked goods are treated to remove riboflavin fluorescence by partitioning both riboflavin and curcumin from butanol into a solution of 0.1*N* NaOH-15% NaCl, acidifying the latter with CH_3COOH, reducing the riboflavin with $Na_2S_2O_4$, and re-extracting curcumin from acid solution into butanol for fluorometric scanning. No interference is caused by annatto, carotene, tartrazine, or egg yolk solids. A spot test may be performed on the butanol extract. The solution is evaporated to dryness, the residue is dissolved in a few drops of ethanol, and the spot test is performed with a boric acid reagent of considerably improved sensitivity.

The use of turmeric by bakers to color bread and rolls yellow, in semblance of the color imparted by egg yolk, has become widespread recently. In the course of the examination of bread samples for this fraud, the official boric acid test for turmeric (1) was found to be unsatisfactory. Results were often inconclusive, as co-extractives appeared to cause interference. Similar difficulties were also encountered in applying the boric acid test to other commodities, such as salad dressings and pickles.

An alternative test reported here is based on the fluorescent property of turmeric. This property is due largely to the fluorescence exhibited by curcumin, the principal pigment of turmeric. A spectrophotofluorometer is used to isolate the specific excitation and emission wavelengths. Details of the method and some of its applications are described.

A modified boric acid reagent is also described. This reagent affords a considerable improvement in the spot test for turmeric.

METHOD

Reagents and Apparatus

(a) *Butanol solution.*—Thoroughly shake 850 ml *n*-butanol with 170 ml water in separatory funnel. Discard excess water.

(b) *Curcumin reference solution.*—3 μg/ml. Dissolve 30 mg curcumin in 100 ml ethanol. Dilute 5 ml of this solution to 500 ml with butanol solution.

(c) *Sodium hydroxide solution.*—Dissolve 150 g NaCl and 4 g NaOH in 1 L water.

(d) *Sodium hydrosulfite.*—$Na_2S_2O_4$, powder.

(e) *Boric acid reagent.*—Dissolve 1 g H_3BO_3 and 5 ml HCl in 95 ml ethanol. Dry over anhydrous Na_2SO_4 and filter.

(f) *Spectrophotofluorometer.*—Aminco-Bowman, or equivalent, with recorder.

(g) *Filter aid.*—Hyflo Super-Cel.

Preparation of Extract

(a) *Salad dressings and pickles.*—Mix 2 g salad dressing or comminuted pickles in beaker with 3 g filter aid to uniform mixture. Add 50 ml butanol solution and stir thoroughly. Let stand 15 min with occasional stirring, filter through Whatman No. 42 paper, and scan. For spot test, purify 20 ml extract as described in (b).

(b) *Bread and rolls.*—Add 10 g comminuted bread in flask containing 50 ml butanol solution, stopper flask, shake well, and let stand 15 min. Reshake and filter extract as above. Transfer 20 ml extract to separatory funnel, add 10 ml NaOH solution, and shake vigorously 1 min. Draw aqueous phase into second separatory funnel, including any red droplets that may collect at interface. Add 1 ml glacial acetic acid and 200 mg $Na_2S_2O_4$. Swirl to dissolve salt, add 20 ml butanol solution, and shake vigorously 1 min. Filter butanol extract and scan within 15 min.

Fluorometric Test

Set fluorometer excitation scale to 435 nm and emission scale to 520 nm. Fill cuvet with curcumin reference solution and adjust slits, meter multiplier, and sensitivity to obtain 100% full scale deflection on recorder. Replace reference solution with sample extract. Keep excitation scale at 435 nm and record emission spectrum of sample extract. In presence of turmeric, emission maximum appears at 520 nm.

Spot Test

Evaporate portion of butanol extract to dryness. Dissolve residue in few drops ethanol and spot sufficient amount on Whatman No. 1 paper to produce distinct yellow spot. Dry paper in 100°C oven 2 min, and, using micropipet, add 3–4 small drops of boric acid reagent to yellow area. In presence of turmeric, red color develops within 2 min at room temperature.

Results and Discussion

The fluorescence of turmeric in ether or alcohol has been previously reported by others (1, 2). For the analytical utilization of this property it was necessary to establish the excitation and emission maxima. As shown in Fig. 1, these were found to be 435 and 520 nm, respectively, in butanol. It was also necessary to provide a reference standard for setting the instrumental parameters suitable for the expected fluorescence range.

Curcumin was selected as a reference material in preference to turmeric. Turmeric is a product of variable composition, while curcumin is available in pure form. Further, its fluorescence maxima are essentially identical with those of turmeric. A solution containing 3 μg curcumin/ml butanol was found to have a fluorescence intensity in the range exhibited by the extracts obtained by the proposed method from samples colored with turmeric; thus this solution was suitable as a reference to adjust the instrument.

Application of the proposed method to pickles and salad dressing is simple and rapid. The sample extract can be scanned without any further treatment. The sample taken is small enough to keep the fluorescent background less than 10% relative intensity, but it is sufficiently large to yield a distinct peak for the amounts of turmeric customarily added to these products for coloring purposes.

No interfering fluorescence was produced by carotene, tartrazine, eggs, paprika, mustard, and annatto. Interfering fluorescence, however, was caused by the riboflavin present in enriched bread and rolls, and additional treatment was required for its elimination.

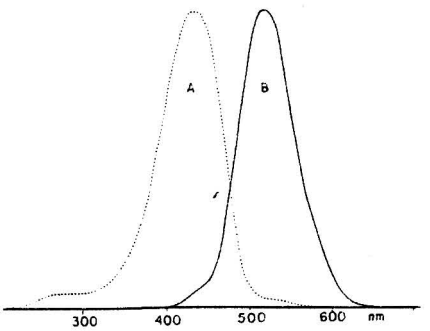

FIG. 1—Fluorescence spectra of turmeric sample: A, excitation; B, emission.

Dilute NaOH solution was found to extract both riboflavin and turmeric from the butanol solution. After acidification of the aqueous phase, the riboflavin present was reduced with sodium hydrosulfite, and from the acid solution turmeric was partitioned back into butanol solution, freed from riboflavin interference. The solution was scanned without undue delay, as in some cases turbidity developed in about 15 min.

It is worth noting that in the course of the first partitioning step, the alkaline solution turns pink in the presence of turmeric and this can be considered a first indication of its presence. This color is transient and gradually changes to light brown.

Application of this method for the quantitative estimation of turmeric in food appeared a possibility only if the fluorescence of turmeric were found to occur within a narrow range. To investigate the probable range, 7 commercial samples of turmeric were extracted with butanol and examined for fluorescence intensity. The apparent curcumin content of the various samples was then estimated from a curve that had been prepared by plotting known curcumin concentrations against relative intensity. The values obtained were variable: 13, 12, 13, 4, 14, 14, and 8%. The quantitation of an unknown product, consequently, is not feasible.

It appeared desirable, however, to test the efficiency of the purification process described for enriched bread by spiking the extract with turmeric. A 10 g sample of enriched bread, spiked with 0.62 mg turmeric, was processed by the pro-

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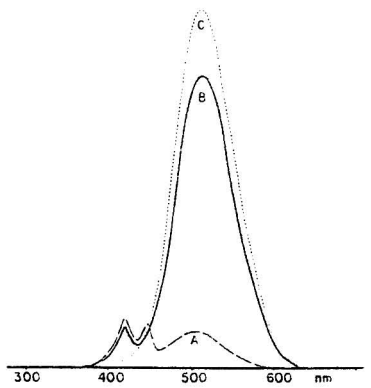

FIG. 2—Recovery of turmeric added to enriched bread: A, unspiked bread; B, bread plus 0.62 mg turmeric/10 g; C, curcumin reference solution, 3 μg/ml.

posed method and the fluorescence of the butanol extract was measured. An equal amount of turmeric was added to 50 ml butanol and its fluorescence was compared with that obtained for the bread. Recovery for 3 trials averaged 90±2%.

The emission curves obtained for the unspiked enriched bread and for one of the spiked samples are shown in Fig. 2. The low relative intensity for the unspiked enriched bread demonstrates the effectiveness of the quenching of riboflavin fluorescence. Fortification at 0.62 mg/10 g, or 62 ppm, produced a reading of 80% relative intensity. This turmeric concentration is in the lower range of commercial usage. Extracts from more highly colored products may require dilution prior to scanning.

The emission curve of the curcumin reference solution was also included in Fig. 2. This was the solution used to calibrate the instrument. Its emission curve, compared with that of turmeric, demonstrates an apparent identity of the 2 spectra.

In the course of the current work, improvement of the AOAC spot test for turmeric (1) was also sought. The approach to improve the test was based on 2 considerations. The first consideration was that the red color, presumably rosocyanin, develops only after thorough drying of the spot. Hence the use of an anhydrous reagent appeared to offer an advantage. The second consideration was that turmeric has been reported to form rubrocurcumin by reacting with boric acid and alcohol (2). An alcoholic reagent therefore seemed preferable to an aqueous solution. The anhydrous alcoholic reagent (e) described in this paper did prove to be considerably more sensitive than the AOAC reagent.

The sensitivity of the 2 reagents was compared. Two series of spots were placed on paper, using varying amounts of an alcoholic extract of turmeric. The amount of turmeric represented in the spots in each of the series ranged from 0.2 to 30 μg. Both papers were dried at 100°C. The spots in one series were then treated with the AOAC reagent and those in the second series with reagent (e).

When the AOAC reagent was used, 20 μg turmeric was required to provide an orange color, a doubtful positive. Reagent (e) produced with 1 μg turmeric a magenta red color, a distinct positive. These results show that reagent (e) is more than 20 times as sensitive as the AOAC reagent.

The improvement in the sensitivity permits the presence of a larger amount of interfering impurities in the sample extract. These co-extractives can be largely eliminated for maximum sensitivity of the test by use of the double-partitioning process described under *Preparation of Extract.*

The time required for either the spot test or the fluorometric test is about 1 hr. An advantage of the fluorometric method is that it provides a graph as a permanent record. This test has been employed for the analysis of well over a hundred samples in the past year.

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This paper was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

EXTRANEOUS MATERIALS

Collaborative Study of a Method for the Extraction of Light Filth from Prepared Horseradish

JOHN S. GECAN and PARIS M. BRICKEY, JR.
Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204

A method has been developed for the extraction of light filth from prepared horseradish. The method involves a direct flotation from 40% isopropanol with a mineral oil-*n*-heptane mixture. Collaborative study showed the method to be rapid and easy to perform, and to yield good recoveries and clean extraction papers. The method has been adopted as official first action.

There is no published AOAC method for the extraction of light filth from prepared horseradish. A method was developed involving a direct extraction of the product. The light filth is floated with a light mineral oil-*n*-heptane flotation liquid from a distilled water-HCl system.

METHOD

Prepared Horseradish—Official First Action

40.C04 ***Determination***

Weigh 100 g sample into 600 ml beaker. Add 200 ml H_2O and transfer to 2 L trap flask with H_2O. Dil. to 1 L, add 50 ml HCl, and stir few sec. Add magnetic stirring bar, 40.002(s), and 50 ml flotation mixt., 40.C01(d), and stir magnetically, 40.004(b), 3 min. Slowly fill flask with distd H_2O by running liq. down stoppered rod while stopper is maintained just above flask contents. After filling flask, gently stir settled material 5–10 sec with stoppered rod. Let stand undisturbed 5 min; then trap off. Add 35 ml flotation mixt., 40.C01(d), stir gently by hand 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with isopropanol and transfer washings to beaker contg trappings. Filter onto ruled paper and examine microscopically.

Collaborative Study

Six collaborators each examined a total of 6 subsamples of prepared horseradish. Each subsample was spiked with 20 insect fragments (0.25×0.25 mm), 20 rodent hairs (1–2 mm), 5 *Tribolium sp.* adults, and 5 *Tribolium sp.* larvae.

This report of the Associate Referee, J. S. Gecan, was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Table 1. Collaborative results for per cent recovery of added insect and rodent filth spikes from prepared horseradish[a]

Coll.	Insect fragments	Rodent hairs	Adult *Tribolium*	Larval *Tribolium*
A	100	85	100	100
	90	95	100	100
	90	90	100	100
	90	90	100	80
	85	95	100	100
	95	75	100	100
B	85	80	100	100
	100	95	100	100
	90	90	100	100
	80	90	80	80
	90	85	100	100
	80	100	100	100
C	100	80	100	100
	100	65	100	100
	100	25	100	100
	100	40	100	100
	100	45	100	100
	100	45	100	100
D	100	100	—[b]	—
	100	85	—	—
	95	85	—	—
	95	90	—	—
	100	80	—	—
	100	85	—	—
E	100	90	100	100
	95	90	100	100
	95	80	100	100
	95	90	80	100
	100	75	80	100
	100	90	100	100
F	100	90	100	100
	100	90	100	100
	85	100	100	100
	95	90	100	100
	100	95	100	100
	100	100	100	100
Av. rec.	95	83	98	98
Coeff. of var., %	6	21	6	5

[a] Each subsample was spiked with 20 insect fragments, 20 rodent hairs, 5 adult *Tribolium*, and 5 larval *Tribolium*.

[b] Spike accidentally omitted from sample.

Results and Recommendations

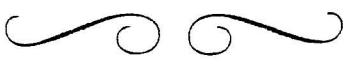
Table 1 shows the collaborators' recoveries of spike materials. Collaborative study of the method resulted in the following average per cent recoveries and coefficients of variation: insect fragments, 95 (6%); rodent hairs, 83 (21%); adults, 98 (6%); and larvae, 98 (5%). All collaborators reported the method to be rapid and easy to perform, and to result in clean filter papers.

The Associate Referee recommends that the proposed method be adopted as official first action.

The recommendation of the Associate Referee was approved by the Referee and Subcommittee F and was adopted by the Association: see (1973) *JAOAC* 56, 408.

Acknowledgements

The Associate Referee wishes to express his thanks to the following collaborators of the Food and Drug Administration: P. DeCamp, Philadelphia; J. Smith, Detroit; J. Thrasher, Washington, D.C.; and R. Trauba, Minneapolis; and J. Miller, Kansas State Department of Health, Topeka; and W. Zimmerman, Wisconsin State Department of Agriculture, Madison.



ATTENTION

Abstracts of papers or reports to be presented at the 87th Annual Meeting of the AOAC, Oct. 9–12, 1973, MUST be submitted on standard, preprinted forms. The forms are available, on request, from the AOAC Editorial Office.

Since 1971 the Abstract Bulletin has undergone two changes. The abstract submitted by the author is now reproduced directly by a photographic process; it is no longer edited and retyped in the Editorial Office. This new procedure makes it imperative that authors provide an informative, carefully prepared abstract typed on the special form, plus 2 additional copies (carbon copies or photocopies). This form includes general rules for preparing satisfactory abstracts. Abstracts not fulfilling these requirements will be returned to the author for correction, if time permits. Improperly prepared abstracts received too late to allow this will not appear in the Abstract Bulletin. The Abstract Bulletin itself is a new size—4 × 9″, the same size as the AOAC program.

In addition to the abstract, five copies of *all* contributed papers and Associate Referee reports must be submitted to the AOAC office. The manuscripts should be double-spaced throughout. Unless otherwise specified, all manuscripts received will be considered for publication in *JAOAC*, pending satisfactory review. Each Associate Referee should also send one copy of his report to his General Referee.

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Write for standard abstract forms (one required for each presentation). Mail your abstract in early.

DEADLINE for receipt of abstracts—August 17, 1973.

Collaborative Study of an Enzymatic Digestion Method for the Isolation of Light Filth from Fresh Pork Sausage

JOHN S. GECAN and PHILIP ALIOTO*
Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204
** General Laboratory Division, Wisconsin State Department of Agriculture, Madison, Wis. 53705*

An alternative method has been developed for the isolation of light filth from fresh pork sausage. The method involves particle size reduction, enzymatic digestion, wet sieving, deaerating, and floating with *n*-heptane-mineral oil from cooled 40% isopropanol. Collaborative study of the method showed the extraction papers and the recovery of light filth elements to be comparable to or better than those obtained for 40.B07. The method has been adopted as official first action as an alternative to 40.B07.

The present official AOAC method for the isolation of light filth from fresh pork sausage, 40.B07, involves mechanical dispersing in a surfactant, wet sieving, solvent dehydrating and defatting, deaerating, and floating with an *n*-heptane-mineral oil mixture from cooled 40% isopropanol. The collaborative study, carried out in 1971, showed the method to yield good recoveries and clean filter papers; however, the mechanical dispersion and solvent defatting operations proved to be quite time consuming. The object of this study was to develop an alternative method for product dispersion and defatting that is less demanding of analytical time.

A method has been developed that greatly reduces the actual analytical time required for product dispersion and solvent defatting; additionally, the method provides the option for either direct transfer of filth from the sieve or flotation, depending upon the amount of spice tissue in the product. The method involves mechanical particle size reduction, optional rapid or overnight enzymatic digestion, wet sieving, deaerating, and either direct transfer of small amounts of sieve retainings or flotation with *n*-heptane-mineral oil from cooled 40% isopropanol.

Experimental

The modified method utilizes a standard meat grinder to reduce particle size in order to present a greater surface area for enzymatic activity. Because of the high fat content of the product, an acid-stable emulsifier (Igepal CO-730) was added to improve the efficiency of digestion. The method specifies the use of either of 2 grades of pepsin. Both grades give comparable results when used as specified; however, if either type has been improperly stored, the efficiency of the digestion may be greatly reduced. The authors found that magnetic stirring, using air- or water-powered submersible stirrers, further improved the digestion; however, this specification was not incorporated into the method in order to limit the equipment requirements and to maintain simplicity. The extensive solvent defatting used in the official method was eliminated by the use of more efficient defatting agents, Igepal CO-730 and Triton X-114, in conjunction with wet sieving. The proposed alternative method, after the wet sieving operation, is identical to the present official method. Since intralaboratory trials by the proposed method resulted in equivalent recoveries of spikes and comparable extraction papers, as compared to the official method, a collaborative study was initiated.

METHOD

40.C01 ***Reagents***

(a) *Igepal CO-730.*—Nonionic surfactant, nonylphenoxypoly(ethyleneoxy) ethanol (GAF Corp., or equiv.).

(b) *Pepsin.*—1:10,000 activity (Difco Laboratories, or equiv.), or NF.

(c) *Triton X-114.*—Alkylaryl polyether alcohol (Rohm and Haas Co., or equiv.).

(d) *Flotation oil.*—*n*-Heptane, 40.003(q), and light mineral oil, 40.003(w), (3+17).

40.C02 ***Determination***

Grind sausage, using meat grinder with end plate having 3/16" holes. Weigh 225 g sample into 1.5–2 L beaker. Add 980 ml warm H_2O and 20 ml Igepal CO-730, (a); stir 5 min. Add 20 ml HCl and stir 1 min. Proceed with overnight digestion, (a), or rapid digestion, (b).

(a) *Overnight digestion.*—Add 0.5 g 1:10,000 pepsin or 2.0 g NF pepsin and stir 1 min. Digest in 50°

H_2O bath 18 hr. Add 5 ml Triton X-114, and stir 1 min. Keep all samples at digestion temp. in bath until ready to sieve. Sieve portionwise on No. 230 sieve. Transfer to ruled filter paper if small amt residue remains on sieve or proceed with flotation, (c).

(b) *Rapid digestion.*—Add 2.0 g 1:10,000 pepsin or 10 g NF pepsin and stir 1 min. Digest in 62° H_2O bath 2 hr. Add 5 ml Triton X-114, and stir 1 min. Keep all samples at digestion temp. until ready to sieve. Sieve portionwise on No. 230 sieve. Proceed with flotation, (c).

(c) *Flotation.*—Wet residue on sieve with 40% isopropanol. Transfer quant. to 2 L trap flask, using 40% isopropanol. Bring vol. to 1 L with 40% isopropanol and add 50 ml HCl. Add magnetic stirring bar and, with gentle stirring, boil 10 min on magnetic stirring hot plate. Cool to room temp. in cold H_2O bath and add 40 ml flotation oil. Stir 3 min with magnetic stirrer at speed at which no splashing occurs and stirring bar is just visible at bottom of vortex. Let oil phase sep. 1 min, and slowly fill flask with 40% isopropanol by letting liq. flow down stoppered rod while top of stopper is maintained just above flask contents. After filling flask, gently stir settled plant material with stoppered rod 5–10 sec. Let stand undisturbed 5 min and immediately trap off. Add 25 ml light mineral oil, stir gently by hand 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with isopropanol and transfer washings to beaker contg trappings. Filter onto ruled paper and examine microscopically.

Results and Recommendation

Five collaborators each examined a total of 6 samples by the proposed method, 3 samples each of bulk and link sausage. The spike material for each sample consisted of 20 elytral fragments (0.3–0.5 mm), 20 rat hair fragments (2–3 mm), 5 *Tribolium sp.* larvae, and 5 *Tribolium sp.* adults.

Table 1 shows recoveries of spiked filth elements from both types of sausage by the proposed method. Rodent hair recoveries were slightly

Table 1. Collaborative results for per cent recovery of added filth elements from bulk and link fresh pork sausage[a]

Coll.	1	2	3	4	5	6	1	2	3	4	5	6
	Adult *Tribolium* (5 added)						Larval *Tribolium* (5 added)					
1	100	100	100	100	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	80	80	80	100	100	80
3	100	100	100	100	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	80	100	100	100	80	80
5	100	100	100	100	100	100	100	100	100	100	100	100
Av. rec						100						94
Coeff. of var., %						0						9
	Insect fragments (20 added)						Rodent hairs (20 added)					
1	100	95	100	90	95	90	65	60	60	90	80	65
2	80	85	85	80	80	50	80	75	85	85	95	25
3	95	80	90	100	90	90	85	90	100	100	95	95
4	90	80	75	85	85	90	75	75	80	80	80	85
5	90	90	80	100	100	95	80	85	75	85	80	75
Av. rec						88						80
Coeff. of var., %						11						18

[a] Samples 1–3 are bulk-type sausage and 4–6 are link-type sausage.

Table 2. Comparison of average per cent recovery and coefficient of variation for added filth elements from fresh pork sausage by the proposed and official methods

	Insect fragments		Rodent hairs		Adult *Tribolium*		Larval *Tribolium*	
Statistic	Off.	Prop.	Off.	Prop.	Off.	Prop.	Off.	Prop.
Av. rec., %	89	88	73	80	86	100	84	94
Coeff. of var., %	12	11	24	18	26	0	28	9

lower and more variable than those obtained during intralaboratory trials. All collaborators obtained clean extraction papers for the link sausage; however, 3 obtained extraction papers containing more plant debris than expected for the bulk sausage. One collaborator reported that the digested sausage was permitted to cool before sieving and that fat droplets were subsequently observed in the sieve retainings. The extraction papers obtained from the analyses of these cooled subsamples contained excessive amounts of plant tissue. A note of caution has been inserted in the method regarding this point.

Table 2 shows a comparison of the average per cent recoveries and per cent coefficients of variation obtained by collaborators for the proposed and the official methods. These data show that the average per cent recoveries and coefficients of variation by the proposed method are equivalent to or better than those by the official method. Individual recovery data for the official method for spiked filth elements obtained during the 1971 collaborative study appear in (1972) *JAOAC* 55, 66–68.

The proposed method is recommended for adoption as official first action as an alternative to 40.B07, (1972) *JAOAC* 55, 441.

Acknowledgments

The authors wish to acknowledge the help of the following collaborators: Russell Proxterman, Kansas State Department of Health, Topeka; V. J. Winkelman, Minnesota Department of Agriculture, St. Paul; and the following members of the Food and Drug Administration: Marvin Carlson, Minneapolis; J. Phyllis Skyrme, Boston; and Joel J. Thrasher, Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee F and was adopted by the Association; *see JAOAC* 56, 408.

This report of the Associate Referee, J. S. Gecan, was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Collaborative Study of the Extraction of Light Filth from Canned Whole and Cream Style Corn

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

An improved method has been developed for canned whole and cream style corn to replace 40.104. The proposed method is unique because the microscopic and macroscopic examinations can be conducted almost concurrently; in the official method the macroscopic examination is dependent upon completion of the microscopic examination. The proposed method is shorter and it eliminates the use of viscous castor oil by specifying extraction in a percolator with paraffin oil from hot 40% isopropanol. Glassware is easily cleaned up after percolator cycles with isopropanol and water rinses. The proposed method yielded improved recoveries of insect and rodent filth spike elements. The method has been adopted as official first action.

The official AOAC method for filth in corn, 40.104, involves a trapping procedure which must be performed first in order to proceed into the macroscopic examination. The 40 min extraction must be carried to completion before the macroscopic examination can be started. The proposed method has a rapid oil separation that permits almost concurrent microscopic and macroscopic examinations.

The objectives of this study were to shorten the official method and to eliminate the use of castor oil as a flotation liquid. Castor oil was undesirable because its high viscosity impedes filtration of the oil-extracted filth and necessitates the use of hot alcohol in the cleanup of glassware. In addition, castor oil is a nonstandardized reagent that deteriorates during extended storage.

In the proposed method, castor oil has been replaced with paraffin oil, a stable standardized reagent, which is not adversely affected by storage. The cleanup procedures are also simplified to alternate rinses of isopropanol and water.

Collaborative Study

Six collaborators were requested to analyze 5 samples of cream style corn and one of whole kernel by the proposed method. In addition, 2 samples of cream style and 1 of whole kernel corn were to be analyzed by the official method. A collaborator was also selected from the Division of Microbiology to analyze 11 cream style and 7 whole kernel corn samples by the official method only.

The filth elements added to each sample consisted of whole insects or equivalent, insect fragments, and rodent hairs as outlined below:

1. 1 adult house fly
2. 1 corn earworm larval head
3. 1 corn earworm larval posterior terminal end
4. 10 corn earworm larval abdominal sections
5. 10 rodent guard hairs exceeding 5 mm

METHOD

Canned Whole and Cream-Style Corn—Official First Action

40.C03 ***Macroscopic and Microscopic Examination***

Place 200 g well mixed sample in 1.5 L beaker and add 1 L 40% isopropanol. Bring to boil, stirring magnetically, 40.004(b), add 50 ml mineral oil, 40.003(w), and boil and stir 3 min more. Transfer immediately to Corning percolator contg ca 100 ml 40% isopropanol and glass or metal rod for forcing corn thru spout. Retain stirring bar in beaker. Rinse bar with undild isopropanol. Add ca 900 ml 40% isopropanol to beaker, stir, and add to percolator. Reserve beaker. After ca 5 min standing, drain percolator to within 3″ of bottom onto 8″ No. 20 sieve nested in large white enamel tray of ca 2 L capacity. Use rod to force corn thru percolator drain spout. Withdraw rod after removing corn from percolator and wash with small amt undild isopropanol into reserved beaker. Discard isopropanol collected in tray. Leave sieve in place with retained corn material. Using reserved beaker, add ca 1.5 L hot tap H_2O (50–70°) to percolator. Let phases sep. ca 3 min and make final drain. Discard all but last 2″ oil-aq. phase. Drain into 600 ml beaker. Wash sides of percolator with alternate isopropanol and H_2O rinses, and collect in same beaker. Add rest of corn from can to corn retained on sieve, sieve portionwise if necessary, and wash with tap H_2O to remove starch and fine particles. Reverse sieve into white enamel tray. Wash corn into tray with forceful spray of H_2O (ca 22°) to 1″ depth in tray. Let corn settle and examine under H_2O for worm-eaten or rotten kernels and whole

Table 1. Collaborative results for the recovery of whole insects (W/E),[a] insect fragments (IF),[a] and rodent hairs (RH)[b] from canned cream style and whole kernel corn by the proposed method and the official method (40.104)

	Proposed Method[c]										Official Method[d]					
	1		2		3		4		5		1		2		3	
Coll.	W/E+IF	RH	W/E+IF	RH	W/E+IF	RH	W/E+IF	RH	W/E+IF	RH	W/E+IF	RH	W/E+IF	RH	W/E+IF	RH
A	13	3	13	8	13	10	13	9	11	9	3	1	2	2	12	10
B	13	2	11	10	13	5	8	10	13	1	12	1	11	10	12	8
C	12	7	13	10	12	7	13	10	12	10	12	0	11	1	12	3
D	13	9	13	8	13	6	13	7	13	5	13	5	13	0	13	10
E	13	7	12	10	13	10	11	9	12	10	12	3	12	4	12	7
F	13	10	13	4	13	4	12	5	13	8	13	9	13	7	13	10
											13	6	10	7	11	4
G[e]											13	5	9	4	13	3
											13	1	12	4	12	1
											12	1	12	1	11	2
											12	3	10	4	11	1
											13	3	12	3	11	8

Av. rec., % (W/E+IF):
Colls. A–F, Prop. 95.3
Colls. A–F, Off. 86.1

Av. rec., % (W/E+IF):
Coll. G, Off. 89.2
Colls. A–G, Off. 86.6

Av. rec., % (RH):
Colls. A–F, Prop. 74.5
Colls. A–F, Off. 50.5

Av. rec., % (RH):
Coll. G, Off. 30.4
Colls. A–G, Off. 42.2

[a] One whole fly, 10 abdominal insect larvae fragments, 1 larval head, and 1 larval posterior terminal end were added. All collaborators found the whole fly except Collaborator A for sample 2 by the official method.
[b] Ten rodent hairs (5 mm guard hairs) were added.
[c] Samples 1–4, proposed method, were cream style corn; Sample 5 was whole kernel corn.
[d] Samples 1 and 2, official method, were cream style corn; Sample 3 was whole kernel corn.
[e] Used official method only.

worms, heads, or large fragments. Add these to trappings previously obtained from percolator. Tip tray and slowly decant H_2O while carefully observing flowing H_2O for insect fragments. Refill tray with 1" H_2O (ca 22°) and repeat decantation, examining closely for objectionable material. Discard pan contents. Filter beaker contents thru ruled filter paper and examine microscopically. If filtration is impeded by excessive starch material, proceed as in 40.004(d).

Results and Recommendation

For statistical purposes, all insect elements were considered together in determining the average recovery. Recoveries of the above filth elements are shown in Table 1.

All 6 collaborators thought the method in general was easy to perform. The average analytical time was 77 min per sample, although the Associate Referee was able to perform the proposed method in about 50 min.

Filth papers were heavier in the proposed method than the official method because no screen was used to filter excess corn tissue. However, the corn tissue that floated in the proposed method was primarily silk and husk and this did not interfere with the microscopic observation of the natural filth elements.

Some difficulty was experienced by 4 of the 6 analysts in filtering the trappings by the proposed method. The Associate Referee suggests that when this occurs, the technique for removing starchy material, 40.004(d), should be followed.

Each analyst was asked to examine one sample for familiarization before carrying out the analysis on the test samples by the proposed method. This sample was spiked with the same elements as the collaborative study. The objective was to determine if the analyst could detect the elements in the macroscopic examination as well as to provide information on the time required to read the filter plate. Average time to read a plate was 22 min.

The Associate Referee has found that if both the percolator cycles and macroscopic examination are performed in a sink, spillage problems and manipulative difficulties are alleviated, thereby expediting the analysis.

Extractions by the proposed method are accomplished by 2 percolator cycles requiring a total of 8 min. By performing the macroscopic examination concurrently with the extractions, the entire method is shortened. Recoveries are also improved. The Associate Referee recommends that the proposed method be adopted as official first action to replace 40.104 for determination of filth in canned whole kernel or cream style corn.

Acknowledgments

The Associate Referee wishes to express his thanks to the following collaborators from the Food and Drug Administration: Jack LaRose, Seattle; Ruth Rupp, Washington, D.C.; G. E. Russell, Detroit; Richard L. Trauba, Minneapolis; and the following State collaborators: Stan Heffley, Kansas State Department of Health, Topeka; B. B. Woodward, Florida State Department of Agriculture, Tallahassee; William Zimmerman, Wisconsin State Department of Agriculture, Madison.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee F and was adopted by the Association; *see* (1973) *JAOAC* 56, 408.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Thin Layer Chromatographic Test for Mammalian Urine on Wheat

JOEL J. THRASHER
Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204

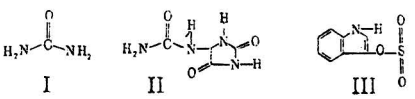
Follow-up experimental work of the 1971 preliminary method study revealed the need for a change in sample size, an improved extract cleanup technique, and the identification of a urine-specific metabolite. A modified method collaboratively tested in 1972 is capable of handling a larger sample in the Soxhlet extractor. The extract is cleaned of interfering substances by chromatography with an appropriate solvent prior to 2-dimensional TLC. The indicator metabolites, urea, allantoin, and indican, are detected by sequential sprays. The collaborative study resulted in 90% correct positive determinations with no false positives. The minimum detection level is approximately 2 μl urine/9 g wheat. The method has been adopted as official first action.

This is the second report on this topic (1). The objectives of the original study were to develop a simple qualitative test for urine beyond the mere presence of urea (I, *see* Fig. 1) and a simple test to identify the source of the urine. The first objective was apparently achieved when we found that allantoin (II, 5-ureidohydantoin) is the second highest amount of material in the urine of most mammals. As a result of the 1971 preliminary studies and additional research, we realized that changes in the method and sample size were necessary.

Experimental

The major changes of the 1972 collaborative study were: increased sample size, modified cleanup, and detection of an additional metabolite. The cleanup procedure presented in the 1971 report was modified to handle a larger size sample extract. In lieu of the multiple solvent cleanup in a test tube, the wheat extract was cleaned directly on the TLC plate, using an appropriate solvent system prior to chromatography.

As a result of the improved cleanup technique, both urea and allantoin were detected in all wheat samples analyzed. The finding of allantoin in cleaned wheat prompted an additional search of the literature. A 1931 article reported finding allantoin as a naturally occurring constituent in 2 varieties of wheat (2). This finding forced the Associate Referee to seek another urine component recoverable from small amounts of urine residue. Two possibilities were found: creatine/creatinine and indoxyl sulfate, more often called urinary indican.



I II III

FIG. 1—Structures: I, urea; II, allantoin; III, indican.

Creatine/creatinine levels in human urine are influenced by sex, according to Kilbrick (3), and levels in mice are also sex-dependent (4). Attempts to recover creatine or creatinine from small amounts of urine residue on wheat gave erratic results.

Mouse urine contains about 0.005% urinary indican (4). Thus, 2 μl mouse urine should contain about 0.1 μg indican (III, shown as the free acid, 3-indoxylsulfuric acid). This amount was detected routinely after proper spraying techniques were developed.

The R_f value of indican is very sensitive to the relative humidity of the chromatographic tank and/or previous handling of the chromatographic plate. At low relative humidity, the indican has a lower R_f value than allantoin. In high relative humidity the R_f value of indican is such that the compound may be completely obscured by a large amount of urea.

Ideally, indican, the smallest of the 3 spots, should be centered between the trailing edge of the large urea spot and the leading edge of the smaller allantoin spot. The center should be about R_f 0.60 with the urea at about 0.75 and 0.55 for allantoin. These R_f values are obtained at about 55% relative humidity, a condition that may be produced by using $NaHSO_4$ solution in presence of excess solid phase in the chromatographic tank.

An intralaboratory study indicated indican may be recovered from small amounts of urine on wheat. An interlaboratory collaborative study was then initiated.

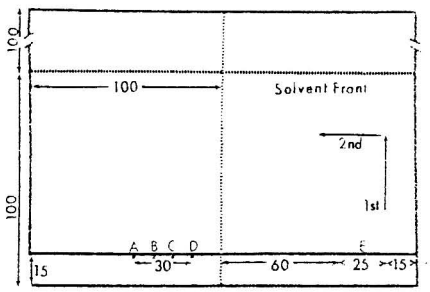


FIG. 2—Suggested spotting guide: 1 μl standard solutions spotted at A, tryptophan; B, allantoin; C, urea; D, indican; and E, sample along 25 mm band. Measurements in mm.

Collaborative Study

Seven collaborators analyzed wheat extracts consisting of 2 blanks, 4 low level spikes (2 μl/9 g), and 2 high level spikes (4 μl/9 g). They also received a suggested spotting guide (Fig. 2).

Sequential sprays were used to detect urea, allantoin, and indican after 2-dimensional thin layer chromatography. Each collaborator indicated on the reporting forms the approximate positions of the spots found after each step in the spray sequence.

The collaborators were asked to determine whether the samples were positive or negative for urine. For the positive determinations, they were asked to distinguish between the high and low levels of contamination.

METHOD

40.C05 *Apparatus*

(a) *Thin layer apparatus.*—*See* 29.006 and 29.007(c).

(b) *Blender.*—High speed, with stainless steel semimicro jar (Arthur H. Thomas Co., No. 4282-F, or equiv.).

(c) *Soxhlet extractor.*—250 ml extn flask; extn chamber 39 mm id × 115 mm with top joint ⅎ 45/50 and bottom joint ⅎ 24/40; 35 × 90 mm thimbles; condenser joint ⅎ 45/50 (Arthur H. Thomas Co., No. 4406-E34, or equiv.).

(d) *Kuderna-Danish concentrator.*—*See* 29.005(e).

40.C06 *Reagents*

(a) *Urea soln.*—20 mg/ml H_2O.

(b) *Allantoin soln.*—2 mg/ml H_2O.

(c) *Indoxyl sulfate (indican).*—Approx. 0.1 mg/ml H_2O (Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178). Store in dark.

(d) *Tryptophan.*—1 mg/ml 50% aq. acetone (used as longwave fluorescent marker with R_f approx. that of urea in developing solv.).

(e) *p-Dimethylaminobenzaldehyde (pDAB) spray.*—Mix 75 ml MeOH and 25 ml HCl, and cool to 70°. Add 2 g reagent grade pDAB to cooled mixt. and dissolve with stirring. (*Caution:* Spray reagent is toxic and corrosive. *See* 46.017.)

(f) *Sodium acetate spray.*—Satd aq. soln.

(g) *Developing solvent.*—*n*-BuOH-HOAc-H_2O (10+5+5); prep. fresh daily.

(h) *Cellulose powder.*—TLC grade, MN 300 (Brinkmann Instruments, Inc.).

40.C07 *Extraction of Sample*

(*Caution: See* 46.011, 46.039, and 46.046.)

Prep. plates for overnight drying, and equilibrate TLC tank before Soxhlet extn of sample (ca 1 hr).

(a) *Soxhlet extraction.*—Ext 18 g sample with 60 ml acetone 1 hr at 3–4 min/siphon. Transfer ext to 100 ml g-s graduate, dil. to vol., and take aliquot equiv. to 9 g sample. Conc. as below.

(b) *Alternative extraction.*—Place 18 g sample in 50 ml beaker or erlenmeyer. Add 1 ml acetone/g sample and boil gently 5 min, avoiding excessive loss of acetone. Decant thru glass wool pad into 100 ml g-s graduate. Repeat acetone boil and decanting 3 addnl times, and dil. to 100 ml. Conc. aliquot equiv. to 9 g as below.

Conc. aliquot to ca 0.2 ml in Kuderna-Danish concentrator as in 29.012; or, alternatively, place empty 13 ml graduated conical centrf. tube in beaker of boiling H_2O. When tube is hot, slowly transfer portions ext, using syringe fitted with long needle, to evapn tube. Let each portion evap. before next is added. Evap. to ca 0.20 ml. Chromatograph without appreciable delay.

40.C08 *Thin Layer Chromatography*

Prep. TLC tank as follows: Add ca 150 ml satd $NaHSO_4$ soln to lined tank; then add ca 15 g addnl solid $NaHSO_4$ to bottom of tank. Place empty solv. trough in bottom of tank and cover tank.

Prep. plates as follows:

(a) *Brinkmann-DeSaga.*—Add 15 g cellulose to 100 ml H_2O in blender operating at ca 90 v setting of variable transformer. Use small spatula to work powder into H_2O. Turn variable transformer to 120 v (line voltage) and blend ≥1.5 min. Apply slurry as 0.375 mm layer to 5 plates and air-dry plates overnight.

(b) *Quickfit plate leveler and spreader.*—Add 12 g cellulose to 75 ml H_2O in blender operating at 90 v setting of variable transformer. Use small spatula to work powder into H_2O. Turn transformer to 120 v (line voltage), add 15 ml H_2O, and blend ≥1.5 min.

Using 0.50 cm slot, spread slurry on 5 plates and air-dry plates overnight.

Spot sample conc. as band ca 25 mm long on line 15 mm up and 15 mm in from edge of plate. Wash sides of evapn tube with ca 50 μl acetone and transfer wash to sample band area. Repeat 50 μl washings and transfers until last transfer is colorless (ca 4 transfers). Spot 1 μl each of std solns (a)–(d) ca 10 mm apart along line 15 mm to left of center of plate and 15 mm from edge of plate.

Place plate in trough contg ether in lined tank presatd with ether. Let ether travel to top of plate. Remove plate and let air-dry. Immediately draw intersecting lines to divide plate in 4 equal sqs. Dry plate 5 min in 80° forced-draft oven. Remove plate from oven and promptly place in dry solv. trough in TLC tank with spotted band down. Close tank and let stand 20 min. Slide top aside just enough to introduce long stem funnel into solv. trough. Slowly add 20 ml developing solv. to trough. Close lid and develop to line of first direction. Dry plate 5 min in 80° forced-draft oven.

Rotate warm plate to place chromatographed stds in upper left quarter of plate and promptly place in dry trough in tank. Let stand 20 min without touching any liq. in closed tank. Then slide cover aside just enough to introduce long stem funnel into solv. trough and slowly add 20 ml developing solv. Let front move to line in this second dimension. Dry plate 5 min in 80° forced-draft oven.

Spray plate with pDAB reagent until distinctly moist but *not* shiny wet and again heat 5 min in 80° forced-draft oven. Strong yellow-to-orange area at R_f 0.75–0.80 is urea. Pale yellow smaller spot at R_f 0.45–0.50 is allantoin. Mark each area as color develops, since colors fade from one step to next. Place under longwave black light, 29.007(c), in darkened room and check for pale yellow fluorescent area between urea and allantoin. Spray satd NaOAc soln (ca 1–2 ml/plate) in space between urea and allantoin until yellow of *both* has faded. Let plate air-dry ca 10 min in hood (do *not* heat), and check plate under longwave black light. Weak fluorescent pink-to-orange color against very pale blue fluorescent background confirms presence of urinary indican.

Results and Recommendations

Most collaborators felt that the method was capable of detecting small amounts of urine residue; however, 2 collaborators reported that they were unable to distinguish between the 2 levels of spiked samples. Several reported that the use of one plate per sample was a limitation to the usefulness of the method for regulatory purposes. A modification is being developed which will permit spotting more samples per plate with 1-dimensional chromatography for 10 cm, but this requires more manipulations and time. This change, once it has been proven reliable, may be incorporated as an option in the method reported above.

Table 1. Collaborative results for the TLC examination of wheat extracts for rodent urine[a]

Coll.	Spiking level: Low	Spiking level: High
A	L L L L	H H
B	L L L L	(B) (B)
C	L L L H	H L
D	L L L L	L L
E	L L L L	L L
F	L L L L	(B) (B)
G	L L L L	b b

[a] L, low level positive result; H, high level positive; B, incorrectly reported as blank; b, not completed. All collaborators examining 8 extracts, A–F, correctly reported the 2 blanks received.

Table 1 shows the results of the collaborative determinations for the presence of urine indicators in extracts of wheat; 90% of the contaminated sample extracts were reported as positive. No false positives were reported in the blank sample extracts.

It is recommended that the method be adopted as official first action, and that the study be extended to other food seeds and food packaging materials.

Acknowledgments

The Associate Referee gratefully acknowledges the work and comments of the following Food and Drug Administration collaborators: Annette Abadie, Washington, D.C.; Sandra Bell, Washington, D.C.; Earl Bloomingdale, Kansas City; J. E. Harrell, Atlanta; J. LaRose, Seattle; Joan Smith, Detroit; and R. L. Trauba, Minneapolis.

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This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee F and were adopted by the Association; *see JAOAC* 56, 408.

Method for Determining Direction of Insect Boring Through Food Packaging Materials

PARIS M. BRICKEY, JR., JOHN S. GECAN, and ALBERT ROTHSCHILD*
Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204
** Petitions Control Branch*

Insect chewing on food packaging materials has been characterized in order to differentiate between entrance and exit bore holes. The characteristics specific to various types of packaging are presented as a method in the form of a descriptive "key" that permits determination of the direction of boring. A collaborative study of the method resulted in 92% correct determinations of boring direction. The method has been adopted as official first action.

A number of court litigations during the past few years have indicated the need for a method for determining the direction in which insects bore or chew through food packaging materials. This is especially important in determining when and where a product became contaminated with whole insects.

Insect infestation of foods may occur before or during processing, while in storage, during transit, or on the dealer's shelf. If there are no holes or tears in the package and all seams appear to be tightly sealed, it is reasonable to assume that the infestation originated before or during processing. When insect-bored holes are present in the packaging, the source of the infestation is more difficult to determine. However, if the direction in which insects bored into the material can be determined, the origin of the infestation may be ascertained. For example, if the hole in the package indicates that insects egressed, then the infestation had to occur before packaging. If the hole indicates entry into the container, then the infestation had to occur after packaging.

The authors initiated a study to characterize insect chewing and to determine the direction of boring from the chewing characteristics.

Experimental

Twelve species of insects were used: *Tribolium confusum*, *T. castaneum*, *Sitophilus oryzae*, *S. granarium*, *Oryzaephilus mercator*, *O. surinamensis*, *Trogoderma inclusum*, *Attagenus piceus*, *Rhyzopertha dominica*, *Lasioderma sericorne*, *Ephestia cautella*, and *Plodia interpunctella*.

Packaging materials consisted of Kraft paper bags, Cellophane, plastic, foil, and combination laminates. Each type of material was sandwiched between 2 glass vials. Ten to twenty insects of one species were placed in the top vial and food was placed in the bottom vial. Upon penetration through the layer of material the insects fell into the bottom vial and therefore were unable to bore back into the top vial. Packaging material was removed for examination when boring was evident. If the insects in a vial died before penetration was accomplished, another group of the same species was placed in a sealed bag constructed of the testing material. The bag was then placed in a glass jar containing uninfested culture media. The bag was removed for examination as soon as boring was evident. The controlled boring experiment yielded packaging materials with specific chewing characteristics that enabled the authors to develop a key of chewing characters for determining the direction of insect boring. Since intralaboratory trials using the boring characteristics key resulted in a high percentage of correct determinations, a collaborative study of the identification method was initiated.

METHOD

40.C09 *Direction of Insect Penetration into Food Packaging*

(a) **Entrance Characteristics**

(*1*) *Kraft paper, paper box.*

(*a*) *Surface fraying.*—Consists of paper fibers cut and lifted from surface of packaging material by mandibular activity. Represents first activity of hole formation. May occur at random on "entrance" surface of packaging materials. *See* Fig. 40:C1 (B).

(*b*) *Terraced depression.*—Consists of "step effect" formed when *secondary depression* is superimposed on *initial depression; see* Fig. 40:C1(D). This terracing may be present around entire perimeter of final hole or at one or more points around it.

This report of the Associate Referee, P. M. Brickey, Jr., was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

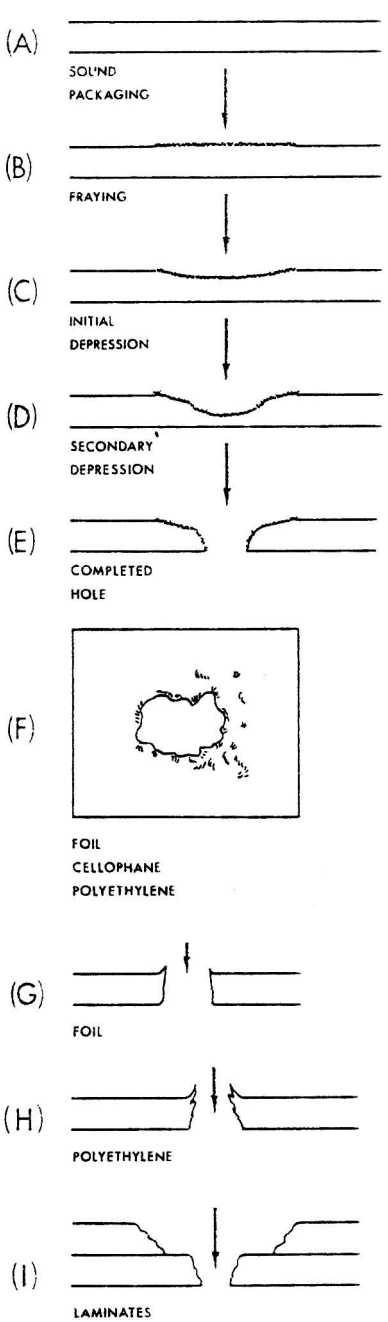

FIG. 40:C1—Insect penetration of packaging.

(*c*) *Tapered hole.*—Diam. of hole is greater on entrance side than exit side. This feature is most obvious on thicker packaging materials. *See* Fig. 40:C1(E).

(*2*) *Foil, Cellophane, polyethylene plastic, waxed paper.*

(*a*) *Mandibular scratches.*—Found on Al foil, Cellophane, and polyethylene plastic. Consist of small, short surface scratches or grooves formed by pincer-like action of mandibles. Frequently observed around perimeter of hole or in localized groups at random on entrance surface. *See* Fig. 40:C1(F).

(*b*) *Upturned edges.*—Present around perimeter of holes in Al foil and polyethylene plastic materials. Appear as continuous irregularly upturned edge in foil (Fig. 40:C1(G)) and generally as upturned fraying of plastic (Fig. 40:C1(H)) in polyethylene materials. Not observed on waxed paper and Cellophane materials.

(*c*) *Roughened surface.*—Observed around perimeter of holes or randomly on surface of polyethylene plastic and waxed paper. Consists of surface fraying or pulled up tufts resulting from mandibular action on material. Distinct mandibular scratches may be observed around or in roughened areas.

(*3*) *Foil/paper, foil/plastic, or other laminates.*

(*a*) *Entrance characteristics.*—*See* specific materials.

(*b*) *Terracing of laminates.*—Quite common on entrance side of these materials. Observed as larger hole bored in laminate material on entrance surface and smaller hole in exit side of material. *See* Fig. 40:C1(I).

(b) Exit Characteristics

All types of packaging materials.

(*1*) Clean-cut hole perimeter.

(*2*) Diam. of hole smaller than on entrance side.

(*3*) No surface fraying, scratches, or depressions (*see* Fig. 40:C1(E)).

Results and Recommendation

Eight collaborators each examined a total of 5 pieces of insect-bored packaging material. Each piece of packaging material was marked with the letter A on one side and B on the other. Using the key of chewing characteristics for entrance and exit holes, the collaborators were asked to determine the direction of boring.

Table 1 shows the results of the collaborators' determination of direction of boring. Five of 8 collaborators made 100% correct determinations. The collaborators generally agreed that the chewing characteristics key was a simple and rapid aid for determining boring direction. The Associate

Table 1. Collaborative results for the determination of direction of insect boring in food packaging materials[a]

Coll.	Heavy paper	Poly-ethylene	Kraft paper	Aluminum foil/poly-ethylene laminate	Aluminum foil/paper laminate	Cellophane	Wax paper
1	− +	+	+	+			
2	+	− +			+	+	
3	+ +	+	+	+			
4	+ +	+		+			
5	+ +	+	+		+		
6	+	+	+	+		−	
7	+ +	+	+	+			
8	+ +	+	+		+		

[a] + = Correct determination of boring direction; − = incorrect determination of boring direction; blank indicates collaborator did not receive this sample.

Referee recommends that the proposed key of insect boring characteristics be adopted as official first action.

Acknowledgments

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The recommendation of the Associate Referee was approved by the General Referee and Subcommittee F and was adopted by the Association; *see* (1973) *JAOAC* 56, 408.

MYCOTOXINS

Preparation of ^{14}C-Labeled Aflatoxin B_1

GRANT L. SCHOENHARD[1], RUSSELL O. SINNHUBER, and DONALD J. LEE
Department of Food Science and Technology, Oregon State University, Corvallis, Ore. 97331

A successful method to obtain ^{14}C-labeled aflatoxin B_1 from *Aspergillus parasiticus* ATCC 15517 was developed. The nitrogen-free resting culture contained mycelia from a 72 hr primary culture, 0.02*M* glucose, 0.005*M* (1 mCi/mmole) acetate-1-^{14}C, salts, and trace metals. It was incubated 12 hr at 30°C. Aflatoxin B_1-^{14}C was isolated from chloroform extracts of the resting culture by column chromatography on silica gel H eluted under pressure with a continuous gradient of chloroform to chloroform-methanol (98+2). The ultraviolet spectrum of the ^{14}C-labeled aflatoxin was examined. The ratios of the absorbances at 220/265 and 362/265 nm compared to established values and a reference standard were used as criteria of chemical purity. Label integrity was verified by chromatography, hydrogenation to the tetrahydrodeoxoaflatoxin B_1, and conversion to the hemiacetal and the epimeric acetates. After purification, 1.37 mg ^{14}C-labeled aflatoxin B_1 of specific activity 744 μCi/mmole was obtained from 2 mCi acetate-1-^{14}C of specific activity 1 mCi/mmole.

Aflatoxin B_1-^{14}C has been obtained by the addition of labeled precursors to mold culture after mycelial growth (1), to mold mycelia in nitrogen-free resting culture (2–6), to rice culture (7, 8), or by catalytic tritium exchange (9). Ayres, Lee, and Sinnhuber (7, 8) reported that the above resting cell preparation yielded a highly radioactive impurity with chromatographic characteristics similar to aflatoxin B_1. Labeled aflatoxin B_1 produced on rice culture had a low specific activity. The labeled precursor was presumably diluted in the complex rice culture.

This communication presents a successful method for the preparation of ^{14}C-labeled aflatoxin B_1 from resting cultures of *Aspergillus parasiticus*. The ratios of the absorbances (nm) of aflatoxin B_1 at 220/265 and 362/265 compared to the published values (10) and a reference standard were used as criteria of chemical purity. Label integrity was verified by chromatography, hydrogenation to the tetrahydrodeoxoaflatoxin B_1 (THDB$_1$), and conversion to the hemiacetal and the epimeric acetates.

[1] The material presented herein is taken in part from a thesis submitted by G. L. Schoenhard in partial fulfillment of the requirements for the degree of Master of Science in 1972.

METHOD

Reagents

(a) *Silica gel.*—MN-silica gel H (Brinkmann Instruments, Inc., Westbury, N.Y.).

(b) *Acetate-1-^{14}C.*—2 mCi of specific activity 25 mCi/mmole (Calbiochem, Los Angeles, Calif.).

(c) *Primary culture stock solutions.*—(*1*) *Glucose.*—50 g/250 ml. (*2*) *Salts (g/100 ml).*—$(NH_4)_2SO_4$, 4.0; KH_2PO_4, 10.0; $MgSO_4.7H_2O$, 2.0. (*3*) *Trace metals (mg/100 ml).*—$Na_2B_4O_7.10H_2O$, 70; $(NH_4)_6Mo_7O_{24}.4H_2O$, 50; $Fe_2(SO_4)_3.6H_2O$, 1000; $CuSO_4.5H_2O$, 30; $MnSO_4.H_2O$, 11; $ZnSO_4.7H_2O$, 1760.

(d) *Resting culture stock solutions.*—(*1*) *Glucose.*—9.0 g/250 ml. (*2*) *Sodium acetate.*—Anhydrous, 0.3936 g/100 ml. (*3*) *Nitrogen-free salts (g/100 ml).*—KH_2PO_4, 5.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5. (*4*) *Trace metals.*—*See* (c)(*3*).

(e) *Liquid scintillation counting fluor solution.*—1 L toluene, 4 g 2,5-diphenyloxazole, and 40 mg 1,4-bis(2-(5-phenyloxazolyl)), or above plus 40 g Cab-O-Sil (Godfrey L. Cabot, Inc., Boston, Mass.).

Apparatus

(a) *Rotary shaker.*—Model 75-732 (Eberbach Corp., Ann Arbor, Mich.).

(b) *Circulator.*—Lauda, Model K2/R (Brinkmann Instruments, Inc.), to maintain temperature in water bath attached to rotary shaker.

(c) *Blender.*—Kenmore, Model 600 (Sears, Roebuck and Co., Chicago, Ill.).

(d) *Flash evaporator.*—Model PF-10DN (Buchler Instruments, Fort Lee, N.J.).

(e) *Metering pump.*—Cheminert, Model CMP-1 (Chromatronix, Inc., Berkeley, Calif.).

(f) *Liquid scintillation spectrometer.*—Nuclear-Chicago.

(g) *Column and column accessories.*—12 × 500 mm id column with 12 × 1000 mm id extender (Kontes Glass Co., Vineland, N.J., No. K-422430).

Primary Culture

For 4 cultures of 100 ml each, combine 50 ml stock salt solution and 0.5 ml stock trace metal solution in 1 L Erlenmeyer flask. Add water to 250 ml, stopper with cotton plug, cap with aluminum foil, and sterilize 15 min at 121°C and 15 psi. Prepare and sterilize 125 ml glucose stock solution in 500 ml Erlenmeyer flask. Add ¼ of volume of above flasks to 4 separate sterile 500 ml Erlenmeyer flasks. Inoculate each medium with 1 ml *A. parasiticus* ATCC 15517 spores (2×10^5/ml). Dilute contents of each flask to 100 ml with sterile water to give following concentrations: g/L—glucose, 50; $(NH_4)_2SO_4$, 4.0; KH_2PO_4, 10.0; $MgSO_4.7H_2O$, 2.0; mg/L — $Na_2B_4O_7.10H_2O$, 0.7; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.5; $Fe_2(SO_4)_3.6H_2O$, 10.0; $CuSO_4.5H_2O$, 0.3; $MnSO_4.H_2O$, 0.11; and $ZnSO_4.7H_2O$, 17.6. Measure pH. If necessary, adjust pH to 4–5 with either sterile dilute HCl or NaOH.

Incubate flasks in 30°C water bath on shaker rotating at 144 rpm for first 24 hr and at 200 rpm for 48 hr more.

Harvest of Mycelia

Collect mycelia from each flask on cheesecloth over Büchner funnel attached to suction flask equipped with trap. Rinse well with distilled water. Place mycelia in blender, add 100 ml distilled water, and chop 10 sec on "blend" setting. Collect mycelia as before on fresh cheesecloth. Rinse well with distilled water. Place 5.0 g mycelial cake in clean indented 500 ml Erlenmeyer flask.

Resting Culture

Add 50 ml each of resting culture stock glucose, acetate, and nitrogen-free salts and 0.5 ml stock trace metal solution to 500 ml Erlenmeyer flask; dilute to 375 ml with water. To 4 separate Erlenmeyer flasks containing mycelia add 75 ml above medium and 0.5 mCi acetate-1-^{14}C (25 mCi/mmole). Dilute contents of each flask to 100 ml, resulting in following concentrations: glucose, 0.02*M*; acetate-1-^{14}C, 0.005*M* (1 mCi/mmole); KH_2PO_4, 5.0 g/L; $MgSO_4.7H_2O$, 0.5 g/L; KCl, 0.5 g/L; and trace metal concentration equal to that in primary culture.

Incubate flasks in 30°C water bath on shaker rotating at 200 rpm in a well ventilated hood. Cease incubation between 12 and 20 hr and before pH decreases below 3.

Extraction

To obtain crude toxins, filter medium contained in each flask through separate cheesecloths in Büchner funnel fitted to suction flask with trap attached. Rinse each flask and mycelial cake with 100 ml $CHCl_3$.

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Combine filtrates in 2 L separatory funnel. Add 300 ml $CHCl_3$, mix, and let phases separate. Draw off lower $CHCl_3$ phase a little at a time and concentrate in flash evaporator at room temperature.

Column Chromatography

Prepare slurry of 10 g activated MN-silica gel H in 200 ml $CHCl_3$. Pour slurry into column with extender attached to top. Remove extender after packing column and connect column to metering pump. Pump solvent through the column at ca 60 ml/hr until height of packing material remains constant. Detach pump connection from top of column, add acid-washed and fired sand to height of 10 mm above silica gel, and remove $CHCl_3$ to surface of sand.

Carry out remaining operations in subdued incandescent light. Carefully apply sample in 1 ml $CHCl_3$ to sand layer, forcing it onto silica gel with pressure.

Attach 1 L Erlenmeyer flask with stirrer, containing 600 ml $CHCl_3$, to metering pump. Add 600 ml $CHCl_3$-methanol (98+2) to second 1 L Erlenmeyer flask and join it to first flask with glass siphon. Apply solvent to column at flow rate of 60 ml/hr.

Collect 10 ml fractions during elution in region of ^{14}C-labeled aflatoxin B_1. Determine tubes containing B_1 by quick examination with hand-held ultraviolet light. Determine exact composition of tubes by analytical thin layer chromatography (TLC) and combine center fractions of ^{14}C-labeled aflatoxin B_1.

Thin Layer Chromatography

See 25.020 (11, 12).

For 2-dimensional TLC, spot in corner of TLC plate as above. Develop first with $CHCl_3$-acetone (9+1); then, after air-drying, turn plate 90° and develop with $CHCl_3$-acetone (9+5). Scrape aflatoxin spot into counting vial, add toluene gel, and count sample in liquid scintillation spectrometer, using optimum gain setting.

Determination of Concentration and Purity by Ultraviolet Spectroscopy

Determine concentration and purity of ^{14}C-labeled aflatoxin by comparing ratios of absorbances (nm) of sample at 220/265 and 362/265 to established values (10,12), 1.77, and 1.76.

Hydrogenation of Aflatoxin B_1

Proceed according to Ayres, Lee, and Sinnhuber (8).

Preparation of Aflatoxin B_1 Derivatives: Hemiacetal and Epimeric Acetates

Prepare hemiacetal and epimeric acetates of ^{14}C-labeled aflatoxin B_1 as described by Pohland *et al.* (13). Scrape each derivative from TLC plate into

counting vial and count as above (9). In addition, scrape remaining portion of plate above and below individual derivatives and count.

Results and Discussion

The specific activity remained constant and no other spots were observed in the TLC determination of purity. Examination of the ultraviolet spectrum indicated that the ratios of the absorbances were in close agreement with the established values (10) and a pure reference standard analyzed in the same manner. In the case of one preparation, 1.37 mg pure ^{14}C-labeled aflatoxin B_1 was isolated from four 100 ml cultures. The specific activity was 744 μCi/mmole.

Over 99% of the original radioactivity was found in the $THDB_1$ when the hydrogenation products were subject to analytical TLC. Preparation of the hemiacetal and epimeric acetates resulted in retention of specific activity. In total, the evidence supports the chemical and radioactive purity of the ^{14}C-labeled aflatoxin B_1.

The method described was based on the concept that aflatoxin B_1 is a secondary metabolite of *Aspergillus parasiticus* (14). Its metabolism was considered to occur in 2 distinct phases (15, 16). During the first stage, trophophase, cell growth occurred. It required a medium of salts and trace metals and an energy and nitrogen source. Idiosyncratic (species peculiar) metabolites including aflatoxin B_1 were produced in the second stage, idiophase. At this time, cell growth and protein synthesis no longer occurred. For this reason nitrogen was not required in the medium.

A number of structurally similar aromatic compounds have been hypothesized to be synthesized from acetate in a manner similar to the formation of fatty acids and are referred to as polyketides (17). They are stabilized by cyclization and therefore are not as reduced as their fatty acid counterparts. For example, the synthesis of alternariol (18), a polyketide, is favored over lipid synthesis by $NADPH_2$ deprivation and the ionic strength of the medium. Lipid synthesis increases with the addition of $NADPH_2$. The level of $NADPH_2$ and the ionic strength could perhaps be an integral part of the control of the transition from trophophase to idiophase. It is at this time (15, 16) that the proteins for aflatoxin B_1 synthesis are produced.

It is necessary to determine whether the level of $NADPH_2$ and ionic strength control the transition from trophophase to idiophase and to verify that aflatoxin B_1 is produced solely during the idiophase. Most recently aflatoxin synthesis has been reported (2) to occur with growing cells. This finding is in contrast to earlier reports (6, 19) and this communication. Furthermore, lipid synthesis has been reported to occur primarily during the growing culture (5). Increasing the pH above 5.0 resulted in a 7-fold increase in lipid synthesis and a subsequent sharp decrease in aflatoxin synthesis in the resting culture.

It should be recognized that yields of crude toxin obtained by various culture parameters are only a preliminary indication of the actual aflatoxin B_1 present. Other aflatoxins and chloroform-soluble materials may also absorb at 360 nm to give spurious results. Consequently the crude toxin should be subjected to further purification and establishment of identity before the yield is determined.

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ASSOCIATE REFEREES—Have you conducted a successful collaborative study, and do you want your method adopted by AOAC at the next annual meeting? Please be sure to observe the following points:

1. Submit five copies of your report to the AOAC Business Office for evaluation by the Subcommittee members.
2. IN ADDITION, send one copy of the report direct to your General Referee so that he can prepare his own recommendations in time for consideration by the Subcommittee.
3. Be very specific in your recommendations. If the method you propose for adoption is to replace a method currently in effect, say so, and recommend that the older method be deleted. If your method is to be adopted for alternative use with another official method, state this specifically. If you are recommending that only certain portions of an official method be revised, write out the sections involved so that no misunderstanding can occur.
4. For more specific details, write or call the AOAC Editorial Office.
5. Deadline for 1973 AOAC Associate Referee reports to be received in the AOAC office: August 17, 1972.

DRUGS

Semiautomated Colorimetric Determination of Methenamine and Methenamine Mandelate in Tablets

MATTHEW L. DOW
National Center for Drug Analysis, Food and Drug Administration, St. Louis, Mo. 63101

A semiautomated chromotropic acid colorimetric procedure was collaboratively studied by 6 laboratories, both as a composite assay and as an individual tablet assay method. Results agreed well with the USP and NF methods. The coefficients of variation ranged from 0.54 to 2.48%. The method has been adopted as official first action for the analysis of coated and uncoated tablets of methenamine mandelate, methenamine, and methenamine with sodium biphosphate.

The compendial titration procedures for methenamine (1, 2) and methenamine mandelate (3) are adaptable to individual tablet analysis (ITA) but not to semiautomation using conventional equipment. The latter is necessary when large numbers of assays must be done. There are few, if any, specific colorimetric reactions for methenamine as such, but the very sensitive chromotropic acid (CTA) test (4) has long been used for formaldehyde (HCHO), one of its hydrolysis products. An automatic analyzer procedure based on this reaction was devised (C. E. Wells, private communication, 1970) and found to be applicable to uncoated, sugar coated, and enteric coated tablets found on the market today.

Validation

The intensity of the violet color obtained was found to be dependent upon the concentration of HCHO, CTA, and H_2SO_4 in addition to temperature and reaction time. In very dilute solution and under the reproducible conditions attainable in an automatic analyzer it was found possible to make the concentration of HCHO the governing variable. This was true in spite of the fact that the reaction does not go to completion in this procedure.

Commercial samples of uncoated 325 mg methenamine tablets, coated 500 mg methenamine mandelate tablets, and coated tablets of 325 mg methenamine plus 500 mg sodium biphosphate were ground to pass a 60-mesh sieve and mixed in a mechanical tumbler. Portions of each were withdrawn and analyzed by the CTA procedure concomitantly and by separate determinations over a period of days. Portions were also analyzed by the official compendial methods. The results summarized in Table 1 were promising enough to recommend collaborative study of the method.

Solutions representing a range of concentrations of standard methenamine and methenamine mandelate were carried through the proposed procedure. All absorbances were linear for values from 40 to 80 mg/L, as methenamine. In addition the methenamine mandelate values converted to methenamine content also fell on the straight line plot for standard methenamine. Because of this, methenamine can be used as the

Table 1. Preliminary validation results for CTA colorimetric procedure

	Automated method		Compendial method (NF XIII)
Statistic	Concomitant assays, % of declared	Individual assays, % of declared	Individual assays, % of declared
Methenamine, 325 mg/tablet			
Av. (10)	100.4	101.2	99.8
Std dev.	0.45	1.28	0.77
Coeff. of var., %	0.45	1.26	0.78
Range, %	1.3	4.7	2.0
Methenamine Mandelate, 500 mg/tablet			
Av. (10)	100.2	101.8	99.3
Std dev.	0.89	1.38	0.66
Coeff. of var., %	0.88	1.35	0.66
Range, %	2.8	3.6	2.1
Methenamine plus Sodium Biphosphate 300/500 mg/tablet			
Av. (10)	97.0	99.0	100.7
Std dev.	0.95	1.00	0.90
Coeff. of var., %	0.98	1.01	0.89
Range, %	3.0	3.3	2.3

standard material for all determinations. The advantages include the ease and accuracy of the titration procedure for standardizing methenamine (2) and the commercial availability of a very pure product, which, after analysis, may be used as a reference standard material.

Standard HCHO solutions carried through the CTA procedure also gave linear absorbances. The slope of the line indicated that the hydrolysis of methenamine was less than half complete. The degree of hydrolysis was accurately determined by the following experiment: An aliquot of standard methenamine was completely hydrolyzed with dilute HCl under reflux, cooled, and diluted to one half of the original concentration. Portions of hydrolyzed and unhydrolyzed methenamine solution were then carried through the CTA procedure. From the relationship, % hydrolysis = 100 (absorbance unhydrolyzed standard)/2 (absorbance hydrolyzed standard), values of 47.3 and 47.6% were obtained. A similar experiment with standard methenamine mandelate solution gave a value of 47.4%. These values were found to vary with the temperature of the heating bath and the efficiency of the water-jacketed coil used to cool the emergent stream. To avoid the possibility of erratic results the water flow rate and temperature should be permitted to equilibrate before proceeding with an analysis.

The analytical peaks obtained in this method approximate 86% of the steady state signal (Fig. 1). The viscosity of the developed color solutions is such that turbulence and schlieren result if abrupt changes in flow rate occur in the flowcells. The internal diameter of the inlet and outlet tubing should be matched approximately to avoid this.

This method does not discriminate between hydrolyzed and undecomposed methenamine or methenamine mandelate. The decomposition product, gaseous HCHO, is also the analytically significant moiety. If any HCHO released by decomposition has become entrapped in the tablet matrix, it will be determined along with the drug present. Most free HCHO will probably volatilize out of the tablet. The official compendial methods (1–3) are likewise deficient in this respect, because they also determine either HCHO or the equally volatile ammonia content of the drugs.

Interferences

Lactose, sucrose, cornstarch, glucose, stearic acid, and magnesium stearate (500 mg each) were carried through the sample preparation step and the CTA colorimetric assay procedure. The only discernible departure from the baseline observed was that of 0.002 absorbance units for sucrose. In addition a commercial methenamine sample was spiked with 200 mg each of sucrose, lactose, glucose, and cornstarch. A recovery of 101.0% of declared was obtained compared to 101.9% for the unspiked sample. A similar comparison using standard methenamine solution gave absorbances of 0.444 and 0.442, respectively. Duplicate portions of the methenamine sample were also spiked with 500 mg of sodium biphosphate, water was added, and the solution was allowed to stand for 1.5 hr. An aliquot of each gave recoveries of 101.9 and 101.2% of declared, indicating that $NaH_2PO_4.H_2O$ has little, if any, effect on this determination.

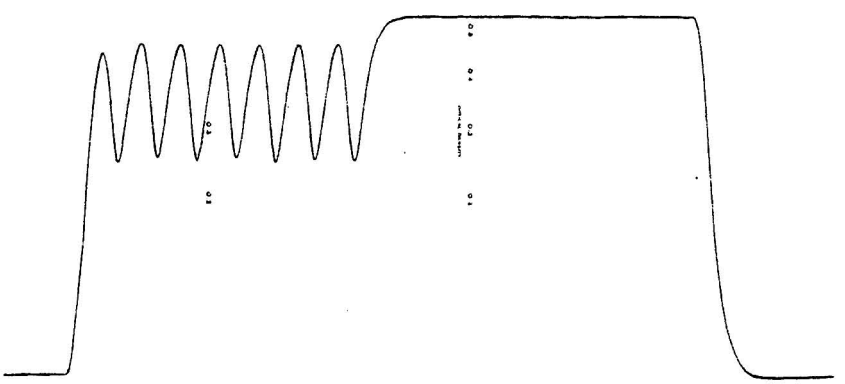

FIG. 1—Steady state signal for standard methenamine solution.

METHOD

Methenamine and Methenamine Mandelate—Automated Method

36.C32 *Principle*

Methenamine is hydrolyzed to HCHO and NH_4^+ in acid soln. Free HCHO condenses with chromotropic acid in strong acid soln to form colorless hydroxydiphenylmethane derivative which is further oxidized to colored *p*-quinoidal compd with max. *A* at 570 nm. Method is applicable to methenamine, methenamine mandelate, and methenamine with NaH_2PO_4.

36.C33 *Reagents and Apparatus*

Use deionized H_2O or equiv. thruout.

(a) *Dilute ammonia.*—Dil. 5 ml NH_4OH to 1 L with H_2O.

(b) *Dilute sulfuric acid.*—72%. Slowly add H_2SO_4 to 600 ml H_2O to total vol. of 1.5 L (when cool).

(c) *Chromotropic acid (CTA) color reagent.*—Suspend 500 mg CTA, di-Na salt (K&K Laboratories, Inc., Eastman Kodak Co., or equiv.) in 20 ml H_2O and *slowly* add 30 ml H_2SO_4 in small portions (overheating produces deep violet color and inactivates reagent). Cool, and mix into 1.5 L 72% H_2SO_4.

(d) *Methenamine std soln.*—Dissolve enough methenamine, previously dried over P_2O_5 and stdzd (NF XIII), in dil. NH_4OH to give concn of std appropriate for dosage level analyzed (Table 36:C1).

(e) *Automatic analyzer.*—AutoAnalyzer with following modules (Technicon Instruments Corp., Tarrytown, NY 10591): Sampler II with 20/hr (2:1) cam; proportioning pump 1; heating bath set at 90° with two 40′ coils, 1.6 mm id; colorimeter with 15 mm tubular flowcell and matched 570 nm filters; recorder with semilog paper; manifold (Fig. 36:C2). Wire down all tube connections carrying H_2SO_4.

36.C34 *Preparation of Sample*

Disintegrate uncoated tablets contg equiv. of 250–500 mg methenamine by intermittent shaking in 100 ml dil. NH_4OH. Crush coated tablets and hard uncoated tablets before addn of solv. Ultrasonic bath may be used to hasten soln. Dil. 1.0 ml sample soln to 50 ml with dil. NH_4OH.

Samples of all dosage levels can be prepd in 4 oz glass, snap-cap vials provided with Parafilm seal if appropriate sampling pump tube is used (*see* Table 36:C1). A 1+50 diln may be accomplished with 1.00 ml Thomas-Seligson vac. diln pipet mounted beneath automatic 50.0 ml delivery pipet. Est. increase in vol. resulting from dissolving 1 tablet in 100 ml solv., using vol. flask and graduated pipet.

36.C35 *Analytical System*

See Fig. 36:C2 and Table 36:C1. Sample solns are withdrawn from sample cups, segmented with air, and dild in manifold with H_2O. CTA soln is added and stream is passed thru beaded coil into 90° heating bath for color development. Stream is cooled in H_2O-jacketed coil and equilibrated at room temp.

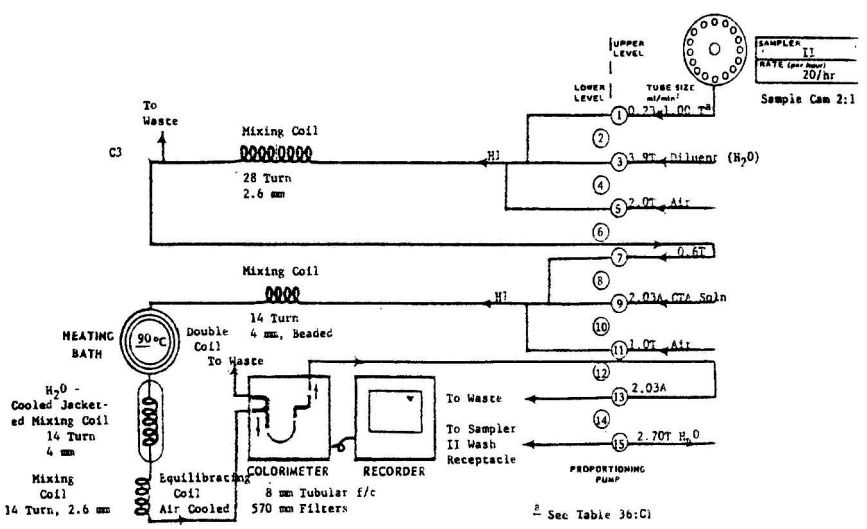

FIG. 36:C2—Flow diagram for automated analysis of methenamine and methenamine mandelate.

Table 36:C1. Sample pump tube sizes and methenamine std concns for various tablet dosage levels

Product	Dosage, mg/tablet	Pump tube size, ml/min	Std, mg/100 ml
Methenamine mandelate	250	1.00	2.50
Methenamine mandelate	500	0.60	5.00
Methenamine	300, 325	0.60	6.00, 6.50
Methenamine	500	0.23	5.00
Methenamine mandelate	1000	0.23	10.00

in mixing coil. Stream is debubbled and passed into colorimeter equipped with 570 nm filters and 15 mm tubular flowcell for A measurement. Inlet and outlet tubing of flowcell should be ca same id.

36.C36 ***Start-Up and Shut-Down Procedures***

Turn on heating bath (3 hr), cooling H_2O in jacketed coil (30 min), and colorimeter (30 min) in advance. Prewash system 5 min with H_2O and then pump all reagents thru their resp. lines. Let equilibrate 20–30 min and adjust colorimeter and recorder to produce steady baseline. To shut-down system, flush 10–15 min with H_2O and pump all lines dry.

36.C37 ***Determination***

Fill 8.5 ml sample cups with prepd solns and aspirate thru 0.034″ stainless steel probe at 20/hr with sample-to-wash ratio of 2:1. Include 1 std soln between each 5 sample solns, and insert 3 std solns at beginning and end of each 10–30 samples. Draw line between baseline at beginning and end of run. Subtract av. baseline A from max. A to obtain net A (ΔA) for each peak. Calc. av. $\Delta A'$ for std solns, disregarding first and last 2 std peaks. Calc. mg methenamine)/unit dose = $(\Delta A/\Delta A') \times C \times D$, where C = mg methenamine/ml std soln and D = diln factor.

Collaborative Study

Commercial samples of uncoated 325 mg methenamine (A), coated 500 mg methenamine mandelate (C), and coated 325 mg methenamine + sodium biphosphate tablets (D) were each ground to pass a 60-mesh sieve and mixed on a mechanical tumbler. A fourth sample (B) was prepared from standard methenamine and magnesium stearate by geometrical dilution and contained 97.38% of drug (325 mg per 0.3337 g). Portions of these samples were sent to 5 collaborators with instructions to analyze 10 single tablet portions from each and to report the individual determinations. Appropriate standard materials were also furnished each participant. The results reported by the collaborators are summarized in Tables 3–6. Those obtained by the Associate Referee are listed in column 1. The averages of the reported results for Samples A, B, C, and D were 100.3, 99.7, 97.7, and 100.6% of declared or added drug, respectively. The corresponding coefficients of variation for the determinations were 1.39, 0.98, 1.22, and 1.02%.

Assay of the methenamine tablet composite by the NF XIII titration procedure (2) yielded an average result of 325 mg drug/tablet. The methenamine mandelate composite assayed 493 mg/tablet by the USP XVIII method (3). No attempt was made to determine methenamine in the product containing sodium biphosphate by an official method.

Conclusion and Recommendation

The first in the series of 10 results for methenamine and methenamine mandelate reported by Collaborator 6 was considerably lower than the subsequent 9 results. This was probably due to the use of an insufficient number of cups of standard solution preceding the sampling pattern. In usual practice, if a sample solution yields a low peak near the beginning of a pattern, the solution is resampled at the end of the run. This would confirm either a correct low result or insufficient equilibration prior to the sample run. The collaborators were not instructed to rerun a sample if such a result occurred.

Other than this only a few minor mechanical difficulties were reported by the collaborators. None of these prevented the participants from getting satisfactory results.

It is recommended that the method be adopted as official first action.

Acknowledgments

The Associate Referee gratefully acknowledges the cooperation of the following collaborators: E. Balzer, Warner-Chilcott Laboratories, Morris Plains, N.J.; Kenneth M. Gordon, Food and Drug Administration, Philadelphia, Pa.; Jerry Hale, Eli Lilly & Co., Indianapolis, Ind.; Thelma Kincheloe, Food and Drug Administration, Chicago, Ill.; and Richard E. Kolinski, National Center for Drug Analysis (NCDA), Food and Drug Administration, St. Louis, Mo.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was adopted by the Association; see (1973) *JAOAC* 56, 395.

Table 3. Summary of results[a] for methenamine tablets

	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6	Av.
	330	327	317	332	327	304	
	322	333	321	328	328	320	
	329	329	328	333	330	325	
	326	332	328	333	316	325	
	330	321	321	333	322	328	
	329	324	328	333	318	328	
	322	331	317	333	330	321	
	321	332	321	334	317	333	
	324	331	321	333	326	329	
	324	330	321	336	324	328	
Av.	326	329	322	332	323	324 (326)[b]	326 (100.7% of declared)
Std dev.	3.6	4.5	4.0	1.8	5.3	8.0 (4.1)	
Coeff. of var., %	1.09	1.36	1.24	0.54	1.65	2.48 (1.27)	1.39 (1.18)
Range, %	2.8	3.6	3.4	2.2	4.3	9.0 (4.0)	4.2 (3.4)

[a] Individual results are in mg/tablet.
[b] Values in parentheses are calculated after dropping first result of Collaborator 6.

Table 4. Summary of results[a] for authentic methenamine

	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6	Av.
	322	325	322	322	325	320	
	321	325	315	327	325	314	
	325	321	315	330	324	319	
	325	324	322	328	323	314	
	324	322	330	330	330	323	
	321	326	323	330	325	320	
	321	321	331	329	325	320	
	326	326	322	330	324	320	
	325	322	328	331	319	323	
	325	324	328	331	319	326	
Av.	324	324	324	329	324	320	324 (99.7% recovered)
Std dev.	2.1	1.8	5.6	2.7	3.0	3.8	
Coeff. of var., %	0.64	0.57	1.74	0.84	0.93	1.17	0.98
Range, %	1.5	1.5	4.9	3.0	3.4	2.8	2.8

[a] Individual results are in mg/tablet.

Table 5. Summary of results[a] for methenamine mandelate

	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6	Av.
	479	483	510	476	493	459	
	480	479	496	472	494	483	
	482	485	496	480	496	486	
	493	485	510	466	493	490	
	483	490	505	477	493	493	
	483	483	510	468	487	493	
	480	488	510	481	500	492	
	479	485	513	474	487	492	
	492	486	513	480	494	493	
	491	489	496	480	493	492	
Av.	484	485	506	476	493	487 (490)[b]	489 (97.7% of declared)
Std dev.	5.6	3.3	7.2	5.3	3.9	10.5 (3.6)	
Coeff. of var., %	1.16	0.67	1.42	1.11	0.80	2.15 (0.73)	1.22 (0.98)
Range, %	2.9	2.3	3.4	3.1	2.6	7.0 (2.0)	3.6 (2.7)

[a] Individual results are in mg/tablet.
[b] Values in parentheses are calculated after dropping first result of Collaborator 6.

Table 6. Summary of results[a] for methenamine and sodium biphosphate

	Coll. 1	Coll. 2	Coll. 3	Coll. 4	Coll. 5	Coll. 6	Av.
	330	330	319	330	330	324	
	329	331	319	326	322	323	
	329	330	317	330	323	326	
	331	330	323	326	329	325	
	331	322	317	323	329	325	
	333	325	323	329	332	324	
	324	328	323	335	334	324	
	325	332	317	330	331	330	
	331	323	317	332	336	326	
	324	330	319	334	331	330	
Av.	329	328	319	330	330	326	327 (100.6% of declared)
Std dev.	3.3	3.4	2.9	3.1	5.0	2.5	
Coeff. of var., %	0.99	1.04	0.90	0.95	1.51	0.75	1.02
Range, %	2.7	3.0	1.9	3.3	5.2	2.1	3.0

[a] Individual results are in mg/tablet.

He also thanks Thomas G. Smith and Larry K. Thornton, NCDA, for their participation in the original validation of this method and Clyde E. Wells and William B. Furman, also of NCDA, for their advice and suggestions during this study.

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This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Differentiating Nonaqueous Titration of Mixtures Containing Acetaminophen and Salicylamide

MARTIN I. BLAKE and LINDA B. SHUMAKER
Department of Pharmacy, University of Illinois at the Medical Center, P.O. Box 6998, Chicago, Ill. 60680

A differentiating nonaqueous titration procedure is described for the determination of mixtures of acetaminophen and salicylamide. Tetrabutylammonium hydroxide is the titrant and a Fisher titrimeter equipped with a calomel-glass electrode system is used. The technique is applied to the individual components, synthetic mixtures, and complex dosage forms.

Acetaminophen is a commonly used analgesic-antipyretic, alone or in combination with other similar drugs in liquid or solid dosage forms. The analysis for acetaminophen content has been the subject of numerous studies. The NF acetaminophen elixir is assayed (1) by a column chromatographic procedure developed by Levine and Hohmann (2). The column is prepared with Celite buffered to pH 10.1 with carbonate. When chloroform is used as the eluting solvent, acetaminophen is separated from basic and neutral components and substances having weaker acidity. Acetaminophen is then eluted with ether and determined spectrophotometrically. The authors point out that acetaminophen may be "sharply" separated from other drugs with which it may be combined in dosage forms by proper selection of buffer pH and solvent systems. Salicylamide and barbiturates, also weak acids, are typical drugs combined with acetaminophen. This procedure was subjected to a collaborative study, reported by Hohmann (3), in which several solid and liquid dosage forms and 2 synthetic tablet mixtures were analyzed for acetaminophen content. Salicylamide was an ingredient in 2 synthetic tablet mixtures and in a commercial tablet. Hamilton (4) reported a collaborative study of the analysis of acetaminophen and salicylamide in tablets and capsules based on the use of strongly acidic and basic Celite columns. Both the Levine-Hohmann and the Hamilton methods were adopted as official by the AOAC. Hall and Levine (5) described a procedure for the separation of barbiturates in combination with salicylamide, using partition chromatography. Only the barbiturate was recovered. Alber, Overton, and Smith (6) used gas-liquid chromatography for the separation and determination of salicylamide, acetaminophen, and caffeine combinations. In the NF assay (1), acetaminophen is analyzed by UV spectrophotometry and the tablets are assayed similarly after extraction of the powdered tablet mass with a chloroform-alcohol (3+1) mixture.

Salicylamide, according to the NF assay procedure (1), is analyzed by visual nonaqueous titration using sodium methoxide as the titrant, dimethylformamide as the titration solvent, and thymol blue T.S. as the indicator. This is based on a procedure reported by Fritz and Keen (7). The tablets are analyzed similarly using an aliquot of the powdered tablet mass. Prior separation of the salicylamide is not required. However, for the NF oral suspension (1) the salicylamide is determined by UV spectrophotometry after preliminary extraction with ether.

Previous reports (4, 8) have reviewed the literature on the analysis of acetaminophen as the free compound, in simple dosage forms, and in combination with other drugs as complex dosage forms.

In the present study combinations of acetaminophen and salicylamide are analyzed by a potentiometric differentiating nonaqueous titration procedure using tetrabutylammonium hydroxide as the titrant and a Fisher titrimeter equipped with a calomel-glass electrode system. The method is applied to the individual components, synthetic mixtures, and complex dosage forms.

METHOD

Apparatus and Reagents

(All chemicals and solvents employed were reagent grade and were used without further purification. All dosage forms were obtained from commercial sources.)

(a) *Titrimeter.*—Fisher Model 36, equipped with glass electrode (Fisher No. 13-639-3) and porous plug-type calomel electrode (Fisher No. 13-639-51).

(b) *Standard solution—0.1N solution of tetrabutylammonium hydroxide in benzene-methanol.*—Prepare according to method of Cundiff and Markunas (9) and standardize potentiometrically against reference

standard benzoic acid dissolved in dimethylformamide. Restandardize standard solution each day of use.

Preparation of Sample

(a) *Synthetic mixtures.*—Prepare mixtures of acetaminophen and salicylamide containing following ratios for each component: 1+0, 1+1, 1+2, 1+3, and 1+4.

(b) *Dosage forms.*—Weigh 20 tablets and grind to fine powder in mortar, or remove contents of 20 capsules of a dosage form as completely as possible and weigh combined contents.

Procedure

Acetaminophen and salicylamide.—Transfer 0.5–1 meq (75–150 mg) salicylamide or acetaminophen powder, accurately weighed, to 150 ml beaker. Dissolve powder in 50 ml dimethylformamide. Stir solution magnetically and titrate potentiometrically with calomel-glass electrode system, using 0.1*N* tetrabutylammonium hydroxide as titrant. (Typical titration curves are shown in Fig. 1 (curves A and B).)

Acetaminophen and salicylamide mixtures.—Transfer mixture containing ≥40 mg each component, accurately weighed, to 150 ml beaker. Dissolve in 50 ml dimethylformamide. Titrate solution as above. (Ratios of components in mixtures analyzed are listed in Table 1; typical differentiating titration curve is shown in Fig. 1 (curve C).)

Dosage forms.—Transfer aliquot of powder mass containing 75–150 mg each component, accurately weighed, to 150 ml beaker. Dissolve in 50 ml dimethylformamide and titrate as above. (Capsule and dosage forms analyzed are listed in Table 2; typical titration curve for a dosage form is shown in Fig. 1 (curve D).)

Calculations

Prepare titration curves by plotting ml titrant against mv. Determine end points in titration from inflections in curve. Calculate per cent recoveries for acetaminophen and salicylamide in mixtures with following equations:

$$\% \text{ Acetaminophen} = (\text{ml titrant} \times \text{normality titrant} \times 151.17 \times 100)/\text{sample wt}$$

$$\% \text{ Salicylamide} = (\text{ml titrant} \times \text{normality titrant} \times 137.14 \times 100)/\text{sample wt}$$

Results and Discussion

Previous reports from this laboratory have concerned the differentiating potentiometric titration of weak acid mixtures, both as simple combinations and as complex mixtures in dosage forms. These have included salicylic acid and benzoic acid (10), aspirin and barbiturates (11, 12), acetaminophen and aspirin (13). In each case the difference in pKa values was sufficiently great to permit a successful differentiating titration.

Acetaminophen and salicylamide are both very weak acids and both appear frequently in analgesic-antipyretic preparations. According to Barlin and Perrin (14), the calculated pKa values for salicylamide and acetaminophen are 8.31 and 9.92, respectively. This difference in pKa value does permit differentiation in the nonaqueous system employed in this study. Typical curves

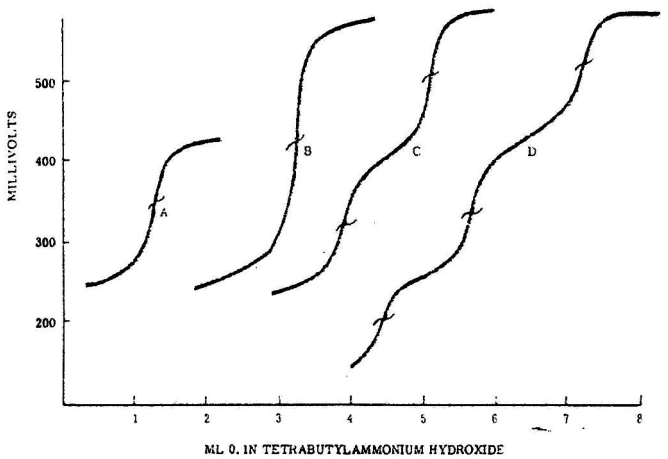

FIG. 1—Typical titration curves: A, acetaminophen; B, salicylamide; C, salicylamide and acetaminophen mixture (1+1); and D, tablet dosage form (tablet A, Table 2).

for the titration of the individual compounds and for synthetic mixtures of the 2 compounds are shown in Fig. 1 (curves A, B, and C). The curves have been displaced for better demonstration of the inflections. In curve C the first inflection is for salicylamide and the second is for acetaminophen. Recovery data are presented in Table 1.

Several commercially available dosage forms containing acetaminophen and salicylamide in addition to other active ingredients were analyzed by the proposed procedure. The composition of the dosage forms and recovery data are listed in Table 2. Per cent recoveries are reported only for the acetaminophen and salicylamide, and are based on label claim values. A typical titration curve is shown in Fig. 1, curve D for dosage form, tablet A of Table 2. The initial inflection in the titration curve is for the ascorbic acid component. The ascorbic acid, and the barbiturates indicated for tablet C and capsule A, did not interfere in the titration of the respective dosage forms. Since they have somewhat lower pKa values than salicylamide, they titrated first and permitted suitable differentiating titration curves for the active ingredients analyzed. Basic components, salts, and excipients did not apparently interfere in the titration. Preliminary extraction of the active components was not necessary.

Table 1. Analysis of mixtures of acetaminophen and salicylamide by differentiating nonaqueous titration

Ratio[a]	Per cent recovery and standard deviation[b]	
	Acetaminophen	Salicylamide
1+0	100.7±0.3	—
0+1	—	100.1±0.3
1+1	101.3±2.1	101.4±1.6
1+2	101.3±1.6	101.1±1.6
2+1	100.7±1.0	100.2±2.1
1+3	102.1±0.5	99.8±1.5
3+1	102.4±2.2	100.9±2.7
1+4	109.6±4.5	93.7±1.1
4+1	99.0±0.7	101.5±2.2

[a] Acetaminophen to salicylamide.
[b] Standard deviation based on ≥4 determinations.

Table 2. Analysis of dosage forms by proposed method

Dosage form	Label claim/unit dose, mg	Label claim found, %[a]
Tablet A		
Salicylamide	250	99.5±1.3
Acetaminophen	250	100.6±1.7
Ascorbic acid	25	
Tablet B		
Salicylamide	250	98.9±2.0
Acetaminophen	250	99.7±2.6
Ascorbic acid	25	
Prednisone		
Tablet C		
Salicylamide	250	97.4±1.0
Acetaminophen	250	98.1±2.6
Hyoscyamine sulfate	0.0518	
Atropine sulfate	0.0097	
Hyoscine HBr	0.0032	
Phenobarbital	8	
Capsule A		
Salicylamide	250	99.6±1.3
Acetaminophen	250	96.8±1.7
Pentobarbital	25	
Capsule B		
Salicylamide	150	99.8±2.6
Acetaminophen	175	99.4±0.9
Caffeine	15	
Phenylpropanolamine	18	

[a] Standard deviation based on ≥5 determinations.

Acknowledgments

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Separation of *dl*-2-(3-Phenoxyphenyl)propionic Acid (Fenoprofen) From Its Intermediates by Thin Layer Chromatography

RAFIK H. BISHARA
Analytical Development Department-Physical, Eli Lilly and Co., Indianapolis, Ind. 46206

Fenoprofen and its synthetic intermediates are resolved on silica gel F_{254} TLC plates developed with chloroform-acetic acid (98+2). The interrelationship between the chemical constitution and the R_f values is discussed. The lower limit of detecting the intermediates in Fenoprofen raw material is 1%.

Several aryl- and alkyl-substituted carboxylic acids have been shown to exhibit anti-inflammatory properties (1–8). A new compound of this series developed in our research laboratories (9) is Fenoprofen, *dl*-2-(3-phenoxyphenyl)propionic acid. This compound exhibited anti-inflammatory and analgesic activities in experimental animals (4) and in man (8, 10–12). The synthesis of Fenoprofen as reported by Marshall (9) is shown in Fig. 1.

This paper describes a thin layer chromatographic (TLC) developing solvent system for the complete resolution of Fenoprofen from its intermediates on precoated silica gel plates. Application of this system to estimate the presence of any intermediate at the level of 1% in Fenoprofen raw material is discussed.

Based on past experience in resolving mixtures of closely related steroids (13–15) and chlorinated pesticides (16, 17), we used commercially available precoated plates in this study.

METHOD

Apparatus

(a) *TLC plates.*—Precoated silica gel F_{254}, 20 × 20 cm, 0.25 mm adsorbent layer (E. Merck, A. G. Darmstadt, Germany, available from Brinkmann Instruments, Inc., Westbury, N.Y.).

(b) *Spotting pipets.*—Disposable, calibrated to contain 1 or 10 µl (Drummond type, distributed by Ace Glass Co., Louisville, Ky.).

(c) *Developing tank.*—Rectangular glass tank (Brinkmann Instruments, Inc., Westbury, N.Y.). External measurements 30 × 10 × 28 cm.

(d) *Ultraviolet light source.*—Chromato-Vue with shortwave (ca 254 nm) lamp (Ultra-Violet Products, Inc., San Gabriel, Calif.).

Reagents

(a) *Fenoprofen and its intermediates.*—*m*-Hydroxyacetophenone (I), 3-phenoxyacetophenone (II), α-methyl-3-phenoxybenzyl alcohol (III), 3-(1-bromoethyl)diphenyl ether (IV), 2-(3-phenoxyphenyl)propionitrile (V), and *dl*-2-(3-phenoxyphenyl)propionic acid (Fenoprofen) (VI). Structures of above compounds are shown in Fig. 1.

(b) *Developing solvent.*—Chloroform-acetic acid (98+2). Mix just before use.

Thin Layer Chromatography

Prepare chloroform solution containing 1 mg each intermediate/ml. Weigh 100 mg fenoprofen sample and transfer to 10 ml volumetric flask. Dissolve and dilute to volume with chloroform.

Score adsorbent layer across TLC plate 15 cm from starting line which is 2.5 cm from the bottom of the plate. Divide plate into 4 equal sections and spot 1 and 10 µg intermediates mixture on section 1 and 3, respectively. Spot 100 µg fenoprofen sample on section 2. Section 4 is plate blank. Place 100 ml developing solvent in TLC tank lined with Whatman No. 1 paper and let equilibrate 30 min before use. Develop plate at room temperature (25°C) until solvent reaches scored line. Remove plate and let solvent evaporate ca 3 min at room temperature. View chromatogram under shortwave UV light and mark quenched spots.

Results and Discussion

At the same concentration for each of the 6 compounds shown in Fig. 1, the amount of quenching appeared in the following order: Compound I > II > III > VI > IV > V. If we assume that the efficiency of quenching is related to the amount of UV absorption by the compound, *m*-hydroxyacetophenone (I) should be the most efficient quencher because of the presence of the auxochromic hydroxyl group (18).

The R_f values for Compounds I, II, III, IV, V, and VI are 9, 54, 22, 69, 59, and 13, respectively. A typical thin layer chromatogram is shown in Fig. 2.

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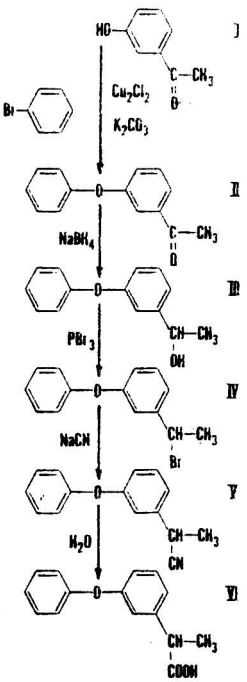

FIG. 1—Synthesis of Fenoprofen (5).

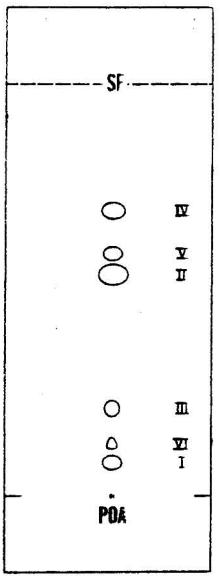

FIG. 2—Thin layer chromatogram of a mixture of Fenoprofen and its intermediates (10 μg per compound) on silica gel F_{254} developed with chloroform-acetic acid (98+2); POA, point of application; SF, solvent front. Roman numerals of compounds correspond to Fig. 1.

The 6 compounds examined chromatographically in this study are generally separated into 2 groups. In the first group are the slow-moving polar compounds which contain a hydroxyl group (Compounds I and III) or a carboxyl group (Compound VI). The nonpolar compounds (IV and V) move faster and form the second group. However, the mobility of Compound II is close to the fast-moving group. This behavior is due to the presence of the less polar carbonyl group (19) relative to the hydroxyl and carboxyl groups.

The structure of Fenoprofen and its 4 intermediates, II–V, contains the diphenyl ether moiety (*see* Fig. 1). On the other hand, the starting material, Compound I, contains a phenolic hydroxyl group. The powerful influence of the free phenol group on the adsorption affinity of compounds to the silica gel thin layer explains the low R_f value of the starting material in comparison to the other 5 compounds. This is also in good agreement with Stahl (19) who reported that phenol ethers showed distinct lower adsorption affinity; hence they move faster than the corresponding phenol.

The mobility of the compounds containing the diphenyl moiety is mainly influenced by the characteristics of the functional groups they contain. Fenoprofen, being the only carboxylic acid in this group of compounds, has the lowest mobility because of the highest adsorption affinity of the carboxyl group (19). Compound III has a hydroxyl group which follows the carboxyl group in order of polarity and adsorption affinity (20). This explains the higher mobility of Compound III in relation to Compound VI. The intermediate mobility of Compound II has been previously discussed and is mainly attributed to the less polar carbonyl group. The nitrile group has been reported to be less polar than the hydroxyl and carbonyl groups (19). This justifies the higher R_f value of Compound V. Compound IV is the fastest moving of all 6 compounds. This high chromatographic mobility is enhanced by the presence of the nonpolar bromine atom, by the absence of any polar group, and by the presence of one carbon atom less than in Compounds V and

VI. The preceding discussion serves the purpose of shedding some light on the interrelationship between the chemical constitution and R_f values of Fenoprofen and its intermediates.

To determine the chromatographic purity of Fenoprofen raw material, any extra spot(s) in section 2 other than the main compound is compared with the spot having the same mobility (R_f value), intensity, and size in sections 1 and 3. Confirmation is positive if the R_f value of the extra spot in the sample lane matches the R_f ($\pm 5\%$) of the standard spot. When the sample of Fenoprofen raw material is between 2 levels of intermediates, namely 1 and 10%, the semiquantitative determination of its chromatographic purity is greatly facilitated. This TLC method detects 1% of any intermediate in Fenoprofen.

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Minicomputer–Automatic Analysis System for Pharmaceuticals

LARRY L. ALBER, MACK W. OVERTON, and DONALD E. SMITH*
Food and Drug Administration, 433 W. Van Buren St., Chicago, Ill. 60607
Department of Chemistry, Northwestern University, Evanston, Ill. 60201

Software has been developed for reduction of automatic analyzer data both for on-line and off-line modes, using a PDP-12 minicomputer with 8K core memory, magnetic tape and disk storage, a multiplexer and 16 channel 10-bit analog-to-digital converter, and a cathode ray tube. The on-line system performs data-logging onto magnetic tape of 16 instruments asynchronously, displaying any 2 analog-to-digital conversion channels on the cathode ray tube in real-time. Spectrophotometers are interfaced to the computer, using economically constructed operational amplifier circuits. Data reduction is aided by a hardware floating-point processor, using an 8-point least-squares fitting algorithm to digitally smooth the data array and determine each peak maximum. Computer results of 3 different systems operating simultaneously are compared to values obtained manually. The off-line system was written using FOCAL-12 conversational language and will accept digital values by Teletype, peak detector paper punch, or TWX; the latter makes the program available to other laboratories through the Federal Telecommunications TWX System.

With the USP (1) and NF (2) requirements for content uniformity testing on many pharmaceutical preparations, regulatory laboratories were faced with an enormous amount of additional assays due to individual tablet analyses (ITA). As clinical laboratories found long ago, large numbers of chemical tests of the same type can be automated. In order to perform these ITA tests, pharmaceutical laboratories have adapted Technicon AutoAnalyzers, with ultraviolet, visible, or fluorescence spectroscopy as the determinative step. In each case, the detector output may be displayed in the form of a recorder trace, absorbance, per cent transmission, or fluorescence units versus time, in which samples and standards appear in a predetermined order. As a result, substantial analytical time is required to interpret graphs, subtract baseline and/or system drift, perform arithmetic operations on calculators, and write reports. Efficient use of analytical time demands some form of automated data manipulation and report handling. The on-line minicomputer is an appealing means to this end (3–5).

Although the major portion of our laboratory computer programming thus far has been directed to gas chromatography (6), as a temporary measure, an off-line program for ITA calculations was written for the minicomputer, using FOCAL-8 language (7). The program performed calculations from net absorbances predetermined manually and entered via Teletype.

A better utilization of the computer hardware and peripherals available involves direct interfacing of the spectrophotometric output for on-line data acquisition and reduction. The feasibility of this approach was tested with a laboratory computer and a conversational language, FOCAL-12 (8, 9).

In addition to mathematical routines, FOCAL-12 provides instructions for performing analog-to-digital conversions (ADC), displaying on a cathode ray tube (CRT), and storing data or subroutines on magnetic tape or disk.[1] Without expanding the disk storage, the system could be used only in a dedicated manner, e.g., it was limited to one instrument at a time.

By modifying an existing GLC program, we developed an on-line system capable of processing data from 16 automatic analyzers simultaneously. The final calculation and report printing routine was written using FOCAL-12. By only slight modification of the latter program, an off-line version to accept digital data by Teletype, peak detector paper punch, or TWX made the program available to instruments not directly interfaced and also to other laboratories connected to the TWX system.

This writing is a qualitative report of the system, software, flow charts, and interfacing, and includes tables of computer results versus manual determinations for 3 different on-line systems running at the same time.

[1] FOCAL-12 disk routines require a minimum of two 32K disk units or one 256K disk unit.

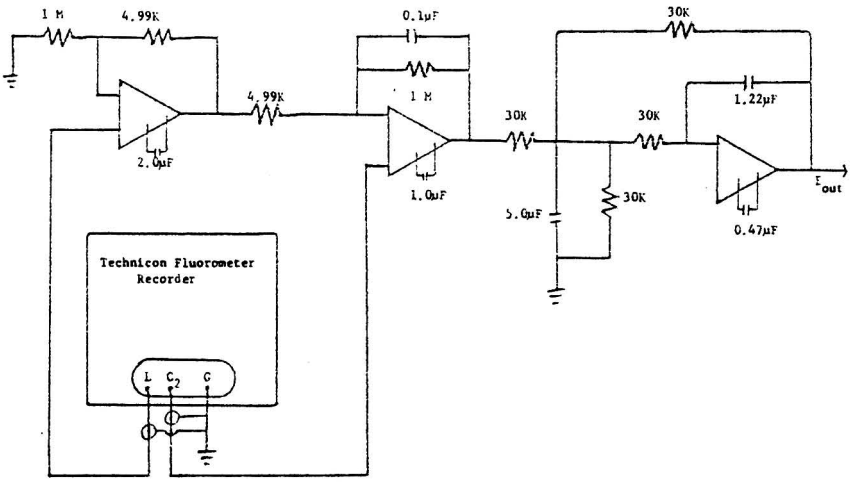

FIG. 1—Schematic of fluorometer II interface.

Experimental

Apparatus

(a) *Computer hardware.*—A PDP-12A digital computer (Digital Equipment Corp., Maynard, Mass.) includes 8K of 12-bit 1 μsec core memory, 2 magnetic tape units, one 32K disk storage device, 6 DPDT relays, 6 sense switches, a multiplexer and 32 channel 10-bit ADC, CRT, programmable clock, and Teletype. The system also utilizes a hardware floating-point processor (FPP-12), which carries all calculations to 28 bits of precision. The numbers can range from 2^{+2047} to 2^{-2048}.

(b) *Interfacing.*—On most modern laboratory instruments, the detector analog output can be amplified as needed for the ADC input to insure maximum computer precision. These circuits have been economically constructed for less than $100 each, using operational amplifiers, and are easily assembled using prewired manifolds[2] (6). These circuits provide high input impedance which prevents detector loading problems and permits normal operation of the recorder without influence by the amplifier. Figure 1 shows the interface schematic of the Technicon fluorometer.

For UV and visible determinations, Beckman DK-2A spectrophotometers were utilized. We modified the gear assembly to provide a continuous scan at constant wavelength. The photomultiplier output was linear only in % T mode; therefore, a 10-turn precision re-transmitting slidewire was mounted on the pen pulley so the absorbance mode could be used. This schematic has been previously reported (8).

(c) *Automatic analyzer equipment.*—Technicon AutoAnalyzer equipment consisting of a liquid sampler II and proportioning pump I (Technicon Corp., Tarrytown, N.Y. 10591) was used with a Beckman DK-2A spectrophotometer with 10 mm flowcell (Arthur H. Thomas, No. 9120N05). The Technicon fluorometer II system consisted of a flowthrough cell, liquid sampler II, continuous filter, proportioning pump I, and recorder.

On-Line Software

(a) *Overall operation.*—The total on-line system is operated in 2 modes: "data-logging" until all automated analyses are finished, and then "batch-processing" each sample one at a time. The data array of each sample is stored on magnetic tape, the location of which is printed on Teletype before proceeding to the next sample run. When all automated systems are finished, the batch-processing is performed with FOCAL-12, using the FPP program as a subroutine. This was accomplished by modifying FOCAL-12 to allow overlay of the FPP program stored on disk. When the FPP completes the storage of peak x and y values on tape for the sample run, FOCAL-12 is returned to core by disk overlay, per cent declared calculations are performed, and results are printed.

(b) *Data-logging.*—A program to sample up to 16 analog instruments asynchronously had already been

[2] Manifold Model No. 603. Operational amplifier No. 3057/01 (Burr-Brown Research Corp., Tucson, Ariz. 85706).

The use of equipment described herein does not constitute an endorsement by the Food and Drug Administration.

developed (10). The program allows ADC rates up to 1 KC and stores data on contiguous magnetic tape blocks for later processing. Sampling rates of 1 data point every millisecond can be achieved, but for the typical automated pumping system, the most concentrated portion of the solution being determined takes about 15 sec to pass through the cell; therefore, sampling each ADC once every 3 sec yields about 5 data points at each peak maximum.

(c) *FPP peak detection.*—Because the concentration of the solution being determined by spectrophotometry is directly proportional to the net absorbance of the peak (maximum absorbance − baseline), programming is considerably easier than determining peak area as in gas chromatography. An additional advantage is that every peak, samples and standards alike, has approximately the same peak shape. In the approach reported here, the only instrumental values required for calculations are the initial baseline, the x and y coordinate of each peak, and the final baseline.

The data are first digitally smoothed by an 8-point least-squares fitting algorithm, using the FPP (11). The symbolic instructions are combined to examine 8 successive 12-bit data words stored anywhere in 32K core, float the value,[3] fit to the best quadratic for the 8 points, smooth the 6 center points to meet the function, fix[4] the floating resultant back to a 12-bit word, and restore into their original core location, advancing by one data point through the array.

The program invokes the standard least-squares fit technique (12) whereby the A, B, and C coefficients of the least-squares quadratic are obtained by simultaneous solution of the equation set:

$$\bar{y} = A + B\bar{x} + C\overline{x^2}$$
$$\overline{xy} = A\bar{x} + B\overline{x^2} + C\overline{x^3}$$
$$\overline{x^2y} = A\overline{x^2} + B\overline{x^3} + C\overline{x^4}$$

where $\bar{y}$, $\bar{x}$, $\overline{x^2}$, $\overline{xy}$, etc., designate average values of the respective quantities associated with the 8-point data array. Rather than require the FPP to perform all operations associated with simultaneous solution of the 3 equations, the determinant expansion was performed by hand in the interest of conserving processor time. Relative x coordinate values of 1–8 are assigned the 8 data points involved in each quadratic fit-smooth operation. This allows assignment of numerical values to $\bar{x}$, $\overline{x^2}$, $\overline{x^3}$, and $\overline{x^4}$ so that the solutions to A, B, and C can be expressed in terms of 3 remaining unknowns, $\bar{y}$, $\overline{xy}$, and $\overline{x^2y}$. One obtains:

$$A = 15.5714\,\bar{y} - 7.28571\,\overline{xy} + 0.714286\,\overline{x^2y}$$
$$B = -7.28571\,\bar{y} + 4.04762\,\overline{xy} - 0.428571\,\overline{x^2y}$$
$$C = 0.714286\,\bar{y} - 0.428571\,\overline{xy} + 0.0476190\,\overline{x^2y}$$

Consequently, the FPP is simply assigned the task of calculating $\bar{y}$, $\overline{xy}$, and $\overline{x^2y}$ for a data set, applying the foregoing equations to obtain A, B, and C, and smoothing the data by assigning values on the quadratic to 6 central data points in the 8-point array.

After all the data have been smoothed, the FPP begins looking for each peak maximum. The average of the first 8 data points is stored as baseline 1. Advancing through the smoothed data, the FPP looks for 8 consecutive positive going points and then checks to see if the first and last of the 8 points exceed a preassigned threshold value. This subroutine compensates for noise peaks on the baseline. After the threshold test, the peak maxima are determined where the first derivative of the 8-point array passes through a slope of zero.

Since the function $dy/dx = 0$ is also satisfied at peak minimum, parameter C of the quadratic fit must be negative to be on peak maximum. The X and Y positions are stored in separate files in proper cup order. The slope must pass through zero before the 8 positive going points test subroutine is reentered. At the end of the array, the last average of 8 points is used as baseline 2. *See* flow diagram, Fig. 2.

The table of X-axis values is written onto one tape block while the table of Y-axis values is written onto the neighboring tape block. For example, location 1 of File X is retention time of cup No. 1, while location 1 of File Y contains the total absorbance (millivolts) of the peak for cup 1.

Since the FPP-12 has its own processor, the PDP-12 CPU can be performing other functions. While the smoothing and peak detection are in progress, the CRT is displaying the data array and the Teletype begins printing the report heading. The whole process takes only a few seconds, but allows the analyst to observe the automatic analysis output before and after treatment by digital filtering. A glance at the CRT will detect any malfunction in the hardware, instrument, plumbing, or interfacing.

(d) *Calculations.*—The software for 16-channel asynchronous ADC with data-logging onto magnetic tape and the FPP peak detection were written in symbolic LAP6-DIAL language which uses all 3 modes of the PDP-12/FPP-12 system. They are the 8-mode (13), the LINC mode (14), and the FPP mode (15).

Although the symbolic language programming is extremely versatile in the types of operations it can implement and in making efficient use of core memory (only 4K used), it is difficult to master and pro-

[3] To float a number in minicomputer terminology is to convert a 12-bit data word into three 12-bit data words consisting of a 12-bit signed binary exponent and a 24-bit signed binary mantissa (mantissa $\times$ $2^{Exponent}$).

[4] To fix a floating point number is the reverse operation, e.g., to reduce a 36-bit data word to a 12-bit word.

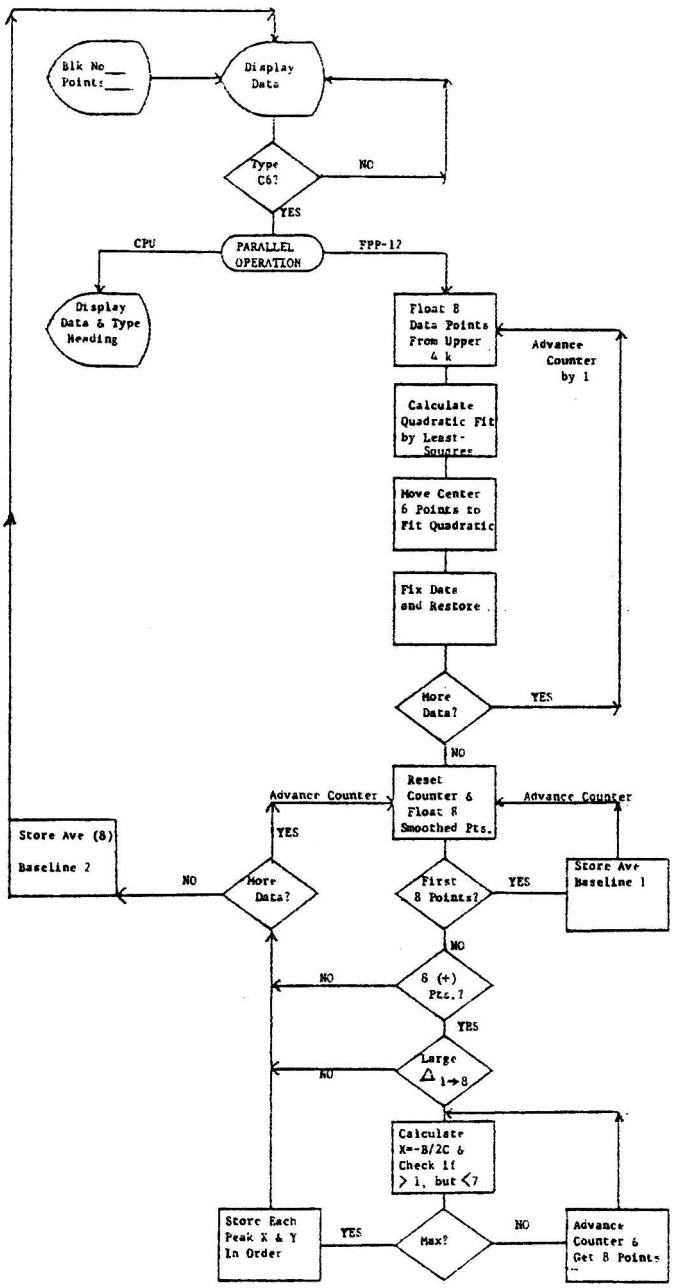

FIG. 2—Flow diagram.

```
C
710
1550
C6
FPP SMOOTHING!
F

SECONDS      NET PKHT    CODE      FOUND     % OF DECLARED


   0     165.0--76.93    S
 121     167.0--77.24    S
 240     168.0--77.55    S
 349     167.0--77.63     U        3.513        100.383
 481     177.0--78.17     U        3.662        104.622
 604     182.0--78.49     U        3.738        106.802
 715     172.0--78.77     U        3.599        102.820
 840     183.0--79.10     U        3.761        107.462
 952     162.0--79.39    S
1082     173.0--79.72     U        3.627        103.618
1202     171.0--80.03     U        3.602        102.924
1303     160.0--80.29     U        3.448         98.521
1428     158.0--80.61     U        3.424         97.833
1561     165.0--80.95     U        3.530        100.843
1686     169.0--81.28     C        3.592        102.636
1791     164.0--81.55    S
1911     170.0--81.86    S
AVE ( 10)                          3.590        102.583

STD DEV OF TABLETS              0.112          3.209
COEFFICIENT OF VARIANCE         3.129
AVE % DEV FROM DECL             3.672

FORMUL: SA/ST X CO X DIL X 1/UNITS = FOUND
INIT BSLN=-  76.9307
FINAL BSLN=-  75.1250
X AT FINAL BSLN= 2616.0000
STD CO X DIL X 1/UNITS =    3.4938
AVG TAB WT./COMP WT.=    1.0002
DECL =    3.5000


RECALC, Y OR N?:N

SPL NO.?:EXHIBIT
DATE?:10AUG72
ANALYST?:AUTHORS
```

FIG. 3—Computer printout of 10 tablet assay.

gram writing takes much longer than that for higher level languages. Consequently, as a temporary expediency, the data manipulation routines were written in FOCAL-12 language. Most of these routines had already been developed (16).

It is this laboratory's routine practice to sequence the order of cup solutions as follows: 3 standards, 5 individual tablets, standard, 5 tablets, etc., composite, 2 standards. The composite consists of a weighed portion of 20 tablets ground to a fine powder, equivalent to one tablet weight. For a 10 tablet assay, there are 17 peaks. For a 30 tablet assay, there are 41 peaks. The program corrects for system drift by calculating the slope between the first and last valid standard reading (the first 2 standards and the last standard are not considered valid). By a linear regression slope correction, each cup reading is adjusted respectively. Additionally, the initial baseline is subtracted from all peaks.

To determine the amount of active ingredient in each tablet, an average of the standard preceding and the one following the tablet cup is used as the standard reading. That resultant is divided by the label declaration and multiplied by 100 to yield per cent of declaration. Once the mean has been determined on the first pass through the data, the program reuses the data on a second pass to calculate the deviation, standard deviation, and coefficient of variance. The resulting computer printout is shown in Fig. 3.

FOCAL-12 INPUT FOR 10-TABLET ASSAY

A. USER INPUT	ON-LINE	OFF-LINE
1. Standard Concentration	0.0250	Same
2. Sample Dilution Factor	10	Same
3. Composite Weight	0.1906	Same
4. Ave. Tablet Weight	0.1907	Same
5. Declared in MG or GR?	MG	1 or 64.8
6. Amount Declared	0.25	Same
7. Total No. Peaks	17	14
8. No. ITA's	10	10
9. Comp. Cup No.	15	13
10. No. Standard Cups	6	3
11. Standard Cup Order	1,2,3,9,16,17	1,7,14
B. AUTO-ANALYZER DATA INPUT	(Millivolts) by FPP-12	(Absorbance) by teletype, TWX or Peak Detector Punch
0. Initial Baseline 1	-179.78	.050
1. First Standard	116.0	Not Entered
2. Second Standard	123.0	Not Entered
3. Third Standard	141.0	.552
4. Tablet No. 1	112.0	.510
5. Tablet No. 2	127.0	.531
6. Tablet No. 3	122.0	.523
7. Tablet No. 4	140.0	.556
8. Tablet No. 5	145.0	.562
9. Fourth Standard	140.0	.553
10. Tablet No. 6	117.0	.517
11. Tablet No. 7	140.0	.553
12. Tablet No. 8	124.0	.533
13. Tablet No. 9	125.0	.527
14. Tablet No. 10	142.0	.557
15. Composite	131.0	.543
16. Fifth Standard	135.0	.546
17. Sixth Standard	132.0	Not Entered
18. Baseline 2	-175.0	Not Entered

FIG. 4—FOCAL-12 input for 10 tablet assay.

Off-Line Software

The original FOCAL-8 ITA program (17) demonstrated that computer calculations and printing of results in worksheet form was the major portion of analytical time saved. Only a small portion of the total time was needed to manually determine the net absorbance of each analytical peak.

The off-line FOCAL-12 calculation routine is identical to the on-line system except for the manner in which the ITA data get onto tape memory. In the latter, the FPP performs the operation automatically; in the former the Teletype input of ITA data is required. Furthermore, FOCAL-12 input was found to be fast enough to accept data by paper tape reader, making the TWX system already in use between laboratories a means of transmitting automated absorbance data to the minicomputer and returning the completed worksheet without any additional hardware or phone connections.

This system assists analysts using instrumentation not directly interfaced. It was also determined that peak detector paper punch output is in acceptable tape input format. Initial user input is needed for both systems as shown in Fig. 4, items 1 through 11.

Results and Discussion

Although the data-logging software can sample as many as 16 instruments, only 3 automatic analyzers are available in our laboratory at present. The results reported in Table 1 were obtained while sampling: A, which was operating in the

Table 1. Results of individual tablet analysis by on-line automatic analyzer system vs. off-line system in per cent of declared

	SYSTEM A		SYSTEM B		SYSTEM C	
Tablet	On-line	Off-line	On-line	Off-line	On-line	Off-line
1	108.7	109.5	91.1	91.5	98.2	98.2
2	103.8	104.1	95.8	95.7	100.8	100.0
3	107.0	106.9	94.2	94.1	94.9	94.8
4	102.6	103.0	99.8	100.7	99.0	98.5
5	100.7	100.2	101.4	102.0	99.5	99.1
6	106.2	106.7	93.6	93.6	99.4	98.9
7	103.6	103.3	88.9	89.2	99.4	98.4
8	95.3	94.0	90.5	90.9	95.8	95.2
9	109.8	109.9	93.9	94.3	93.5	92.2
10	103.5	104.0	97.0	97.1	90.0	89.6
11			96.7	97.2	92.7	91.7
12			96.7	96.8	100.6	99.6
13			94.5	94.4	95.8	95.3
14			94.5	95.1	96.7	96.0
15			95.8	96.5	100.0	99.6
16			95.0	95.1	97.8	95.9
17			96.8	97.3	100.5	100.0
18			90.9	90.8	97.3	97.2
19			97.8	98.3	95.4	95.3
20			93.4	93.4	95.8	95.5
21			96.5	96.9	93.4	92.9
22			92.1	92.8	97.4	96.9
23			94.6	95.0	93.7	93.3
24			89.9	90.7	94.6	94.7
25			96.5	96.3	94.6	94.3
26			94.4	94.3	92.7	91.5
27			101.7	101.5	100.0	99.6
28			96.7	97.6	96.4	95.8
29			97.0	96.4	100.3	99.7
30			102.4	102.4	97.2	96.5
Composite	101.8	101.3	98.9	99.7	96.8	95.8
Av.	104.1	104.1	95.3	95.6	96.8	96.2
σ	1.261	1.398	0.009	0.008	0.003	0.003
Coeff. of var.	4.036	4.473	3.526	3.501	3.054	2.986

UV range, analyzing sodium pentobarbital; B, which was analyzing reserpine in the visible region by an acid-dye complexing method; and C, which was operating in the fluorescence mode for reserpine analysis (18).

The table shows that absolute differences in per cent of declared units did not exceed $\pm 1\%$, except for 2 tablets which varied less than $\pm 2\%$. These differences are within the uncertainty levels normally associated with the automated systems used in our laboratory. The results of Table 1 show that coefficients of variance range from 2.9 to 4.5 for the 3 samples. However, the coefficient of variance of the system is usually less than 2.0 as determined by analyzing 10 portions of the same composite solution. Consequently, there is no evidence for degradation of data fidelity due to utilization of the on-line computer system. Linearity of the operational amplifier interface and the ADC data acquisition has been previously reported (8).

Conclusions

With a minicomputer hardware system costing less than $50,000, software has been developed to process data from 16 automatic analyzers operating simultaneously. In addition, the calculation routine has been made available to many other laboratories and analysts in an off-line mode by using an already established TWX communication system. Calculation time for a 30 tablet analysis, including standard deviation and coefficient of variance, is reduced from a 2 hr manual operation to 8 min for the off-line mode. A further reduction to 2 min is realized by using the on-line system, most of which is the time necessary for the Teletype to print the report.

There are several limitations with the present minicomputer system, some of which can be overcome by software modification, others by hardware additions. With only 8K core memory, the program is restricted to 4K core to allow a data buffer of 4096 points (10) in the second 4K mem-

ory bank. Using FOCAL-12 for the calculation steps also restricts the system from operating on a time-sharing basis, i.e., to be able to data-log from several instruments while processing data from another. A time-sharing system for automatic analyzers can be achieved by writing the calculation steps in symbolic language so that the sampling program and calculation program can be swapped from the disk between ADC interrupts. Expanding the core memory could even allow foreground/background system operations.

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Copies of the software and documentation are available through DECUS (Digital Equipment Users Society), 146 Main St., Maynard, Mass. 01754.

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Determination of Propoxyphene and Aspirin in Combinations

CATHERINE G. CUNNINGHAM and SUSAN BARKAN
Division of Drug Chemistry, Food and Drug Administration, Washington, D.C. 20204

Propoxyphene is assayed by a partition chromatographic method in which it is eluted as the ion pair with $CHCl_3$ from a sulfamic acid-Celite column and determined spectrophotometrically. Napsylate anion present in some formulations is retained on a $NaHCO_3$-Celite trap column.

The official method of assay for propoxyphene HCl and aspirin capsules in the *National Formulary* XIII (1) makes use of a conventional extraction procedure to separate the 2 drugs. The concentration of each is then determined by IR spectroscopy.

Recently, propoxyphene napsylate and aspirin tablets have appeared on the market. We report here a fast, simple separation by partition chromatography. The concentration of each compound is then determined by UV spectrophotometry.

A definite number of coated tablets are dissolved in chloroform, filtered, diluted, and chromatographed through sodium bicarbonate-Celite. Aspirin is retained on the column, and propoxyphene is converted to the free base. This compound is trapped on a sulfamic acid-Celite layer, eluted as the ion pair with chloroform (2), and determined spectrophotometrically.

Aspirin in capsules may be determined by elution of the column with acetic acid-$CHCl_3$ as previously described (3). A separate, smaller aliquot on another bicarbonate column is necessary for determination of aspirin in tablets, due to interferences caused by either the film coating or other excipients.

METHOD

Apparatus and Reagents

(a) *Sodium bicarbonate.*—1*M*.

(b) *Sulfamic acid.*—5% aqueous solution.

(c) *Acid-alcohol.*—Concentrated HCl-methanol (1+49).

(d) *Sulfuric acid.*—0.1*N*.

(e) *Solvents.* — Chloroform (water - saturated), ether (water-saturated), anhydrous ether.

(f) *Chromatographic columns.*—250 × 25 mm id.

(g) *Diatomaceous earth.*—Celite 545, acid-washed.

(h) *Propoxyphene HCl standard solution.*—0.50 mg/ml. Dissolve 50 mg, accurately weighed, USP Reference Standard in 100 ml 0.1*N* H_2SO_4.

(i) *Aspirin standard solution.*—0.05 mg/ml. Immediately before use, dissolve 100 mg, accurately weighed, USP Reference Standard in $CHCl_3$ and dilute with 1% glacial acetic acid in $CHCl_3$ to proper concentration.

Preparation of Column

Place small wad of glass wool in base of 250 × 25 mm column. *Lower layer.*—Mix 3 g Celite with 2 ml 5% sulfamic acid, transfer to column, and tamp firmly. *Upper layer.*—Mix 4 g Celite with 3 ml freshly prepared 1*M* $NaHCO_3$, transfer to column, and tamp firmly. Place wad of glass wool on top of column. Wash column with 50 ml water-saturated $CHCl_3$, let drain, and wash with 50 ml water-saturated ether.

Preparation of Sample

(a) *Propoxyphene HCl and aspirin in capsules.*—Remove as completely as possible the contents of 20 capsules and determine average net contents. Mix powder and pass through 60 mesh sieve. Transfer to 100 ml flask containing 2 ml acid-alcohol, an accurately weighed portion of powder equivalent to ca 130 mg propoxyphene HCl. Add 60 ml $CHCl_3$, and mix thoroughly by shaking. Dilute to volume with $CHCl_3$, and mix.

(b) *Propoxyphene napsylate with aspirin in tablets.*—Place 5 tablets (equivalent to 500 mg propoxyphene napsylate) in 250 ml glass-stoppered Erlenmeyer flask. Cover tablets with $CHCl_3$ and let stand until coating swells (ca 10 min). Using glass rod, puncture coating so that $CHCl_3$ can come in contact with inner core. Place stoppered flask on mechanical shaker until cores are completely disintegrated. Quantitatively wash contents through fluted fast filter paper into 250 ml volumetric flask containing 5 ml acid-alcohol. Rinse flask and filter with several portions $CHCl_3$ and dilute to volume with $CHCl_3$.

Separation of Propoxyphene from Aspirin

Dilute 10.0 ml sample preparation with 30 ml water-saturated ether in 100 ml beaker. Transfer solution to column. After all solution has passed into column, rinse beaker with four 5 ml portions water-saturated ether, letting each portion pass into column before adding next portion. Rinse tip of column

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and discard eluate. Elute propoxyphene with 75 ml water-saturated $CHCl_3$ into 250 ml separatory funnel. Proceed without delay with determination of aspirin below.

Dilute $CHCl_3$ in funnel with ca 150 ml anhydrous ether and extract propoxyphene with 9, 9, and 4 ml portions 0.1*N* H_2SO_4, collecting the portions in 25 ml volumetric flask. Dilute combined extracts to volume with 0.1*N* H_2SO_4, mix, and determine absorbance of this solution and of standard solution at maximum at ca 257 nm, using 0.1*N* H_2SO_4 as blank.

mg Propoxyphene HCl/10 ml aliquot of sample preparation = $(A_u/A_s) \times C \times 25$, where A_u and A_s = absorbance of sample and standard solutions, respectively; C = concentration (mg/ml) of propoxyphene HCl standard solution. For propoxyphene napsylate, multiply by a factor of 1.5.

Determination of Aspirin

Sample preparation (a).—Immediately elute aspirin from column with solution of 1 ml glacial acetic acid in 10 ml $CHCl_3$, followed by ca 90 ml 1% glacial acetic acid in $CHCl_3$, into 100 ml volumetric flask. Dilute to volume, and mix. Transfer 5.0 ml this solution to 50 ml volumetric flask, and dilute to volume with 1% acetic acid in $CHCl_3$. Determine absorbance of sample and standard solutions at maximum at ca 280 nm, using $CHCl_3$ as blank.

Sample preparation (b).—Determine aspirin as soon as possible after preparation of sample solution. Use 5.0 ml aliquot of sample preparation (b) and proceed as directed in the *U.S. Pharmacopeia* XVIII for the assay of aspirin in tablets (4).

Discussion and Results

Initially in the determination of propoxyphene HCl and aspirin in capsules, the sulfamic acid layer was placed above the bicarbonate layer. The sample preparation, diluted with ether, was chromatographed, and the propoxyphene was eluted with chloroform as its sulfamate ion pair. However, we found that propoxyphene napsylate is highly extractable with chloroform. Significant amounts of propoxyphene were eluted as the napsylate, rather than sulfamate, ion pair, with the result that the UV spectrum was essentially that of napsylate. By reversing the acid-base layers, the napsylate was readily removed by bicarbonate from the less polar ether-chloroform solvent; propoxyphene free base passed through this layer but was retained by the sulfamate column. It was then eluted free of napsylate by chloroform. This procedure worked equally well with propoxyphene HCl.

Standard mixtures of (*1*) propoxyphene HCl and aspirin and (*2*) propoxyphene napsylate and aspirin were assayed using this method. Recoveries were (*1*) 99.9, 101.0% for propoxyphene HCl, 99.8, 99.3% for aspirin; and (*2*) 99.7, 99.8% for propoxyphene napsylate, and 99.0% for aspirin on a separate aliquot.

Two commercial samples of propoxyphene HCl and aspirin capsules were analyzed by the method. The results were 98.9 and 96.5% for propoxyphene HCl and 97.8 and 96.4% for aspirin. One commercial sample of propoxyphene napsylate with aspirin tablets was assayed by the method, using 2 different sample preparations of 5 tablets each. The results for propoxyphene napsylate were 92.9 and 94.6%. Aspirin determined on separate aliquots from the 2 preparations was 97.0 and 93.0% (aspirin partly hydrolyzed—95% was calculated as the total).

Acknowledgment

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Nuclear Magnetic Resonance Analysis of Pharmaceuticals. IX. Methenamine and Methenamine Mandelate in Tablets

JOHN W. TURCZAN and BRUCE A. GOLDWITZ
Food and Drug Administration, 850 Third Ave., Brooklyn, N.Y. 11232

An NMR procedure is described by which methenamine and mandelic acid are simultaneously determined in methenamine and methenamine mandelate tablets. Maleic acid was chosen as the internal standard and dimethylformamide-acetone-acetonitrile (10+25+65) as the solvent. The solvent system was selected to resolve the problems of solubility of methenamine and its salt with mandelic acid, overlapping of the resonance signals of the components, and potential decomposition of methenamine. Known standard and commercial preparations were analyzed and the results were compared to those of official USP and NF procedures. The NMR technique, when applied to the determination of methenamine and methenamine mandelate in tablets, is rapid, simple, and specific and can provide an assay with an accuracy of 1–2%.

Methenamine (I) and its salt, methenamine mandelate (II), are both useful as urinary antibacterials. Unique among official therapeutically active salts, II derives its activity from both of its constitutive ions, the protonated methenamine and the mandelate. Assay procedures for both I and II are available from the literature.

The most widely discussed technique for the determination of I is based on its hydrolysis to formaldehyde and ammonia in aqueous solutions of strong acids (1–3). The reaction is quantitative and is the foundation for numerous methods of analysis (4–9). The formaldehyde can be removed by several hours of heating (7) and the excess acid may be back-titrated with alkali (6), or the formaldehyde itself may be determined by using Nessler's reagent (4, 9).

Other techniques employed in the determination of I are argentimetric (10) and nonaqueous titrimetry (11, 12), polarography (13), complexation with subsequent titrimetry (14, 15), and colorimetry (16–19) using the chromotropic acid technique (20, 21).

An alternative indirect method of analysis for II relies on the measurement of mandelic acid (III). The oxidation of the α-hydroxy function of III by Ce^{+4} species has been reported. Helmstaeder (22) applied the Verma and Paul cerimetric titration of III (23) to the analysis of II, a procedure which required refluxing with excess ceric sulfate in sulfuric acid. The method proved to be nonspecific for III in that formaldehyde liberated from methenamine base was also oxidized. Chafetz and Gaglia (24), noting that the reaction rate of the oxidation of mandelic acid by Ce^{+4} was retarded by bisulfate and hydrogen ion (25), used cerium ammonium nitrate in dilute nitric acid and found that at room temperature only III was oxidized.

Methods for the determination of both I and II in combination dosage forms include titrimetry (26–31), gas chromatography (32, 33), and column partition chromatography (34). Baum and Goodman (35) reported a nuclear magnetic resonance (NMR) method for the assay of I in thermosetting molding compounds, utilizing an initial extraction with chloroform.

The official approaches to the measurement of I (as such and as II) vary in the *National Formulary* and the *U.S. Pharmacopeia*. The NF XIII assay (36) for I in tablets involves the hydrolysis of I with excess standard sulfuric acid (to produce formaldehyde and ammonium sulfate), followed by titration of the residual acid. The USP XVIII procedure for methenamine mandelate (37) is based on hydrolysis to produce formaldehyde which is then reacted with a modified Nessler reagent to yield metallic mercury. The latter is oxidized with excess standard iodine (to yield the Hg^{+2} species), followed by titration of the residual iodine with thiosulfate. In the monograph for pure II, the USP XVIII requires a further analysis. A separate specification for the III content of II requires that a sample be titrated with standard base which is actually the titration of the protonated I. Thus the III content is deduced rather than directly measured. Interference from other acidic materials is obvious.

As has been demonstrated previously, NMR

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spectroscopy is an advantageous technique for the determination of many high dosage pharmaceuticals. With the addition of an internal standard and subsequent extraction with a propitious solvent, both I and II can be rapidly assayed by this technique. In addition to quantitative results, the NMR spectrum furnishes an identification of the active ingredient, thereby contributing to the specificity of the method.

Experimental

Apparatus and Reagents

(a) *Apparatus.*—Varian A-60 NMR spectrometer equipped with V-6031 variable temperature probe having 6-turn insert. All spectra were scanned at probe temperature of 42°C.

(b) *Standards.*—Methenamine (hexamethylenetetramine) and methenamine mandelate, K & K Laboratories, Inc., Plainview, N.Y.

(c) *Internal standard.*—Maleic acid, Eastman Organic Chemicals, Rochester, N.Y.

(d) *Solvent.* — Acetonitrile - acetone - dimethylformamide (65+25+10).

(e) *Samples.*—Methenamine and methenamine mandelate, 250, 500, and 1000 mg tablets from various commercial sources.

Procedure

Weigh and finely powder ≥20 tablets in mechanical grinder and pass through 60 mesh sieve. Accurately weigh portion of powder containing ca 70 mg methenamine or 150 mg methenamine mandelate into glass-stoppered centrifuge tube. Add ca 350 mg accurately weighed maleic acid internal standard. Fill tube to ca 3 ml mark with solvent mixture. Stopper, shake ca 2 min, and centrifuge.

Transfer ca 0.4 ml clear solution to analytical NMR tube. Place in spectrometer and record spectrum, adjusting spin rate so that no spinning sidebands occur between 4.7 and 5.3 ppm, 6.1 and 6.5 ppm, and 7.1 and 7.7 ppm, on delta scale. Reference all peak field positions to tetramethylsilane at 0 ppm. Integrate peaks of interest at least 5 times.

Calculate amount of I, II, and III as follows:

$$\text{mg Methenamine/tablet} = (IV_M \times 11.68 \times \text{mg IS} \times \text{av. tab. wt})/(IV_{IS} \times 58.04 \times \text{sample wt})$$

Methenamine mandelate can be calculated from methenamine content by a simple formula weight conversion: 292.3/140.19.

$$\text{mg Mandelic acid/tablet} = (IV_{MA} \times 30.43 \times \text{mg IS} \times \text{av. tab. wt})/(IV_{IS} \times 58.04 \times \text{sample wt})$$

where IV_M, IV_{MA}, and IV_{IS} = integral value of signals representing methenamine, mandelic acid, and maleic acid; 11.68 = formula weight of methenamine/12; 30.43 = formula weight of mandelic acid/5, 58.04 = formula weight of maleic acid/2; and IS = internal standard (maleic acid).

Results and Discussion

Initially, the logical choice of solvent for I and II appeared to be water, since both the solubility and stability of I and II in this solvent are very good. However, water presented a problem because its resonance signal occurs at approximately the same chemical shift as that of the methylene protons of I, and it interfered with the integration of the peak of interest. Attempts to overcome this problem with a shift of the water signal further downfield by acidifying the aqueous solution proved unsuccessful because the stability of I rapidly decreases with decreasing pH.

To overcome these difficulties, a ternary solvent mixture was evolved: acetonitrile-acetone-dimethylformamide (65+25+10). Although acetone and dimethylformamide individually appeared to dissolve the solutes, the use of a mixture presented advantages. Acetone increased the maleic acid internal standard solubility, whereas the dimethylformamide aided the solubility of I and II. Also, the slightly basic nature of dimethylformamide provided a medium in which I would be stable. Finally, the presence of acetonitrile, the major solvent component, caused a desirable separation of the singlet due to I from the singlet ascribable to the III methine proton. The I resonance signal is shifted upfield an additional 7 cps relative to the separation of the 2 signals obtained by solubilizing II in an acetone-dimethylformamide (90+10) solvent mixture. This effect thus permits a more accurate integration of the signals. The combination proved to be satisfactory for the assays of I and II in both the pure powder and in the tablet dosage form.

The stabilities of solutions of I and II in the solvent mixture were observed for 2 hr under the experimental conditions described above. No noticeable changes in the shapes of the resonance signals due to I and II were observed, nor were there any significant changes in the recoveries of either the pure standards or the tablet dosage forms. On this basis, it may be concluded that drug stability should be no problem in the described procedure.

A single internal standard, maleic acid, was found to be satisfactory for both I and II in that it is easily soluble in the selected solvent system,

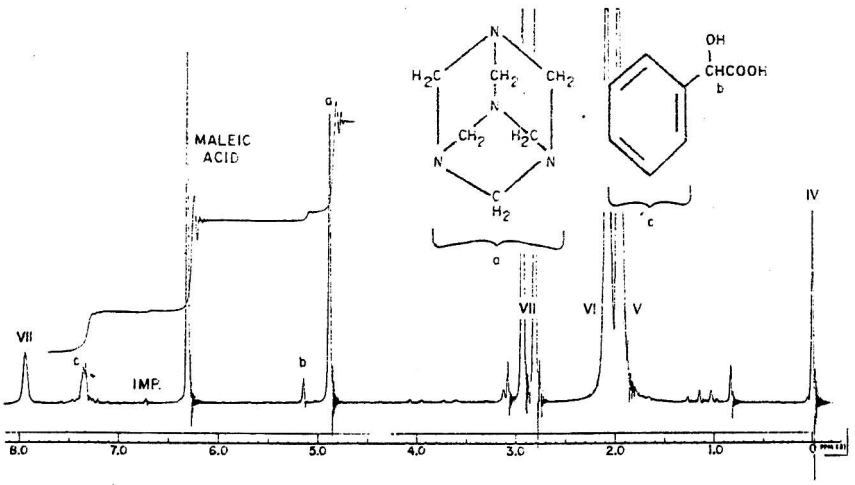

FIG. 1—NMR spectrum of methenamine mandelate in dimethylformanide-acetone-acetonitrile (10+25+65): IV, tetramethylsilane; V, acetonitrile; VI, acetone; VII, dimethylformanide.

and it provides a single resonance signal at a convenient downfield position.

A 60 MHz NMR spectrum of II obtained in the analysis is exhibited in Fig. 1. The resonance signals used in the actual calculations are the singlet at about 4.90 ppm due to the 12 methylene protons of I, the multiplet at about 7.35 ppm resulting from the 5 phenyl protons of III, and, finally, the singlet at about 6.30 ppm ascribable to the 2 olefinic protons of the maleic acid internal standard.

Other peaks are noted in the spectrum. A small fumaric acid impurity present in the maleic acid exhibits a singlet at about 6.73 ppm, downfield from the maleic acid signal. However, this impurity presents no problem since the total integral value for maleic and fumaric acids can be used in the calculations or a purity correction for maleic acid can be substituted. The other signals in the spectrum are the singlet at about 5.15 ppm due to the methine proton of III and the broad region between 0.7 and 3.2 ppm resulting from the solvent mixture. Dimethylformamide also exhibits a broad singlet at about 7.95 ppm attributed to its formyl proton.

A group of known I and II standard mixtures were analyzed by this technique and the results are summarized in Table 1, along with those obtained by the NF XIII for I and by the USP XVIII for II. In addition, the relative proportions of I and II to the maleic acid internal standard show no significant bearing on the accuracy of the determination for the range of preparations. The procedure proved to be both accurate and precise with a mean of 99.3% and a standard deviation of ±0.7, and compares favorably with the mean from the USP procedure.

Approximately 9 commercial tablet formulations of I and II have been determined, implementing the above method with no evidence of interference from excipients present. The results

Table 1. Determination of methenamine mandelate in standard mixtures by NMR

Std	Maleic acid int. std. mg	Methenamine mandelate Added, mg	Found,[a] mg	Rec., %	Mandelic acid found mg	%
1	355.0	153.4	152.9	99.7	84.5	55.1
2	329.4	143.0	142.7	99.8	79.9	55.9
3	352.7	148.1	146.9	99.2	78.9	53.3
4	200.4	152.1	151.2	99.4	80.5	52.9
5	356.1	145.3	144.9	99.7	77.3	53.2
6	346.7	139.7	139.4	99.8	75.0	53.7
7	502.3	151.4	150.9	99.7	81.3	53.7
8	355.9	148.3	145.2	97.9	77.6	52.3
9	347.1	147.7	145.3	98.4	80.6	54.6
10	344.7	147.8	146.3	99.0	78.0	52.8
Av.				99.3		53.6
Std dev.				0.7		1.1
USP				98.6		52.8

[a] Based on methenamine content.

Table 2. Determination of methenamine and methenamine mandelate in commercial tablets by NMR

Sample	Declared, mg/tab.	NMR[a] mg/tab.	NMR[a] %	Official procedure[b] mg/tab.	Official procedure[b] %	Mandelic acid by NMR mg/tab.	Mandelic acid by NMR %
Methenamine mandelate							
1	500	479	95.8	490	98.0	256	51.2
2	1000	940	94.0	954	95.4	498	49.8
3	500	486	97.2	492	98.4	256	51.2
4	250	249	99.6	248	99.2	130	52.0
5	1000	992	99.2	988	98.8	526	52.6
6	500	494	98.8	492	98.4	238	47.6
Methenamine							
1	500	483	96.6	478	95.6	—	—
2	500	489	97.8	491	98.2	—	—
3	500	492	98.4	490	98.0	—	—

[a] Based on methenamine content.
[b] USP XVIII for methenamine mandelate samples and NF XIII for methenamine samples.

presented in Table 2 are in good agreement with both the declared dosages and those results obtained by the official NF and USP procedures. The NMR results are, on the average, within 1.0% of the USP assay figures and within 0.6% of the NF method values.

Another useful combination of I and II results when sodium dihydrogen phosphate is included in the formulation. The procedure as described can also be used for determination of I and II in this mixture since the phosphate in no way interferes.

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ATTENTION

Abstracts of papers or reports to be presented at the 87th Annual Meeting of the AOAC, Oct. 9–12, 1973, MUST be submitted on standard, preprinted forms. The forms are available, on request, from the AOAC Editorial Office.

Since 1971 the Abstract Bulletin has undergone two changes. The abstract submitted by the author is now reproduced directly by a photographic process; it is no longer edited and retyped in the Editorial Office. This new procedure makes it imperative that authors provide an informative, carefully prepared abstract typed on the special form, plus 2 additional copies (carbon copies or photocopies). This form includes general rules for preparing satisfactory abstracts. Abstracts not fulfilling these requirements will be returned to the author for correction, if time permits. Improperly prepared abstracts received too late to allow this will not appear in the Abstract Bulletin. The Abstract Bulletin itself is a new size—4 × 9″, the same size as the AOAC program.

In addition to the abstract, five copies of *all* contributed papers and Associate Referee reports must be submitted to the AOAC office. The manuscripts should be double-spaced throughout. Unless otherwise specified, all manuscripts received will be considered for publication in *JAOAC*, pending satisfactory review. Each Associate Referee should also send one copy of his report to his General Referee.

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Collaborative Study of a Spectrophotometric Method for the Determination of Dienestrol in Pharmaceuticals

JOSEPH H. GRAHAM
Division of Drug Chemistry, Food and Drug Administration, Washington, D.C. 20204

A highly specific, sensitive, and rapid method for the determination of dienestrol was studied by 13 collaborators. Dienestrol is separated from excipient matter, degradation products, and other drugs present by column partition chromatography, converted to a substituted indene by acid-induced isomerization, and determined by ultraviolet spectrophotometry. Standard deviations obtained for the 4 samples used in the study, containing approximately 0.1 and 0.5 mg dienestrol, ranged from 1.81 to 3.04. The most critical step in the method is the use of suitable ether. This method has been adopted as official first action.

A method for the determination of dienestrol in pharmaceuticals was presented at the 1971 meeting of the AOAC (1). The method was shown to be highly specific, sensitive, and rapid, presenting several advantages over the current official methods in the *National Formulary* (2). Because of these advantages, it was recommended that the method be subjected to a collaborative study.

Collaborative Study

Thirteen collaborators were each supplied with 4 samples and a suitable quantity of dienestrol reference standard. Samples A, B, and C were commercial coated tablets declared to contain 0.1, 0.5, and 0.5 mg dienestrol/tablet. Sample D was a specially prepared uncoated tablet declared to contain 0.1 mg dienestrol/tablet.

The collaborators were requested to perform a single assay on each sample, but, if time permitted, multiple assays could be performed provided that all results were reported. The collaborators were also given the option to deviate from the requirement to use an amount of sample equivalent to 400 μg dienestrol; they could take any amount from 100 μg to 10 mg dienestrol provided that (*a*) the sample weight did not exceed 1.3 g and (*b*) the assay preparation was diluted to the 7.5 to 15 μg/ml range. Due to the possible deterioration of ground tablet composites, the collaborators were urged to prepare them as close to the time of assay as practical. The risks stemming from the use of impure ether were pointed out and an ether suitability test was provided. All raw data and spectra were requested from the collaborators, as well as their comments and suggestions.

METHOD

Dienestrol—Official First Action

(*Caution: See* 46.011, 46.018, 46.045, 46.054, 46.062, and 46.066.)

36.C10 ***Reagents***

(a) *Dienestrol std soln.*—Approx. 15 μg/ml. Accurately weigh NF Ref. Std Dienestrol, dissolve in MeOH, and serially dil. to concn. Store in low-actinic vol. flask.

(b) *Methanolic sulfuric acid.*—Carefully add, with swirling, 50 ml H_2SO_4 to 50 ml cold MeOH, while continuously chilling mixt. in ice-H_2O. Use reagent at room temp. Reagent is stable 3–4 days in g-s flask.

(c) *Ethyl ether.*—Test as follows on day of use: Evap., with gentle heat and air stream, mixt of 10.0 ml dienestrol std soln in ca 200 ml H_2O-washed ether. Dissolve residue in 10.0 ml MeOH. Proceed as in 36.C13, using this soln and 5.0 ml dienestrol std soln. Resulting solns should be clear and exhibit single max. at ca 303 nm, and corrected *A*, 36.C14, should differ ≤3%.

(d) *Diatomaceous earth.*—Celite 545, acid-washed.

36.C11 ***Preparation of Columns***

Trap column.—Mix 4 g Celite and 3 ml *0.25M KOH* and transfer to 200 × 22 mm glass chromatgc tube contg glass wool plug. Tamp mixt. tightly and top with glass wool pad. Prewash column with 25 ml H_2O-washed ether, followed by 25 ml benzene.

Sample column.—Accurately weigh freshly ground sample contg ca 400 μg dienestrol into 150 ml beaker. Add 3 ml *0.3M* K_3PO_4 and wet sample completely. Add 5 g Celite and mix thoroly with spatula.

Quant. transfer sample mixt. to 200 × 22 mm glass chromatgc tube contg glass wool pad in 2 equal portions, tamping each portion moderately tight. Dry-rinse beaker with 1–2 g Celite and add rinse to

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column. Wipe tamper, spatula, and beaker with glass wool pad and add pad to top of column.

36.C12 *Chromatography*

Arrange columns so that eluate from sample column passes into trap column. Add 25 ml benzene to trap column; then add 175 ml benzene-isooctane (9+1) to sample column, using several portions to rinse sample beaker. Maintain layer of eluant over trap column. (To maintain this reservoir in trap column, connect the 2 columns with air-tight stopper, i.e., hollow No. 4 Nalgene stopper with hole drilled to accommodate stem of sample column.) Discard sample column when elution is complete. Wash trap column with addnl 25 ml benzene-isooctane (9+1) and discard eluates.

Elute dienestrol from trap column with 225 ml H_2O-washed ether into 250 ml g-s conical flask contg 10 ml absolute alcohol. Without delay evap. to near dryness, using air stream and gentle heat. Rinse flask walls with small amt absolute alcohol and evap. soln to dryness. Pipet 25 ml MeOH into flask, stopper tightly, and let stand several min with frequent vigorous swirling.

36.C13 *Isomerization*

Into sep. 25 ml g-s conical flasks pipet 5 ml dienestrol std soln, 5 ml sample prepn, and 5 ml MeOH as reagent blank. Add 5.0 ml methanolic H_2SO_4 to each flask with swirling (solns will become warm). Stopper flasks tightly and shake vigorously; then let cool $\geq$25 min at room temp.

36.C14 *Determination*

Det. A of sample and std solns between 400 and 240 nm in 1 cm cells against reagent blank. Correct A at ca 303 nm by subtracting A at 360 nm.

mg Dienestrol/tablet = $[(A/A') \times C \times V \times W]/Q$, where A and A' refer to sample and std solns, resp.; C = exact concn of std in mg/ml; V = ml sample diln (25 ml); W = av. tablet wt (g); and Q = sample wt (g).

Results and Recommendation

No collaborator reported any serious difficulty in following the method except that of finding suitable ether. Several collaborators found it necessary to test several brands and grades of ether before they found a lot that did not either cause distortion of the spectrum of the isomerized dienestrol or produce an excessive background. Freshly opened reagent grades as well as the popular distilled-in-glass variety commonly used in pesticide analyses were found to be unsuitable. One collaborator's comment in this regard called attention to the fact that due to the possible rapid deterioration of ether upon exposure to air it is necessary to reconfirm the suitability of the ether on the day of use and not allow the ether eluates to stand overnight.

Some comments were received in regard to the clarity of some of the phraseology employed. These suggestions and those with reference to the

Table 1. Collaborative results for the determination of dienestrol (mg/tablet)

	0.1 mg/tablet				0.5 mg/tablet			
	Sample A		Sample D		Sample B		Sample C	
Coll.	Found	% of Decl.	Found	% of Decl.	Found	% of Decl.	Found	% of Decl.
1	0.0875	87.5	0.0974	97.4	0.467	93.5	0.494	98.8
2	0.0955	95.5[c]	0.1061	106.1[a]	0.492	98.4[c]	0.510	102.1[a]
3	0.0914	91.4	0.0990	99.0	0.482	96.4	0.491	98.2
4	0.0950	95.0	0.0950	95.0	0.467	93.4	0.478	95.6
5	0.0876	87.6	0.0941	94.1	0.474	94.8	0.483	96.3
6	0.0903	90.3	0.0992	99.2	0.475	95.0	0.482	96.4
7	0.0913	91.3	0.0998	99.8	0.481	96.2	0.473	94.6
8	0.0890	89.0	0.0770	77.0[b]	0.447	89.4[b]	0.456	91.2[b]
9	0.0842	84.2	0.0948	94.8	0.460	92.0	0.473	94.6
10	0.0880	88.0	0.1010	101.0	0.484	96.8	0.469	93.8
11	0.0850	85.0	0.0936	93.6	0.438	87.6[c]	0.438	87.6[c]
12	0.0889	88.9	0.0995	99.5	0.487	97.4	0.495	99.0
Av.	0.0889	88.9	0.0973	97.3	0.475	95.0	0.482	96.4
Std dev.		3.04		2.72		1.81		1.92
Coeff. of var.		3.42		2.79		1.90		1.99

[a] Result not included in statistical treatment due to excessive error in standard.
[b] Sample spectrum distorted; result not included in statistics.
[c] Rejected as outlier (3).

ether problems were incorporated into the above method.

The analytical results reported by 12 collaborators and the statistical treatment are presented in Table 1.

In view of the desirable features of this method in comparison to the current official *National Formulary* procedures (2), its precision and accuracy as established in the initial report of the method, and the results of its interlaboratory durability as shown in this study, it is recommended that this method be adopted as official first action.

Acknowledgments

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee B and was adopted by the Association; *see* (1973) *JAOAC* 56, 397.

Collaborative Study of a Column Chromatographic Method for Chlorothiazide, Methyclothiazide, and Polythiazide

F. RAYMOND FAZZARI
Division of Drug Chemistry, Food and Drug Administration, Washington, D.C. 20204

Chlorothiazide is eluted from a K_2HPO_4 column with acetic acid-ether solvent and extracted from the organic phase into HCl for the spectrophotometric determination. Methyclothiazide and polythiazide are eluted from a $NaHCO_3$ column with $CHCl_3$ and measured directly. The method was collaboratively studied by 10 analysts. The average per cent recovered and standard deviations for preparations of chlorothiazide and methyclothiazide were 100.2±0.67 and 99.8±1.64, respectively. The method for chlorothiazide and methyclothiazide has been adopted as official first action.

A method for the determination of some thiazide compounds have been previously reported (1). This presentation is a modification of that method. The original method used NaOH as the immobile phase in the partition chromatographic step. This cannot be used in the assay of chlorothiazide, methyclothiazide, and polythiazide because they degrade quite rapidly in strongly alkaline solution: Chlorothiazide and polythiazide degrade to 4-amino-6-chloro-1,3-benzenedisulfonamide and methyclothiazide to 4-amino-6-chloro-N^3-methyl-1,3-benzenedisulfonamide. As alternatives to NaOH it was found that K_2HPO_4 was satisfactory for chlorothiazide and $NaHCO_3$ was satisfactory for methyclothiazide and polythiazide.

The official *National Formulary* XIII methods for chlorothiazide and methyclothiazide are very similar polarographic procedures. Such procedures are very susceptible to interferences, not all of which can be satisfactorily explained.

The NF XIII procedure for polythiazide involves separation by thin layer chromatography and spectrophotometric measurement of the isolated material. Standards and blanks are treated similarly to the sample.

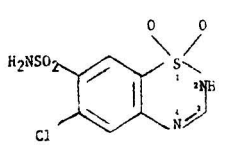
Other methods involve hydrolysis and colorimetric measurement after diazotization and coupling with compounds such as thymol blue or chromotropic acid (2–5). Titrimetric methods have also been applied to these compounds.

The method reported here is simpler, more direct, more specific, and free of the analytical problems associated with the aforementioned methods. Chlorothiazide is eluted from a 0.2*M* K_2HPO_4-Celite column with acetic acid-ether, extracted from the organic phase into 0.2*N* HCl, and determined by UV spectrophotometry. Methyclothiazide and polythiazide are eluted from a 0.1*M* $NaHCO_3$-Celite column with $CHCl_3$ and measured directly by UV spectrophotometry.

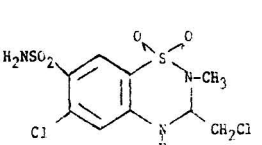
To test the validity of the method a collaborative study was initiated. Chlorothiazide and methyclothiazide were used in the study. Because of the close similarity of methyclothiazide and polythiazide we did not feel it was necessary to include both compounds.

The collaborators received simulated and authentic commercial preparations of both compounds. The commercial chlorothiazide preparation was labeled to contain 250 mg/tablet while methychlothiazide was labeled to contain 2.5 mg/tablet. The synthetic mixes of methyclothiazide and of chlorothiazide contained 1.25 and 67.5%, respectively, in tablet excipients, simulating the commercial products.

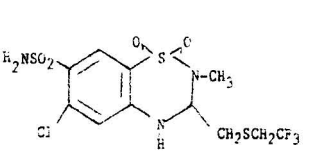
Ten collaborators assayed the samples; 6 collaborators made duplicate assays while 4 made



Chlorothiazide



Methyclothiazide



Polythiazide

triplicate assays. Results were reported on a per cent basis.

For uniformity and convenience the method studied this year is combined with the official first action method for benzthiazide, hydrochlorothiazide, and hydroflumethiazide, 36.B27–36.B31.

METHOD[1]

36.C38 *Principle*

Benzthiazide, hydrochlorothiazide, or hydroflumethiazide is eluted from 0.2*N* NaOH-Celite column with HOAc-ethyl ether, extd into 0.2*N* NaOH, and detd by UV spectrophotometry. Chlorothiazide is eluted from 0.2*M* K_2HPO_4-Celite column with HOAc-ethyl ether, extd into 0.2*N* HCl, and detd by UV spectrophotometry. Methyclothiazide is eluted from 0.1*M* $NaHCO_3$-Celite column with $CHCl_3$ and measured directly by UV spectrophotometry.

36.C39 *Apparatus and Reagents*

(a) *Chromatographic tube and tamping rod.*—*See* 36.015(a) and (b).

(b) *Diatomaceous earth.*—Celite 545, acid-washed.

(c) *Dipotassium phosphate solns.*—0.2 and 0.1*M*, 35.64 and 17.42 g K_2HPO_4/L, resp.

(d) *Benzthiazide, hydrochlorothiazide, and hydroflumethiazide std solns.*—Prep. with ether-satd 0.2*N* NaOH. (*1*) *Benzthiazide.*—3.0 mg NF Ref. Std/200 ml. (*2*) *Hydrochlorothiazide.*—2.0 mg USP Ref. Std/200 ml. (*3*) *Hydroflumethiazide.*—2.0 mg NF Ref. Std/200 ml.

(e) *Chlorothiazide std solns.*—(*1*) *Stock soln.*—1.4 mg/ml. Accurately weigh ca 70 mg NF Ref. Std Chlorothiazide into small beaker, add 2 ml *dimethylsulfoxide* (DMSO), and mix with glass rod until dissolved. Transfer quant. to 50 ml vol. flask, using 0.2*M* K_2HPO_4, and dil. to vol. with same solv. (*2*) *Working soln.*—0.014 mg/ml. Dil. 2.0 ml stock soln to 200 ml with 0.2*N* HCl.

(f) *Methyclothiazide std solns.*—(*1*) *Stock soln.*—0.2 mg/ml. Accurately weigh 20 mg NF Ref. Std Methyclothiazide into 100 ml vol. flask and dil. to vol. with MeOH. (*2*) *Working soln.*—0.01 mg/ml. Dil. 10 ml stock soln to 200 ml with $CHCl_3$.

36.C40 *Preparation of Sample*

Finely powder to pass No. 60 sieve.

(a) *Benzthiazide, hydrochlorothiazide, or hydroflumethiazide.*—Transfer portion contg 75 mg benzthiazide or 50 mg hydrochlorothiazide or hydroflumethiazide to 50 ml vol. flask, using 0.2*N* NaOH. Shake to dissolve completely and dil. to vol.

(b) *Chlorothiazide.*—Transfer portion contg ca 70 mg to small beaker and add 2.0 ml DMSO. Mix thoroly 2–3 min with glass rod to dissolve completely. Transfer to 50 ml vol. flask, using 0.2*M* K_2HPO_4, and dil. to vol. with same solv. Mix thoroly.

(c) *Methyclothiazide.*—Transfer portion contg ca 2 mg to 150 ml beaker. Add 2 ml MeOH and mix thoroly. Add 2 ml 0.1*M* $NaHCO_3$ and mix.

36.C41 *Preparation of Columns*

(a) *Benzthiazide, chlorothiazide, hydrochlorothiazide, or hydroflumethiazide.*—(*1*) *Lower layer.*—Mix 2 g Celite with 1 ml 0.2*N* NaOH (1 ml 0.1*M* K_2HPO_4 for chlorothiazide) in 150 ml beaker, transfer to tube, and tamp to uniform mass.

(*2*) *Upper layer.*—Mix 3 g Celite with 2 ml sample soln, transfer to tube, and tamp. Dry-wash flask contg sample mixt. with 1 g Celite and 2–3 drops H_2O; transfer to column and tamp. Add glass wool plug to column.

(b) *Methyclothiazide.*—(*1*) *Lower layer.*—Mix 3 g Celite with 2 ml 0.1*M* $NaHCO_3$ in 150 ml beaker, transfer to tube, and tamp to uniform mass.

(*2*) *Upper layer.*—Proceed as in (a)(*2*), except use 4 g Celite.

36.C42 *Determination*

(Use H_2O-satd solvs thruout.)

(a) *Benzthiazide, hydrochlorothiazide, and hydroflumethiazide.*—Pass 50 ml $CHCl_3$, followed by 50 ml ether, thru column; discard eluate. Using 250 ml separator as receiver, elute column with 0.1 ml HOAc in 100 ml ether. Wash tip of column with ether. Add 65 ml isooctane to eluate and ext org. phase with three 50 ml portions 0.2*N* NaOH; combine NaOH soln in 200 ml vol. flask and dil. to vol.

Det. *A* of sample and std solns in 1 cm cells with spectrophtr against 0.2*N* NaOH as ref.

(b) *Chlorothiazide.*—Proceed as in (a), except use 0.25 ml HOAc in 100 ml ether, 50 ml isooctane, and 0.2*N* HCl. Use 0.2*N* HCl as ref. solv.

(c) *Methyclothiazide.*—Pass 75 ml isooctane-ether (9+1) thru column; discard eluate. Use 200 vol. flask as receiver and elute column with 100 ml $CHCl_3$. Wash tip of column with ether. Add 10 ml MeOH and dil. to vol. with $CHCl_3$. Use $CHCl_3$ as ref. solv.

Wavelength of max. *A* and *a* of individual compds are as follows:

Compd	λ Max., nm	Absorptivity
Benzthiazide	295	29.6
Chlorothiazide	278	32.4
Methyclothiazide	268	51.8
Hydrochlorothiazide	273	49.1
Hydroflumethiazide	273	45.4

[1] The method does not include analyses for polythiazide. The procedure for this drug is the same as that for methyclothiazide.

36.C43 *Identification*

(a) *Benzthiazide, hydrochlorothiazide, and hydroflumethiazide.*—Acidify portion sample soln with 1*N* HCl and ext with 50 ml ether. Evap. ether to dryness, add 5 ml alcohol, and evap. again. Compare IR spectrum in KBr matrix of residue with that of ref. std previously recrystd from alcohol.

(b) *Chlorothiazide.*—Transfer 5 ml prepd sample, 36.C40(b), to 125 ml separator, add 10 ml H_2O, acidify with 1*N* HCl, and ext with 75 ml ether. Evap. ether to dryness. Add 5 ml alcohol to residue and evap. to dryness. Compare IR spectrum in KBr matrix of residue from 400 to 600 cm^{-1} with that of ref. std previously recrystd from alcohol.

(c) *Methyclothiazide.*—Transfer portion sample contg ca 4 mg active ingredient to 125 ml separator, add 20 ml 0.1*M* $NaHCO_3$, and ext with ca 75 ml ether. Proceed as in (b).

Results and Discussion

The results of the collaborative study are presented in Tables 1 and 2. Statistical treatment is also summarized in these tables. As previously stated, this study did not include polythiazide. However, a limited collaborative study on polythiazide performed by 4 analysts in this laboratory produced an average recovery of 99.8± 1.45% for 12 analyses of a simulated mix.

The method is rapid, reasonably specific, and employs well established techniques of quantitative separation and determination. It is a simple modification of the thiazide method previously reported (1). As previously mentioned these compounds will degrade quite rapidly in strongly alkaline solvent (0.1*N* NaOH). It was therefore necessary to select an aqueous immobile phase that would not degrade the compounds, yet would retain them on a Celite column.

In the case of chlorothiazide, 0.2*M* K_2HPO_4 at pH 10 was found to be highly satisfactory. At higher pH (10–11) some degradation begins to occur, and at lower pH the compound is not

Table 1. Collaborative results of analysis of chlorothiazide

	Sample A[a]		Sample B[b]
Coll.	Found, mg	Recd, %	Found, mg
1	67.2	99.6	68.5
	66.8	98.9	68.3
2	66.5	98.5	67.4
	67.5	100.0	67.3
3	67.2	99.6	66.9
	67.7	100.3	67.4
	67.3	99.6	67.7
4	67.7	100.3	66.8
	67.7	100.3	67.2
	67.3	99.6	68.0
5	68.3	101.2	66.8
	68.3	101.2	68.9
6	68.0	100.7	66.8
	67.6	100.1	67.4
	67.4	99.9	67.4
7	67.4	99.9	67.5
	67.6	100.1	68.0
8	68.0	100.7	68.5
	68.5	101.4	68.6
9	68.0	100.7	67.5
	67.8	100.4	67.5
	67.3	99.6	67.3
10	67.7	100.3	67.7
	67.7	100.3	67.2
Av.	67.6	100.1	67.6
Coeff. of var.	0.71	0.67	0.90
Std dev.	0.48	0.67	0.61

[a] Synthetic chlorothiazide, 67.5%.
[b] Commercial chlorothiazide, 250 mg/tablet.

Table 2. Collaborative results of analysis of methyclothiazide

	Sample C[a]	Sample D[b]	
Coll.	Found, mg	Found, mg	Recd, %
1	1.26	1.23	98.4
	1.27	1.22	97.6
2	1.29	1.27	101.6
	1.31	1.28	102.0
3	1.26	1.25	100.0
	1.28	1.24	99.2
	1.27	1.25	100.0
4	1.27	1.24	99.2
	1.27	1.24	99.2
	1.24	1.26	100.8
5	1.21	1.19	95.6
	1.21	1.21	96.8
6	1.27	1.24	99.2
	1.25	1.26	100.8
	1.27	1.24	99.2
7	1.23	1.21	96.8
	1.24	1.21	96.8
8	1.26	1.26	100.8
	1.24	1.25	99.2
9	1.29	1.25	100.0
	1.30	1.24	99.2
	1.29	1.26	100.8
10	1.33	1.27	101.6
	1.34	1.29	103.2
Av.	1.27	1.24	99.8
Coeff. of var.	2.4	1.8	1.61
Std dev.	0.031	0.024	1.60

[a] Commercial methyclothiazide, 2.5 mg/tablet.
[b] Synthetic methyclothiazide, 1.25%.

retained on the column. Methyclothiazide and polythiazide showed some degradation, even in 0.2*M* K_2HPO_4. Therefore slightly basic (pH 8) 0.1*M* $NaHCO_3$ immobile phase was used.

The stability of these compounds under the conditions of the assay was studied by assaying for the disulfonamide decomposition product. Solutions of the thiazides in acid (0.1*N* HCl) and alkali (0.2*M* K_2HPO_4) were allowed to stand overnight. Examination by a modification of the test for diazotizable substances described in the NF XIII detected approximately 0.1% diazotizable material. This same percentage was detected in the pure standard. Hence, we conclude no decomposition is taking place during the analysis.

Recommendations

It is recommended that the proposed method for the determination of chlorothiazide and methyclothiazide be adopted as official first action. Furthermore, in view of the close similarity between these compounds and polythiazide it is recommended that the method also be adopted as official first action for polythiazide.

The recommendations of the Associate Referee were approved by the General Referee and Subcommittee B (except for the adoption of the method for polythiazide) and were adopted by the Association; see (1973) *JAOAC* 56, 395. Subcommittee B recommended additional study of the interference of vanillin excipient in the determination of polythiazide.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

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Collaborative Study of a Colorimetric Determination of Benztropine Mesylate in Tablets and Injections

MARSHALL C. STRICKLIN
Food and Drug Administration, 50 Fulton St., San Francisco, Calif. 94102

A collaborative study was conducted on simulated tablet and injection preparations at 2 concentration levels each and a 20 tablet composite of a tablet simulating presently marketed dosage forms of benztropine mesylate. Analysis involves the extraction of benztropine from 0.2*N* H_2SO_4 with bromophenol blue in $CHCl_3$. Absorbance of the dye complex formed is measured at about 410 nm. Average recoveries and standard deviations submitted were: 98.9±2.54 and 99.2±2.58, 100.1±2.78 and 100.0±2.19, and 100.1±2.23%, respectively. The method has been adopted as official first action.

Previous work by Stricklin and Smith (1) involved ion exchange chromatography for separation and direct spectrophotometric measurement of benztropine mesylate. The purpose of this study was to develop a colorimetric method to increase sensitivity of measurement and to develop a method suitable for tablets and injections.

Auerbach (2) suggested a general method for determination of various alkaloids by reaction with dyes containing sulfonic acid groups. Other analysts have used dyes with tropane alkaloids to form salt-like addition products which are extractable into water-immiscible solvents. Durick *et al.* (3) determined tropane alkaloids by using bromocresol purple. Bromophenol blue was similarly used by Booth (4) to determine reserpine. The study consisted of 2 simulated tablet preparations, as powders, 2 simulated injections of different concentrations, and a composite of 20 tablets from a total of about 1900, prepared by the University of North Carolina. The powders had the following excipient matter composition: 96.2 g NF dibasic calcium phosphate, 6.5 g Solka Floc (dev. 2030), 19.1 g USP lactose, 6.5 g USP starch, 1.3 g USP magnesium stearate. The method, preparations and reagents, bromophenol blue, and benztropine mesylate standard were submitted to 11 collaborators. The composition of the benztropine mesylate preparations is shown in Table 1. The simulated tablet powder was taken as a portion equivalent to 1 mg benztropine mesylate, 36.C22(b), and 2.0 ml injection was the appropriate aliquot after dilution to 200.0 ml, 36.C23(b).

METHOD

Benztropine Mesylate—Official First Action

(Not applicable in presence of compds reacting with bromophenol blue, e.g., quaternary ammonium compds)

36.C21 ***Principle***

Benztropine is extd from acid soln by bromophenol blue-$CHCl_3$ soln, forming dye complex with max. *A* at ca 410 nm.

36.C22 ***Reagents and Apparatus***

(a) *Dye soln.*—Weigh 100 mg reagent bromophenol blue into 1 L vol. flask, add ca 750 ml $CHCl_3$, stir mech. 10 min to dissolve, and dil. to vol. with $CHCl_3$. Filter thru small pad of glass wool. Dil. 50 ml to 500 ml with $CHCl_3$. Prep. fresh daily.

(b) *Benztropine mesylate std soln.*—1 mg/100 ml. Weigh 100 mg Ref. Std into 100 ml vol. flask and dissolve and dil. to 100 ml with 0.2*N* H_2SO_4. Dil. 10 ml aliquot to 100 ml with 0.2*N* H_2SO_4 and further dil. 10 ml dild soln to 100 ml with 0.2*N* H_2SO_4. Prep. fresh daily.

(c) *Spectrophotometer.*—Recording, with 5 cm matched cells.

36.C23 ***Preparation of Sample***

(a) *Tablets.*—Transfer accurately weighed ground portion contg ca 1 mg benztropine mesylate to 100 ml vol. flask, using ca 70 ml 0.2*N* H_2SO_4. Shake mech. 15 min and filter thru Whatman No. 541 paper wetted with 0.2*N* H_2SO_4 into 100 ml vol. flask. Rinse flask and filter with three 5 ml portions 0.2*N* H_2SO_4, rinse filter with several small portions 0.2*N* H_2SO_4, adding rinses to soln, and dil. to vol. with 0.2*N* H_2SO_4.

(b) *Injections.*—Transfer aliquot contg ca 1 mg benztropine mesylate to 100 ml vol. flask and dil. to vol. with 0.2*N* H_2SO_4.

36.C24 ***Determination***

Perform detn on same day sample and std solns are prepd. Place 25 ml each sample soln and std soln and 0.2*N* H_2SO_4 for blank into sep. 250 ml separators and treat similarly. Add 50 ml dye soln and

shake vigorously 1 min. Let sep. and drain lower layer into 125 ml separator contg 25 ml 0.2N H_2SO_4. Wash by inverting 5 times and let stand ca 20 min. Filter lower $CHCl_3$ layer thru glass wool wetted with $CHCl_3$ into 100 ml vol. flask, covering funnel with watch glass. Re-ext aq. soln in 250 ml separator with 50 ml dye soln, shake vigorously 1 min, drain into same 125 ml separator, and wash and filter as before, rewetting glass wool with $CHCl_3$ if necessary. Dil. to vol. with $CHCl_3$, mix, and place in dark 40 min.

Record spectra of std and sample solns against blank in matched 5 cm cells, and det. A at max. ca 410 nm.

mg Benztropine mesylate/100 ml = $(A/A') \times C$, where A and A' refer to sample and std solns, resp; and C = concn std soln in mg/100 ml.

Table 1. Composition of benztropine mesylate preparations

	Simulated tablet powder, mg (250 mg excipients added)		20 Tablets, g (0.5 mg + excipients/ tablet)	Simulated injection, mg/ml (in 0.9% NaCl)	
Coll.	A	B	C	D	E
1	0.900	1.28	2.6701	0.867	1.217
2	0.914	1.15	2.6265	0.8424	1.221
3	0.852	1.244	2.6249	0.832	1.221
4	0.812	1.14	2.6036	0.832	1.221
5	0.994	1.318	2.6256	0.832	1.221
6	0.840	1.171	2.5812	0.832	1.221
7	0.908	1.177	2.6561	0.832	1.221
8	0.905	1.166	2.6532	0.832	1.221
9	0.922	1.22	2.6253	0.832	1.221
10	0.887	1.202	2.6575	0.832	1.221
11	0.830	1.14	2.6415	0.832	1.221

Results and Recommendation

Table 2 lists results of collaborators. The per cent recovery and standard deviation of the tablet composite, C, were calculated from the mean value of duplicate assays by the proposed method, on each 20 tablet composite sent for collaboration: 0.539±0.012 mg/tablet. The results of 9 individual tablets assayed by the USP method (5) averaged 0.5269±0.0215 mg. Results of 2 collaborators were discarded because of procedural errors. One result was determined to be an outlier by the method of Dean and Dixon (6), and it is not included in the mean value and standard deviation calculation.

The data tabulated show mean percentage recoveries of 98.9±2.54 and 99.2±2.58 for the simulated tablet powder, 100.1±2.23 for the tablet, and 100.1±2.78 and 100.0±2.19 for the simulated injections. As 60–73%, average 64%, of the individual results fall within one standard deviation of their mean assay values and all results except one are within two standard deviations of their mean assay value, results adhere to the normal error curve. Results of 4 collaborators show a trend to vary from mean values positively

Table 2. Results of collaborative study on benztropine mesylate, per cent recovery

	Simulated tablet powder				Tablet		Simulated injection			
	A		B		C[a]		D		E	
Coll.	mg	%	mg	%	mg/tablet	%	mg/ml	%	mg/ml	%
1	0.904	100.4	1.262	98.6	0.534, 0.543	99.1, 100.7	0.879	101.4	1.205	99.0
2	0.908	99.3	1.117	97.1	0.547, 0.541	101.5, 100.4	0.854	101.4	1.223	100.2
3	0.664[b]	77.9	1.20[b]	96.4	0.530, 0.550	98.3, 102.0	0.842	101.2	1.230	100.7
4	0.821	101.1	1.135	99.6	0.534, 0.531	99.1, 98.5	0.825	99.2	1.209	99.0
5	0.985	99.1	1.261	95.7	0.537, 0.537	99.6, 99.6	0.727[c]	87.4	1.200	98.3
6	0.844	100.5	1.192	101.8	0.540, 0.533	100.2, 98.9	0.847	101.8	1.225	100.3
7	0.924	101.8	1.173	99.7	0.560, 0.559	103.9, 103.7	0.852	102.4	1.236	101.2
8	0.871	96.2	1.136	97.4	0.521, 0.526	96.7, 98.0	0.794	95.4	1.170	95.8
9	0.886	96.1	1.266	103.8	0.531, 0.567	98.5, 105.2	1.34[b]	161.1	1.268	103.8
10	0.835	94.1	1.165	96.9	0.524, 0.529	97.2, 98.1	0.795	95.6	1.205	98.7
11	0.831	100.1	1.159	101.7	0.551, 0.539	102.2, 100.0	0.853	102.5	1.254	102.7
Mean, %		98.9		99.2		100.1		100.1		100.0
Std dev., %		2.54		2.58		2.23		2.78		2.19
Coeff. of var., %		2.57		2.60		2.23		2.78		2.19

[a] Per cent recovery calculated from mean assay value, 0.539±0.012 mg/tablet.
[b] Results discarded, procedural error.
[c] Outlier result.

(Collaborators 7 and 9), or negatively (Collaborators 8 and 10), indicating intralaboratory bias possibly due to systematic error. Overall results indicate reproducibility over a considerable range of benztropine mesylate present in the 3 preparations.

The majority of collaborators expressed the opinion that the method was straightforward and/or convenient and was performed with ease. Suggestions and/or questions regarding procedural matters were noted and considered. The collaborative study of this method has indicated its effectiveness in the assay of benztropine mesylate in tablets and injections. It is recommended the method be adopted as official first action.

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The recommendation of the Associate Referee was approved by the Referee and Subcommittee B and was adopted by the Association; see (1973) *JAOAC* 56, 396.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Gas Chromatographic Determination of Ethanol and Isopropanol or Acetone in Drugs

NICHOLAS FALCONE
Food and Drug Administration, 2nd and Chestnut Sts., Philadelphia, Pa. 19106

A gas-solid chromatographic technique, using flame ionization detection and a Porapak Q column, is presented for the identification and determination of ethanol and isopropanol or acetone in pharmaceutical products. The technique involves direct injection of an aqueous dilution of the product and is therefore simple and direct. The commercial products and 3 simulated mixtures, at various concentration levels, were analyzed by 9 collaborators. Recoveries and standard deviations for all 3 components were satisfactory. The method has been adopted as official first action.

The present official AOAC methods for acetone, ethanol, and isopropanol are qualitative (1). The standard technique for identifying these compounds involves the determination of the physical constants of the compounds and the preparation of a suitable derivative. The isolation in a pure state of each of the components of a dilute aqueous solution containing acetone, isopropanol, and ethanol is time consuming. A quick, reliable, and convenient method was needed for the quantitation of the 3 components. Although the literature contains a number of reports and gas-liquid chromatographic methods for alcohols, none met all the desired requirements; many are limited to only one component, e.g., Tarlin (2) and Wells C. W. (1970, private communication).

The official compendia (3, 4) include gas chromatographic techniques for the quantitative determination of ethanol or isopropanol and specific gravity quantitative determination for acetone. However, the above methods are applicable only to the determination of one component in the absence of the other 2. The 2 compendia specify chromatography on 20% polyethylene glycol on calcined silica packing material.

Piechocki (5) presented a comparison study between Porapak Q and Carbowax 400 gas chromatographic packing materials for the quantitative determination of ethanol. Porapak Q has a number of very significant advantages over commonly coated columns for gas chromatography. For example, water is not adsorbed by the polymer beads of Porapak; therefore, water can be separated quickly and easily from other components, and it can also be used as solvent. The study showed that, for a variety of products, Porapak Q and Carbowax 400 give comparable quantitative results for ethanol and, in addition, ethanol is separated from methanol. A study was therefore initiated in our laboratory to develop a method that would quantitatively determine any of the 3 components in pharmaceutical products and would replace the current AOAC qualitative tests (1).

The gas chromatographic technique with flame ionization detection and Porapak Q packing material was investigated and found to be very satisfactory. The packing beads (available commercially from Waters Associates as Porapak Q) more easily solved the problem of separating low molecular weight alcohols, acids, and glycols. Porous polymer beads have many advantages: They need no liquid phase, they are hard and do not break during packing, they require no pretreatment, they have a large number of theoretical plates per unit volume, and they are conditioned in a relatively short time (approximately 1 hr). Porapak Q does not retain water. When an aqueous solution is injected, water will be eluted in the solvent peak or will be the first compound eluted from the Porapak Q column.

METHOD

Ethyl Alcohol, Isopropanol, and Acetone—Official First Action

(Applicable to liq. prepns contg ethanol with isopropanol or acetone or individual compds)

36.C01 *Reagents and Apparatus*

(a) *Ethyl alcohol std stock soln.*—(*1*) *2% (v/v).*—Dil. 5.0 ml absolute alcohol to 250 ml with H_2O. (*2*) *0.02% (v/v).*—Dil. 10.0 ml soln (*1*) to 100 ml with H_2O.

(b) *Isopropanol std stock soln.*—2% (v/v). Dil. 5.0 ml isopropanol to 250 ml with H_2O.

(c) *Acetone std stock soln.*—2% (v/v). Dil. 5.0 ml acetone to 250 ml with H_2O.

(d) *Acetonitrile internal std stock soln.*—2% (v/v). Dil. 5.0 ml CH_3CN to 250 ml with H_2O.

(e) *Gas chromatograph.*—With 6′ × 4 mm id glass column, packed with 80–100 mesh Porapak Q (Waters Associates, 61 Fountain St, Framingham, MA 01701) and H flame ionization detector. Approx. operating conditions: temps (°)—column 135, detector 155, injection port 165; N carrier gas flow rate 120 ml/min. CH_3CN peak should elute in 5 min. Adjust H and air flow rates and electrometer sensitivity so that 5 μl 0.2% EtOH std soln gives 50–70% scale deflection.

36.C02 ***Preparation of GC Column***

Carefully plug column exit with small tuft of glass wool. Apply vac. to exit and slowly add packing material thru inlet, tapping very gently to pack firmly. Pack to within 1 cm of area heated by injection port. Plug with glass wool and condition overnight at 235° with slow N stream.

36.C03 ***Preparation of Sample***

(a) *Ethyl alcohol.*—Prep. soln contg ca 2% (v/v) alcohol by stepwise diln with H_2O. Proceed as in **36.C04**.

(b) *Isopropanol.*—Prep. soln contg ca 2% (v/v) isopropanol by stepwise diln with H_2O. Proceed as in **36.C04**.

(c) *Acetone.*—Prep. soln contg ca 2% (v/v) acetone by stepwise diln with H_2O. Proceed as in **36.C04**.

If acetone concn is unknown, prep. 50% diln of product with H_2O, prep. acetone std soln, and inject sample and std as in **36.C04**. To det. amt acetone, adjust product and std dilns to give comparable peak hts; % internal std added to the 2 solns should be equal to % acetone present in std soln.

36.C04 ***Determination***

Pipet 10 ml sample soln into 100 ml vol. flask. Pipet 10 ml each std stock soln needed into sep. 100 ml vol. flask. Pipet 10 ml internal std stock soln into each flask and dil. to vol. with H_2O.

Inject 5 μl sample and std solns, each in duplicate, using 10 μl syringe. Approx. retention times of peaks relative to CH_3CN internal std peak are as follows: alcohol, 0.76; acetone, 1.32; isopropanol, 1.40.

Calc. % EtOH, acetone, or isopropanol in sample as $\% C = C' \times (H/H') \times (I'/I) \times f$, where C and C' = % component in sample and std, resp., H = av. sample peak ht or area in sample chromatogram, H' = av. std peak ht or area in std chromatogram, I and I' = resp. values for internal std, and f = sample diln factor.

Experimental

The experimental work was conducted on an F&M Scientific 5750 gas chromatograph. Pure specimens of the 3 components were chromatographed in order to determine optimum conditions. Thirteen different commercial products containing declared amounts of ethanol or isopropanol were analyzed. The results are shown in Table 1.

Since commercial products containing a combination of either ethanol and isopropanol or ethanol and acetone were not available on the market, simulated mixtures were prepared by adding known different concentrations of the components to the commercial products. Table 2 shows the recoveries obtained for commercial products containing a declared amount of ethanol and fortified with different amounts of isopropanol, ranging from 2 to 70%. Table 3 shows the recoveries obtained for commercial products containing ethanol fortified with known amounts of acetone. The detection limit of the method was determined and a solution containing any of the 3 components as low as 0.002% can be analyzed successfully.

The commercial products analyzed ranged from free-flowing to viscous liquids. Direct dilution of these products in water and analysis of the water solution showed no difficulties. An internal standard was used to compensate for variations in injection volume and instrument response, for greater accuracy in the determinations.

Collaborative Study

Six unknown solutions were prepared for the collaborative study. The unknowns were designated A to F. Of these, 3 were commercial products and 3 were simulated products, all containing

Table 1. Analysis for ethanol in commercial products containing ethanol or isopropanol

Product type	Ethanol		
	Label deci., %	Found,[a] %	% of Declared
Brown mixture	10	8.75	87.5
Diphenhydramine	5	5.0	100.0
B-G Phos.	17	17.0	100.0
Cheralin	3	3.4	113.3
Freezone	20	19.3	96.5
Tincture opium camphorate	44–46	44.5	98.9
3-Bromide elixir	4	4.10	102.5
Kay Ciel elixir	4	4.25	106.2
Elixophyllin-K1	10	9.85	98.5
Elixir butalan	7	7.02	100.3
Phenobarbital elixir USP	14	13.75	98.2
Ipecac sirup USP	2	2.07	103.5
Druco isopropyl alcohol	70	67.5	96.4

[a] Average of 2 analyses.

Table 2. Analysis of commercial products containing ethanol and fortified with isopropanol

Product	Isopropanol			Ethanol		
	Decl., %	Found,[a] %	Rec., %	Decl., %	Found,[a] %	Rec., %
Druco isopropyl alcohol	70[b]	67.5	96.4	29[c]	29.7	102.4
Brown mixture	42[c]	41.80	99.5	10[b]	8.75	87.5
B-G Phos.	35[c]	34.62	98.9	17[b]	17	100.0
Cheralin	21.0[c]	20.90	99.6	3.0[b]	3.4	113.3
Kay Ciel elixir	14.5[c]	14.20	98.0	4.0[b]	4.25	106.2
Phenobarbital elixir	7.5[c]	7.52	100.3	14.0[b]	13.75	98.2
Ipecac sirup	2.2[c]	2.16	98.0	2.0[b]	2.07	103.5

[a] Average of 2 determinations.
[b] Declared.
[c] Fortified.

Table 3. Analysis of commercial products containing ethanol and fortified with acetone

Product	Acetone			Ethanol		
	Added, %	Found,[a] %	Rec., %	Decl., %	Found,[a] %	Rec., %
Diphenhydramine	2.5	2.48	99.3	5.0	5.0	100.0
Tincture opium camphorate	13.5	13.35	98.9	44–46	44.5	98.9
3-Bromide elixir	25.0	24.8	99.2	4.0	4.10	102.5

[a] Average of 2 determinations.

known or declared amounts of each of the components. The unknowns were prepared in such a way as to contain a wide range of concentrations of the 3 components. Two unknowns were fortified with trace amounts of acetone and isopropanol for identification of contaminants by retention times.

Unknown A: A commercial product which contained a declared amount of ethanol, fortified with a small amount of isopropanol. The ethanol was determined quantitatively and the isopropanol was simply identified.

Unknown B: A commercial product containing a declared amount of ethanol, fortified with a known amount of acetone. Both components were determined quantitatively.

Unknown C: A commercial product containing a declared amount of isopropanol which was determined quantitatively.

Unknown D: A simulated mixture containing a known amount of ethanol and a small amount of acetone. The ethanol was determined quantitatively and the acetone was simply identified.

Unknowns E and F: Simulated mixtures containing known amounts of ethanol and isopropanol; both were determined quantitatively.

Ten collaborators were supplied with the 6 unknowns, a copy of the method, and instructions for the study. They were instructed as follows: Obtain and submit a linearity curve in order to determine instrument response; use pure analytical grade solvents available in the laboratory as reference standards and internal standards; perform a trial run of the method by analyzing solutions with known amounts of ethanol and acetone and report the recoveries.

Results

The results ranged from 98.4 to 101.6% recovery with a mean of 100.2% and a coefficient variation of 0.996% for ethanol. The range for acetone was 97.6 to 102.0% recovery with a mean of 100.0% and a coefficient variation of 1.20%

All collaborators used 6′ × 4 mm id columns. Collaborators 1, 2, and 5 used Packard instruments; 3, 7, and 8 used F&M instruments; 4, 6, and 9 used Barber-Colman instruments. Collaborators 5 and 7 determined peak height by an electronic integrator; the rest determined peak height manually.

One collaborator reported problems with the gas chromatographic column and consequently could not finish the study. Results from the other 9 collaborators are reported in Tables 4 and 5. Table 4 shows the results obtained for the determination of ethanol, isopropanol, and acetone. Table 5 shows the per cent recovery obtained for the simulated mixtures of ethanol, isopropanol, and acetone. The 9 collaborators successfully

Table 4. Collaborative results for analysis of 9 unknown commercial products and simulated mixtures[a]

	Ethanol, %					Isopropanol, %			Acetone, %
Coll.	A	B_I	D	E_I	F_{II}	C	E_{II}	F_I	B_{II}
1	3.52	44.30	3.83	40.05	35.86	67.65	4.61	66.28	4.31
2	3.39	44.35	3.85	40.13	35.74	67.31	4.25	64.50	4.17
3	3.49	43.40	3.77	40.20	34.90	67.30	4.55	64.70	4.32
4	3.64	43.61	3.75	40.69	34.10	69.15	4.62	64.83	4.24
5	3.50	44.36	3.85	39.89	35.05	67.41	4.47	64.00	4.29
6	3.57	45.70	3.92	41.10	36.10	69.80	4.41	67.00	4.00
7	3.57	44.40	3.79	40.68	35.06	68.40	4.38	64.50	4.20
8	3.65	43.60	3.89	40.10	35.40	67.40	4.69	66.40	4.40
9	3.52	43.84	3.97	42.33	34.37	67.76	4.61	64.38	4.32
Mean, $\bar{X}$	3.54	44.17	3.85	40.57	35.18	68.02	4.51	65.18	4.25
Range, %	3.39–3.65	43.40–45.70	3.75–3.92	39.89–42.33	34.10–36.10	66.39–69.80	4.25–4.69	63.40–67.00	4.00–4.40
Std dev., %	0.080	0.691	0.072	0.766	0.671	0.906	0.142	1.079	0.117
Coeff. of var., %	2.27	1.57	1.87	1.89	1.91	1.33	3.15	1.66	2.75

[a] Subscripts refer to samples in which 2 components were determined quantitatively.

Table 5. Calculated recoveries based on the simulated solution, with a known concentration[a]

	Ethanol						Isopropanol				Acetone	
	D (3.80%)		E_I (40.0%)		F_{II} (35.0%)		E_{II} (4.60%)		F_I (65.0%)		B_{II} (4.40%)	
Coll.	Found, %	Rec., %	Found, %	Rec., %	Found, %	Rec., %	Found, %	Rec., %	Found, %	Rec., %	Found, %	Rec., %
1	3.83	100.8	40.05	100.1	35.86	102.5	4.61	100.2	66.28	102.0	4.31	98.0
2	3.85	101.3	40.13	100.3	35.74	102.1	4.25	92.4	64.50	99.2	4.17	94.8
3	3.77	99.2	40.20	100.5	34.90	99.7	4.55	98.9	64.70	99.5	4.32	98.2
4	3.75	98.7	40.69	101.7	34.10	97.4	4.62	100.4	64.83	99.7	4.24	96.4
5	3.85	101.3	39.89	99.7	35.05	100.1	4.47	97.2	64.00	98.5	4.29	97.5
6	3.92	103.2	41.10	102.8	36.10	103.1	4.41	95.9	67.00	103.1	4.00	90.9
7	3.79	99.7	40.68	101.7	35.06	100.2	4.38	95.2	64.50	99.2	4.20	95.5
8	3.89	102.4	40.10	100.3	35.40	101.1	4.69	102.0	66.40	102.1	4.40	100.0
9	3.97	104.5	42.33	105.8	34.37	98.2	4.61	100.2	64.38	99.0	4.32	98.2
Mean, $\bar{X}$		101.2		101.4		100.5		98.0		100.3		96.6
Std dev., %		1.90		1.91		1.92		3.09		1.670		2.66
Coeff. of var., %		1.88		1.88		1.91		3.15		1.67		2.75

[a] Subscripts refer to samples in which 2 components were determined quantitatively.

identified the 2 unknown contaminants in Solutions A and D, and reported no difficulties in interpreting the method during the course of the analysis. Two collaborators reported that they had to change the carrier gas flow rate in order to obtain better separation. Figure 1 shows the chromatographic separations used for quantitative purposes in this method; acetone and isopropanol can be clearly discriminated.

No change or correction in the method was reported by the collaborators.

The results were statistically evaluated on the basis of the laboratory ranking test as described by Youden (6). Total scores of all laboratories are within the limits of acceptance.

Recommendations

The overall results clearly demonstrate the applicability of the method to the assay of ethanol in combination with isopropanol or acetone. This method is recommended for adoption as official first action.

Acknowledgments

The author wishes to thank Trean Korbelak, Research Coordinator, Food and Drug Administration, Philadelphia, Pa., for her assistance throughout the entire study and the following Food and Drug Administration collaborators: Janet L. Booth, Seattle; Jerome Elkins, Dallas; Seymour Fishman, New York; Frank W. Kerner,

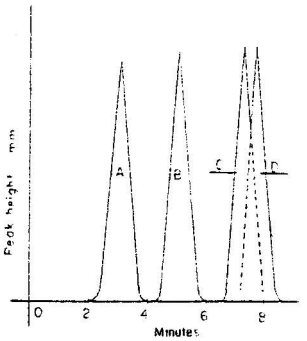

FIG. 1—Gas chromatographic separation of A, ethanol; B, acetonitrile (internal standard); C, acetone; D, isopropanol. For each, 5 μl of about 0.2% solution was injected.

St. Louis; John Nikelly, Science Advisor, Philadelphia; Sharon R. Reid, Detroit; Radmilo Ristich, Buffalo; Bruce Ross, Washington, D.C.; Georgia A. Voytush, Philadelphia; Floyd E. Yarnall, Kansas City.

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The second joint symposium of the Society of Cosmetic Chemists (SCC) and the Association of Official Analytical Chemists (AOAC) will be held at the Marriott Motor Hotel in Washington, D.C. on Oct. 9, 1973.

We are currently seeking papers to be presented at this symposium. These papers should consist of original (15 min maximum) or review (30 min maximum) papers on various analytical techniques in the analysis of *cosmetic products*, both finished products and raw ingredients.

Please call or write if you are interested in presenting a paper at this symposium, giving its tentative title, a brief abstract of the subject, and its approximate length. Formal details can be submitted at a later date if your paper is accepted for presentation.

Sal Di Bianca, Program Chairman
The Mennen Co.
E. Hanover Ave.
Morristown, New Jersey 07960
(201) 538-7100, Ext. 374

Collaborative Study of the Method for the Analysis of Trisulfapyrimidine Preparations by Thin Layer Chromatography

CHARLOTTE A. BRUNNER
Division of Drug Chemistry, Food and Drug Administration, Washington, D.C. 20204

The TLC method for trisulfapyrimidine preparations reported earlier has been collaboratively studied. Fifteen collaborators reported results for total trisulfapyrimidine as well as individual results for sulfadiazine, sulfamerazine, and sulfamethazine for 3 samples (commercial composite, commercial suspension, and simulated tablet mix). The coefficients of variance for the total sulfur content of the 3 samples were 1.65, 1.25, and 1.55. For the determination of the individual sulfur components, the coefficients of variance ranged from 0.76 to 1.66. The method has been adopted as official first action.

A collaborative study has been performed on the assay of trisulfapyrimidines by the TLC procedure which was presented at the 85th Annual Meeting of the AOAC ((1972) *JAOAC* 55, 194–196). Three samples, a composite of ground commercial tablets, a known mixture simulating the commercial tablets, and a commercial suspension, were sent to 15 collaborators. Each collaborator was asked to perform a single analysis. However, several collaborators reported duplicate results. In the preparation of this report the first set was considered to be a trial run and the second was used in the statistical analysis. The reports of several of the collaborators indicated that the instructions were not entirely clear in several areas. This resulted in the use of an incorrect sample dilution in one stage of the assay, necessitating another analysis when the obvious error was noted. The method, edited to clarify these points, is described below:

METHOD

Trisulfapyrimidines—Official First Action

Total Trisulfapyrimidines

36.C44 ***Principle***

Total trisulfapyrimidines in sample are detd by coupling with *N*-1-naphthyl ethylenediamine.2HCl (NED), recording spectra of samples and stds between 660 and 480 nm. Individual sulfonamides are sepd by thin layer chromatography and their ratios detd spectrophtic after coupling with NED.

36.C45 ***Reagents***

(a) *Ammonia-methanol soln.*—Dil. 5 ml NH_4OH to 100 ml with MeOH.

(b) *Sulfamerazine std soln.*—Approx. 6 μg/ml acid soln. Accurately weigh calcd amt USP Ref. Std Sulfamerazine previously dried and dissolve in NH_4OH-MeOH soln; dil. quant. and stepwise with MeOH to obtain soln contg ca 120 μg/ml. Transfer 5.0 ml to 100 ml vol. flask and dil. to vol. with 0.12*N* HCl. Acidic soln is stable ≥1 month.

(c) *Dilute ammonia soln.*—Dil. 400 ml NH_4OH to 1 L with H_2O.

(d) *N-1-naphthyl ethylenediamine dihydrochloride (NED) soln.*—0.1%. Prep. fresh before use.

36.C46 ***Preparation of Sample***

(a) *Tablets.*—Accurately weigh finely powd portion contg ca 180 mg total sulfonamides and transfer to 50 ml vol. flask, using 10 ml dil. NH_4OH, (c). Let stand ca 15 min, mixing occasionally, dil. to vol. with MeOH, and centrf. portion to clarify (Soln I). Dil. 5.0 ml clarified soln to 250.0 ml with H_2O; dil. 4.0 ml of this soln to 50.0 ml with H_2O (Soln II).

(b) *Suspensions.*—Shake in original container to ensure homogeneity, let stand long enough for entrapped air to rise, and invert carefully just before removing portion for weighing. Det. sp gr by weighing 100 ml in tared 100 ml vol. flask. Thoroly mix and weigh portion contg 180 mg total sulfonamides and proceed as in (a).

36.C47 ***Determination***

Pipet 5.0 ml aliquots sulfamerazine std soln and prepd Soln II into sep. 10 ml vol. flasks. Add 1.0 ml HCl (1+1) to each flask, mix, and cool. (Solns must be at room temp. for quant. results.) Add 1.0 ml 0.1% $NaNO_2$, mix well, and let stand 2 min. Add 1.0 ml 0.5% NH_4 *sulfamate*, and mix. After 2 min, add 1.0 NED soln, (f). Mix and adjust to vol. with H_2O. Record spectra of samples and stds against H_2O between 660 and 480 nm (peak ca 545 nm) within 15–60 min. Correct A by subtracting A at 660 nm from peak A at ca 545 nm.

Calc. wt total sulfapyrimidines in samples as mg total sulfapyrimidines = $(A/A') \times 31.25C$, where A and A' = corrected A of dild assay soln and sulfamerazine std soln, resp., and C = μg sulfamerazine/ml std soln.

Ratio of
Sulfadiazine:Sulfamerazine:Sulfamethazine

36.C48 *Reagents and Apparatus*

(a) *Chromatographic identification standards.*—Prep. sep. solns of USP Ref. Std Sulfadiazine, Sulfamerazine, and Sulfamethazine in NH_4OH-MeOH soln, 36.C45(a), to contain ca 1 mg/ml each.

(b) *Developing solvent.*—$CHCl_3$-MeOH-NH_4OH (30+12+1).

(c) *Thin layer plate.*—20 × 20 cm, coated 0.25 mm thick with silica gel GF (Brinkmann Instruments, Inc.). Divide into 2 approx. equal parts by scraping thin vertical line thru coating.

36.C49 *Thin Layer Chromatography*

Line suitable chromatgc tank with blotting paper. Wet bottom of tank and paper with developing solv., seal tank, and let equilibrate 30 min. Apply ca 50 µl centrfd Soln I to starting line of thin layer plate in streak ca 8 cm long (not to extend within 1 cm of plate edge or center line), using N stream. (It is not necessary to spot accurately measured vol.) On other half of plate, spot sep. 10 µl chromatgc identification stds, evenly spaced. Develop plate in tank equilibrated ½ hr, letting solv. migrate 10–15 cm above starting line. Air-dry plate, locate bands under shortwave UV light, and circle with stylus. Remove silica gel from each band by scraping onto glazed weighing paper, and transfer into sep. 50 ml g-s centrf. tubes. Add 10.0 ml 0.1*N* NaOH to each tube, shake 3 min, and centrf. Transfer 5.0 ml aliquots of each supernate to 10 ml vol. flasks. Add 1.0 ml HCl (1+1) to each flask, mix, and cool. (Soln must be at room temp. for quant. results.) Develop color and record spectra as in 36.C47.

36.C50 *Calculations*

Calc. fraction of each sulfapyrimidine in total sulfapyrimidines as follows:

Sulfadiazine fraction = $0.947A_d/T$

Sulfamerazine fraction = A_r/T

Sulfamethazine fraction = $1.053A_m/T$,

where A_d, A_r, and A_m = corrected A of the sulfadiazine, sulfamerazine, and sulfamethazine bands, resp., and $T = 0.947A_d + A_r + 1.053A_m$.

Results and Discussion

Previous chromatographic assays of trisulfapyrimidines required the application of measured volumes of sample solution to the chromatographic plate. In this procedure, the chromatogram is used only to determine the relative amounts of the individual compounds. The total content is determined in a separate analysis. The original instructions to the collaborators may not have made this entirely clear, since several collaborators applied the precise volumes, thereby missing one of the major advantages of this method. This point has now been clarified.

Several collaborators suggested that the chromatogram should be developed 15 rather than 10 cm in order to increase the separation. However, this also increases the time for plate development. The results obtained using either distance were satisfactory; therefore, the instructions have been changed to specify a development distance of 10–15 cm.

Commercial plates were used in some instances but most of the collaborators used laboratory-made plates. One collaborator who used commercial plates reported he achieved only fair separation.

There were no outliers among the data reported; therefore, all were used and none was discarded in the statistical evaluation (except the trial runs as noted above).

Even though the total sulfa assay has no bearing on the success or failure of the individual sulfa determination by TLC, the collaborators were asked to perform both procedures. The collaborative results for the total sulfapyrimidines are found in Table 1. The reported results for the

Table 1. Collaborative results for total sulfapyrimidine content

Coll.	Simulated com. mixt., % rec.	Com. composite, % of label	Com. suspension, % of label
1	101.8	100.1	100.2
2	100.1	103.6	105.0
3	103.6	100.8	101.7
4	100.6	100.8	103.7
5	101.2	99.7	102.0
6	99.3	99.4	103.8
7	101.0	101.6	102.4
8	97.5	100.4	101.7
9	100.5	100.2	103.0
10	102.0	102.8	103.4
11	102.4	101.5	104.3
12	102.7	100.9	102.7
13	102.1	101.6	99.5
14	102.3	102.0	104.3
15	99.6	99.1	99.8
Av.	101.1	100.9	102.5
Std dev.	1.57	1.26	1.69
Coeff. of var., %	1.55	1.25	1.65

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Table 2. Collaborative results for the ratio of sulfapyrimidines

Coll.	Sulfadiazine	Sulfamerazine	Sulfamethazine
Simulated commercial mixture			
1	0.343	0.347	0.310
2	0.341	0.347	0.312
3	0.346	0.348	0.306
4	0.345	0.346	0.309
5	0.350	0.348	0.302
6	0.351	0.342	0.308
7	0.354	0.343	0.303
8	0.346	0.346	0.308
9	0.343	0.353	0.303
10	0.358	0.341	0.301
11	0.350	0.345	0.305
12	0.343	0.348	0.308
13	0.345	0.345	0.309
14	0.346	0.346	0.308
15	0.341	0.348	0.311
Av.	0.3468	0.3462	0.3069
Actual composition	0.346	0.347	0.303
Std dev.	0.0049	0.0029	0.0034
Coeff. of var., %	1.41	0.840	1.10
Commercial composite			
1	0.327	0.330	0.343
2	0.338	0.330	0.332
3	0.333	0.332	0.335
4	0.332	0.331	0.336
5	0.329	0.331	0.340
6	0.341	0.336	0.323
7	0.333	0.332	0.335
8	0.332	0.330	0.338
9	0.322	0.341	0.337
10	0.332	0.333	0.335
11	0.333	0.333	0.334
12	0.329	0.333	0.339
13	0.333	0.334	0.333
14	0.324	0.333	0.343
15	0.337	0.328	0.335
Av.	0.3317	0.3325	0.3359
Label composition	0.333	0.333	0.333
Std dev.	0.0050	0.0031	0.0049
Coeff. of var., %	1.51	0.922	1.44
Commercial suspension			
1	0.334	0.339	0.327
2	0.335	0.336	0.332
3	0.343	0.320	0.337
4	0.334	0.337	0.329
5	0.330	0.340	0.325
6	0.336	0.334	0.330
7	0.329	0.340	0.331
8	0.328	0.340	0.332
9	0.322	0.344	0.335
10	0.328	0.339	0.333
11	0.332	0.337	0.331
12	0.332	0.336	0.332
13	0.332	0.337	0.332
14	0.330	0.340	0.330
15	0.322	0.344	0.334
Av.	0.331	0.3375	0.3316
Label composition	0.333	0.333	0.333
Std dev.	0.0052	0.0056	0.0025
Coeff. of var., %	1.57	1.66	0.763

(Continued)

ratio of the sulfapyrimidines are presented in Table 2. The 3 columns in the table represent the fraction of individual sulfapyrimidine found.

Recommendation

It is recommended that the proposed thin layer chromatographic method for the analysis of trisulfapyrimidine preparations be adopted as official first action.

Acknowledgments

The Associate Referee wishes to thank the following collaborators: Daniel Rosenbaum, Sandoz Pharmaceuticals, Hanover, N.J.; Marvin Pruzansky, Squibb & Sons, New Brunswick, N.J.; and the following Food and Drug Administration personnel: Richard Baldwin, Philadelphia; Earl Bloomingdale, Kansas City; Anthony Clarke, New York; Paul Flinn, St. Louis; Fred R. Krick, Seattle; Sal Marchese, Boston; Victor S. Marino, Detroit; John G. Meyer, Minneapolis; Edith C. Peavey, Atlanta; Bobby R. Rader, Los Angeles; H. S. Scroggins, New Orleans; Paul V. Smith, Chicago; Norlin Tymes, Cincinnati.

The Associate Referee would also like to thank Joseph Levine and James B. Kottemann for their assistance during this collaborative study.

The recommendation of the Associate Referee was approved by the Referee General and Subcommittee B and was adopted by the Association; see (1973) *JAOAC* 56, 395.

Collaborative Study of a Semiautomated Method for the Analysis of Sodium Warfarin and Dicumarol Tablets

RICHARD E. KOLINSKI
National Center for Drug Analysis, Food and Drug Administration, 1114 Market St., St. Louis, Mo. 63101

A semiautomated extraction and UV determination for sodium warfarin and dicumarol tablets was collaboratively studied by 7 laboratories. Collaborators were supplied with 4 composites of tablets of different dosage levels (3 sodium warfarin composites and 1 dicumarol composite). Results agreed well with the USP method. For sodium warfarin, coefficients of variation ranged from 0.35 to 2.10%. For dicumarol, coefficients of variation ranged from 0.39 to 2.19%. The method has been adopted as official first action.

The semiautomated method described here is an adaption of the compendial assay for sodium warfarin and dicumarol (bishydroxycoumarin) tablets (1, 2). It is designed to save time and labor in both potency assays on tablet composites and individual tablet assays.

A series of validation tests was performed on the system as constructed in Fig. 1. The response of the system was linear when tested with 5 concentrations of warfarin standard from 0.02 to 0.2 mg/ml. A standard solution containing 0.1 mg warfarin/ml gave reproducible peaks which were approximately 100% of the steady state signal (Figs. 2 and 3). Twenty individual tablet portions of composites were assayed. The composites prepared from commercial samples of each drug were not the same composites sent to collaborators. The data in Table 1 show the automated system to be precise.

Portions of tablet composites equivalent to single tablets were analyzed by the proposed semiautomated method and the USP XVIII procedure. These composites were not the same ones used for collaborative samples. Ten determinations were performed using the semiautomated method, one on each of 10 consecutive days. Ten consecutive determinations were performed by the USP XVIII method. The data in Table 2 show close agreement between methods.

Mixtures of common tablet excipients were prepared to test for interference in the proposed method. Aliquots of standard solution were added to an amount of excipient mix equivalent to a single tablet, and this simulated sample was subjected to the sample preparation and the automated procedure. A 99.2% recovery of sodium warfarin was obtained at the 10 mg/tablet level with lactose, starch, magnesium stearate, and stearic acid as excipients. Recovery of dicumarol at the 50 mg/tablet level was 100.1%, with corn starch, magnesium stearate, and dibasic calcium phosphate as excipients. The above results indicate that there is no interference from tablet excipients, based on recoveries from simulated mixtures.

METHOD

Dicumarol (Bishydroxycoumarin) and Sodium Warfarin—Official First Action

36.C25 ***Principle***

Basic soln of drug is acidified and extd with $CHCl_3$ for Na warfarin or $CHCl_3$-pyridine-propylene glycol (90+5+5) for dicumarol, and A of extd material is read in flowcell at 308 nm.

36.C26 ***Apparatus***

(a) *Automatic analyzer.*—AutoAnalyzer with following modules (Technicon Instruments Corp., Tarrytown, NY 10591): Sampler II with 20/hr (2:1) cam; Proportioning Pump 1; manifold (Fig. 1).

(b) *Spectrophotometer.*—Double-beam spectrophtr which records A at fixed wavelength, equipped with 10 mm flowcell (Arthur H. Thomas Co., No. 8495-L10) for Na warfarin and 2 mm flowcell (Beckman Instruments, No. 565411) for dicumarol.

(c) *Ultrasonic generator.*—Model 11, 150 watt (Heat Systems-Ultrasonic, Inc., Plainview, NY 11803).

(d) *Filter.*—Fill 50 mm × 5 mm id glass tubing completely, but loosely, with glass wool.

36.C27 ***Reagents***

(a) *Chloroform.*—H_2O-washed and filtered thru paper. Prep. fresh for use in Na warfarin assay.

(b) *Chloroform-pyridine-propylene glycol soln.*—Mix 50 ml pyridine with 50 ml propylene glycol and dil. to 1 L with $CHCl_3$. Use in dicumarol assay.

(c) *Warfarin std soln.*—0.1 mg/ml. Accurately weigh ca 25 mg USP Ref. Std Warfarin into 250 ml

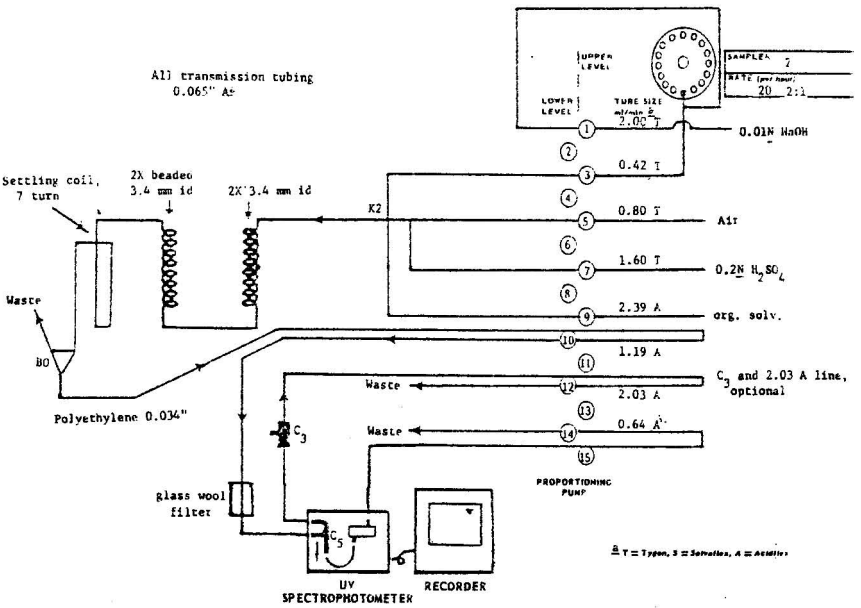

FIG. 1—AutoAnalyzer flow diagram for determination of sodium warfarin and dicumarol in tablets.

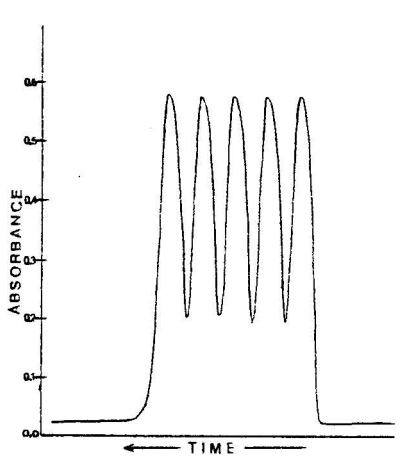

FIG. 2—Reproducibility curves for warfarin, 0.1 mg/ml.

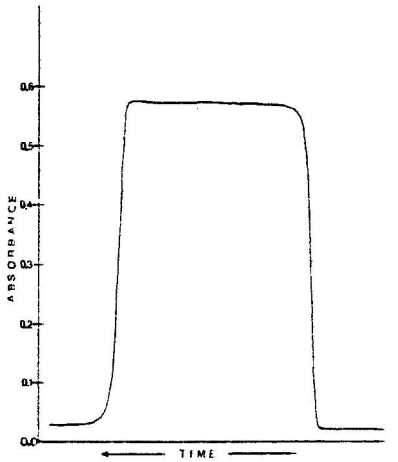

FIG. 3—Steady state curve for warfarin, 0.1 mg/ml.

vol. flask, and dissolve and dil. to vol. with 0.01*N* NaOH.

(d) *Dicumarol (bishydroxycoumarin) std soln.*—0.25 mg/ml. Accurately weigh ca 25 mg USP Ref. Std Bishydroxycoumarin in 100 ml vol. flask. Dissolve in ca 50 ml 0.01*N* NaOH with aid of ultrasonic generator and dil. to vol. with same solv. Prep. fresh daily.

36.C28 *Preparation of Sample*

(a) *Sodium warfarin.*—Disintegrate individual tablet or disperse weighed composite in accurately measured vol. 0.01*N* NaOH to give drug concn of 0.1 mg/ml. Use ultrasonic generator ≥ 1 min to assure tablet disintegration. Let suspension stand 1.5 hr with occasional mixing.

(b) *Dicumarol.*—Disintegrate individual tablet and capsule or disperse weighed composite in accurately measured vol. 0.01*N* NaOH to give drug concn of 0.25 mg/ml. (Most tablets and capsules require 15–20 min of ultrasonic treatment for complete disintegration.) Let suspension stand 1.5 hr with occasional mixing.

36.C29 *Analytical System*

Sample is withdrawn, segmented with air, and acidified with 0.2*N* H_2SO_4. Org. solv. is added, mixed in beaded coil, and phases are sepd in BO fitting. Org. phase contg extd drug is debubbled, and *A* of soln at 308 nm is measured in 10 mm flowcell for warfarin and 2 mm flowcell for dicumarol.

36.C30 *Start-Up and Shut-Down Procedures*

Pump alcohol thru org. solv. line 10 min; then pump org. solv. (a) or (b) thru line 5 min. Place remaining tubes in their resp. solns and let system equilibrate 20–30 min. Calibrate recorder to spectrophtr at 2 or 3 absorbances. Adjust mask in ref. compartment of spectrophtr to set desired baseline.

Place acid, base, and sampling lines in H_2O, leave org. solv. line in its reservoir, and pump 5 min. Remove acid, base, and sampling lines from H_2O and continue pumping 5 min to purge system of H_2O. Place org. solv. line in alcohol and pump 5 min. Remove line and pump system dry.

36.C31 *Determination*

Fill sample cups in following order: 4 cups std soln, 5 cups sample soln, 1 cup std soln, 5 cups sample soln, etc. Place 2 cups std soln at end of each run. (Extra cups of std solns at start and end of sampling pattern are used to overcome carryover effect in transitions from wash soln to std soln and vice versa. Three extra cups at beginning and 1 extra cup at end should suffice, but det. exact number needed for equilibrium by experiment. System should give uniform response for at least final pair of extra std cups before sample pattern is started.) Start Sampler II. After last cup has been sampled, let system operate until steady baseline is obtained. Draw tangent to initial and final baselines. Subtract baseline to det. net *A* and *A′* for each sample and std peak, resp. Discard values for first 2 and last std peaks and calc. av. std *A′*. Calc. Na warfarin in portion taken as mg Na warfarin = $1.071 \times (A/A') \times C \times D$, where 1.071 = ratio of molecular wts of Na warfarin to warfarin; *C* = concn of std in mg/ml, and *D* = diln factor. Calc. dicumarol in portion taken as mg dicumarol = $(A/A') \times C \times D$.

Table 1. Precision data for proposed automated method

Statistic	Sodium warfarin, 10 mg/tablet	Dicumarol, 25 mg/tablet
Mean (20)[a]	10.05	24.95
Std dev.	0.075	0.14
Coeff. of var., %	0.75	0.56

[a] Results of mean in mg/tablet.

Table 2. Comparison of automated method with USP XVIII method

	Sodium warfarin, 10 mg/tablet		Dicumarol, 25 mg/tablet	
Statistic	Automated	USP XVIII	Automated	USP XVIII
Mean (10)[a]	10.25	10.24	24.75	24.75
Std dev.	0.11	0.06	0.14	0.30
Coeff. of var., %	1.11	0.59	0.56	1.20

[a] Results of mean in mg/tablet.

Table 3. Results of composite assays at individual tablet levels by automated method

	Sodium warfarin			Dicumarol,
	2.5 mg	5.0 mg	10.0 mg	50.0 mg
	2.54	5.08	9.87	50.4
	2.54	4.98	9.88	50.9
	2.53	5.08	9.84	50.9
	2.53	5.09	9.80	50.8
	2.51	5.05	9.83	50.1
	2.51	5.03	9.82	50.7
	2.54	5.05	9.83	50.7
	2.53	5.04	9.82	51.0
	2.50	5.04	9.72	51.1
	2.53	5.03	9.72	50.7
Mean	2.53	5.05	9.81	50.7
Std dev.	0.0143	0.0320	0.0544	0.280
Coeff. of var., %	0.566	0.634	0.554	0.551

Collaborative Study

Three dosage levels of sodium warfarin were selected: 2.5, 5, and 10 mg/tablet. The dosage level selected for dicumarol was 50 mg/tablet. Two hundred fifty tablets from each dosage level were ground, sieved, and mixed. Each composite was analyzed 10 times by the semiautomated method. Each determination was performed on a

weighed portion of composite equivalent to one tablet to simulate an individual tablet assay. *See* Table 3. Each composite was also assayed by the USP XVIII methods for sodium warfarin and dicumarol in tablets. Results for the 2.5, 5, and 10 mg sodium warfarin tablets were 2.57, 5.06, and 9.97 mg/tablet. Assay for the 50 mg dicumarol tablet was 49.5 mg/tablet.

Composites and standards were sent to 7 collaborators with assay instructions as described above. Collaborators' results are tabulated in Tables 4 and 5. Collaborator 6 reported 5 results per composite instead of 10. The collaborative results from each collaborator were combined to give a total of 65 results for each composite. The mean, standard deviation, and coefficient of variation were calculated for each group and are listed in the tables.

Table 4. Collaborative results of analysis of sodium warfarin[a]

	Collaborator							Combined results
	1	2	3	4	5	6[b]	7	
2.5 mg/tablet composite								
	2.43	2.51	2.47	2.43	2.52	2.45	2.56	
	2.48	2.56	2.49	2.48	2.50	2.47	2.51	
	2.46	2.52	2.48	2.45	2.50	2.54	2.51	
	2.45	2.52	2.51	2.45	2.49	2.51	2.53	
	2.55	2.48	2.49	2.56	2.50	2.49	2.53	
	2.60	2.51	2.49	2.44	2.48		2.52	
	2.48	2.55	2.51	2.42	2.46		2.52	
	2.50	2.50	2.52	2.42	2.42		2.53	
	2.52	2.52	2.51	2.44	2.45		2.52	
	2.45	2.48	2.50	2.43	2.46		2.51	
Mean	2.49	2.51	2.50	2.45	2.48	2.49	2.52	2.49
Std dev.	0.052	0.026	0.016	0.042	0.030	0.035	0.015	0.039
Coeff. of var., %	2.10	1.03	0.63	1.71	1.21	1.40	0.60	1.56
5 mg/tablet composite								
	4.78	5.01	5.01	4.89	4.98	5.09	5.00	
	4.78	5.03	5.04	4.71	4.97	5.11	5.00	
	4.76	5.02	4.99	4.71	5.05	5.14	4.98	
	4.71	5.08	5.00	4.81	4.95	5.11	5.03	
	4.89	5.07	5.01	4.81	4.95	5.11	5.03	
	4.85	5.10	4.93	4.90	4.95		5.03	
	4.83	5.01	4.99	4.84	4.90		4.99	
	4.92	4.97	5.00	4.84	4.94		5.04	
	4.88	5.06	5.00	4.88	4.92		5.01	
	4.85	5.01	4.96	4.90	4.95		5.04	
Mean	4.82	5.03	4.99	4.83	4.95	5.11	5.01	4.96
Std dev.	0.066	0.040	0.030	0.071	0.040	0.018	0.021	0.10
Coeff. of var., %	1.37	0.79	0.60	1.47	0.81	0.35	0.42	2.10
10 mg/tablet composite								
	9.51	9.72	9.69	9.55	9.60	9.83	9.81	
	9.47	9.65	9.72	9.70	9.58	9.83	9.71	
	9.60	9.81	9.64	9.46	9.56	9.89	9.77	
	9.70	9.83	9.67	9.45	9.55	9.85	9.88	
	9.62	9.79	9.69	9.51	9.55	9.93	9.92	
	9.51	9.76	9.60	9.48	9.72		9.87	
	9.45	9.83	9.69	9.68	9.46		9.95	
	9.71	9.80	9.66	9.52	9.56		9.96	
	9.53	9.90	9.79	9.52	9.53		9.93	
	9.58	9.84	9.64	9.24	9.56		9.86	
Mean	9.57	9.79	9.68	9.51	9.57	9.87	9.87	9.67
Std dev.	0.090	0.070	0.052	0.128	0.065	0.043	0.081	0.16
Coeff. of var., %	0.94	0.71	0.53	1.34	0.68	0.44	0.83	1.62

[a] Results in mg/tablet.
[b] Five results received from Collaborator 6.

Table 5. Collaborative results of analysis of dicumarol[a] (50 mg/tablet composite)

	Collaborator							Combined results
	1	2	3	4	5	6[b]	7	
	47.7	50.1	50.5	50.2	49.3	51.1	49.5	
	51.3	50.4	50.4	49.3	49.6	51.3	49.7	
	49.5	50.0	50.5	50.2	49.2	51.6	49.6	
	50.1	49.7	51.0	50.3	49.5	51.5	49.8	
	50.4	50.6	49.6	50.7	49.6	51.5	50.0	
	49.2	50.0	50.5	51.4	49.6		49.9	
	50.2	50.0	50.6	49.9	49.5		50.2	
	51.3	50.7	50.6	51.1	49.0		49.8	
	48.9	50.0	51.0	50.0	48.8		49.6	
	49.8	50.2	50.5	50.1	48.7		49.2	
Mean	49.8	50.2	50.5	50.3	49.3	51.4	49.7	50.1
Std dev.	1.09	0.31	0.38	0.61	0.34	0.20	0.28	0.77
Coeff. of var., %	2.19	0.62	0.76	1.21	0.69	0.39	0.56	1.53

[a] Results in mg/tablet.
[b] Five results received from Collaborator 6.

Discussion and Recommendation

Some collaborators had to modify their systems because of lack of certain equipment. One collaborator did not have a 7-turn settling coil and had to substitute a 14-turn coil. Another collaborator did not have a K2 fitting and used a combination G3 and DO. A third collaborator had to use No. 18 gauge Teflon tubing instead of 0.034″ id polyethylene tubing. Collaborators used a variety of spectrophotometers such as the Beckman DK-2A, Hitachi-Perkin-Elmer 124, Perkin-Elmer 402, and Cary 14.

One collaborator suggested that the construction of the glass wool filter be described in the method. The method has been changed to include assembly of the filter.

Two collaborators stated that the C3 debubbler fitting and the 2.03 ml/min Acidflex pump tube can be eliminated since the system works as well without it. This is true and was not realized at the time the system was validated. The C3 debubbler and waste line have been marked as optional on the flow diagram (Fig. 1).

Analysis of the data submitted by the collaborators indicates the system to be precise and accurate. Considering the variations in construction of the system and use of various spectrophotometers, the semiautomated system has been shown to give useful results. It is recommended that the semiautomated method for analysis of sodium warfarin and dicumarol be adopted as official first action.

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Thanks also go to W. Furman, L. Jones, R. D. Kirchhoefer, and J. W. Myrick, Food and Drug Administration, St. Louis, for their advice and suggestions during this study.

References

(1) *United States Pharmacopeia* (1970) 18th Rev., Mack Publishing Co., Easton, Pa., p. 80
(2) *United States Pharmacopeia* (1970) 18th Rev., p. 677

The recommendation of the Associate Referee was approved by the Referee and Subcommittee B and was adopted by the Association; *see* (1973) *JAOAC* 56, 395–396.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

FLAVORS AND NONALCOHOLIC BEVERAGES

Collaborative Study of the Quantitative Determination of Ethanol by Gas-Solid Chromatography

GLENN E. MARTIN and DONALD M. FIGERT
Bureau of Alcohol, Tobacco and Firearms, U.S. Treasury Department, Washington, D.C. 20224

The determination of ethanol by gas-solid chromatography was collaboratively studied for nonbeverage alcohol samples by 10 collaborators representing 6 laboratories. In one sample, the alcohol was determined by pycnometer, hydrometer, fluid densitometer, and gas-solid chromatography and the results obtained by these procedures compare favorably. The standard deviation for the 7 samples range between 0.133 and 1.311. This method is simple and fast, and the interference from other compounds is essentially eliminated. The proposed procedure for the quantitative determination of ethanol has been adopted as official first action.

We studied the use of gas-solid chromatography for the determination of ethanol in flavors and extracts. The major advantages of gas-solid chromatography over hydrometer are: (*1*) distillation is not required; (*2*) the efficiency obtained by gas chromatography allows ethanol to be determined as a single entity; (*3*) after simple distillation the specific gravity obtained by hydrometer represents the composite of all volatile materials; (*4*) the formation of azeotropes with ethanol as carried out in simple distillation is eliminated; (*5*) the extraction and re-extraction of the sample with an organic phase, as described in *U.S. Pharmacopeia* (1), is eliminated and thereby the loss of ethanol due to these manipulations is avoided.

Seven samples of nonbeverage alcohol were analyzed including hop extract, chloroform liniment, perfume, proprietary solvent, lemon extract, ethanol standard, and sauterne flavor, ranging from approximately 4 to 85% ethanol. These samples were sent to 10 collaborators representing 6 laboratories. Collaborators were asked to analyze each sample in triplicate. In addition each collaborator was asked to determine the slope of the line from the area of ethanol divided by the area of 1 ml *n*-propanol as the ordinate and per cent ethanol as the abscissa.

METHOD

The following method for the gas-solid chromatographic determination of alcohol was adopted official first action for flavors, and as an alternative first action method for lemon, orange, and lime extracts and flavors, 19.050 (oil, alcohol, and water only); almond extract, 19.091 and 19.092; cassia, cinnamon, and clove extracts, 19.102 and 19.103; peppermint, spearmint, and wintergreen extracts, 19.110 and 19.111; and other extracts and toilet preparations:

19.C01 *Apparatus and Reagents*

(a) *Gas chromatograph.*—With flame ionization detector (F&M Model 400, or equiv.). Column 4′ × 2 mm id glass packed with 100–120 mesh Chromosorb 102; column temp. 160° (isothermal), detector and inlet 200°; He flow rate 50 ml/min; relative retention times: EtOH 1.00 (≤100 sec), *n*-propanol 2.06, and tetrahydrofuran 3.04.

(b) *Integrator.*—Hewlett-Packard Model 3370A, or equiv.

(c) *Alcohol std solns.*—Dil. 5, 10, 15, and 20 ml absolute alcohol at 60°F to 100 ml with H_2O, adjusting final vol. at 60°F, to prep. 5, 10, 15, and 20% solns.

19.C02 *Determination*

Pipet 25 ml of each EtOH std soln into sep. flasks or bottles and add 1.00 ml *n*-propanol internal std. Cap immediately with rubber stoppers, shake 3 min, and let stand 10 min at room temp. Inject 0.1 µl portions from 1 µl microsyringe. Det. peak areas with integrator, calc. ratio (R) of areas of EtOH to *n*-propanol, and plot % EtOH as abscissa against R as ordinate. Slope should be 0.195±0.006.

Perform detn on sample as above but shaking 10 min. If sample contains >20% EtOH, pipet 5 ml sample, 20 ml *tetrahydrofuran* (Fisher Scientific Co.), and 1 ml *n*-propanol into flask or bottle. Analyze ≥1 std EtOH–*n*-propanol soln daily as check on performance.

% EtOH = (EtOH peak area/*n*-propanol peak area) × (1.00/slope) × F, where F = 1 for undild samples and 5 for dild samples.

Table 1. Per cent ethanol obtained by 10 collaborators with gas-solid chromatographic method

Coll.	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
1	4.76	46.75	75.90	80.71	85.40	20.01	11.16
2	4.78	47.18	76.35	81.44	84.56	19.98	11.28
3	4.77	46.49	74.77	80.10	84.72	19.98	11.19
4	4.81	45.68	75.09	80.71	85.40	19.95	11.16
5	4.66	46.44	72.84	80.20	86.36	19.12	10.87
6	4.57	48.36	76.26	79.00	83.77	19.91	10.89
7	4.76	47.43	76.58	79.56	86.21	19.89	11.12
8	4.36	44.02	75.48	78.46	84.77	20.01	10.16
9	4.71	46.70	77.86	79.7	84.0	19.87	11.37
10	4.63	47.39	75.91	78.98	86.09	19.90	11.22
Av.	4.68	46.64	75.69	79.88	85.02	19.86	11.04
Std dev.	0.133	1.172	1.311	0.924	0.812	0.266	0.346

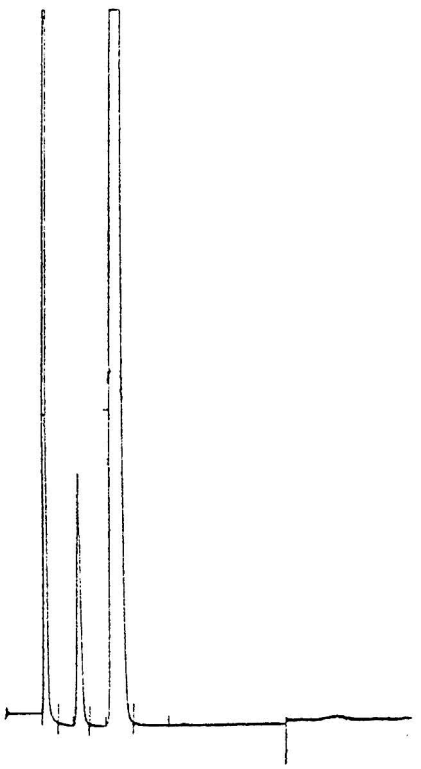

FIG. 1—Typical chromatogram of ethanol, n-propanol, and tetrahydrofuran, respectively.

Discussion

We selected tetrahydrofuran as a solvent because it could be used as a diluent in both polar and nonpolar solutions. In addition, tetrahydrofuran eluted after ethanol and *n*-propanol with no resulting interference. One collaborator performed a series of determinations of ethanol. The analyses were repeated 5 times with each standard solution. He obtained a straight line for the plot of ethanol peak area: *n*-propanol peak area versus per cent ethanol for the range studied, 2–20% ethanol. The deviation between experiments averaged 0.2%. The mean value of the slope for the 9 standard solutions was 0.193, and the average deviation from this value was ±0.0002.

Results and Recommendation

The retention times for ethanol, *n*-propanol, and tetrahydrofuran, relative to ethanol, are 1.000, 2.062, and 3.038, respectively. A typical chromatogram is shown in Fig. 1. The average value obtained for the slope by the 10 collaborators is 0.195, while the standard deviation is 0.0041. If the slope is divided by 1.000, the factor is 5.12.

The average per cent ethanol found in samples 1–7 was 4.68, 46.64, 75.69, 79.88, 85.02, 19.86, and 11.04, respectively; the standard deviation for these samples was 0.133, 1.172, 1.311, 0.924, 0.812, 0.266, and 0.346, respectively (see Table 1).

The per cent ethanol obtained by gas-solid chromatography compares favorably with the calculated values of the formulated samples 1 through 7 (see Table 2). In sample 6 the per cent ethanol was determined by 4 different procedures: pycnometer (2), hydrometer (3), fluid densitometer, and gas-solid chromatography; the results were 19.94, 19.86, 19.86, and 19.86%, respectively.

The recommendation contained in this paper was approved by the General Referee and by Subcommittee D and was adopted by the Association: see (1973) *JAOAC* 56, 402.

Table 2. Description and composition of samples 1–7

Sample	Composition	Ethanol, %
1 Hop extract	4.7% ethanol 1.0% hop extract 94.3% water	4.7
2 Soap liniment	70% soap liniment (67% ethanol) 30% $CHCl_3$	46.9
3 Lemon extract	75% ethanol 2% lemon oil 23% water	75
4 Perfume	80% ethanol 20% oil	80
5 Special industrial solvent	86% ethanol 3.5% methanol 9.0% isopropanol 1.5% methyl isobutyl ketone	86
6 Standard	20% ethanol 80% water	20
7 Sauterne flavor	11% ethanol 1% sauterne flavor 88% water	11

The method is fast and reproducible and eliminates interferences from other compounds. It is recommended that the proposed method for ethanol be adopted as official first action for flavors; and as an alternative method for extracts, 19.050, 19.091–19.092, 19.102–19.103, 19.110–19.111.

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The Associate Referee wishes to express his sincere appreciation to collaborators who participated in this study, without whose assistance and criticism this study would have been impossible:

Andrew Johnson and Donald J. Murphy, George Washington University, Washington, D.C.; Matthew Thompson, University of Louisville, Louisville, Ky.; and the following personnel of the Bureau of Alcohol, Tobacco and Firearms: Mike Byron, Atlanta, Ga.; James Cherolis, Cincinnati, Ohio; Ewell Davis, Philadelphia, Pa.; Randolph H. Dyer, Washington, D.C.; Marijane C. Lawson, Philadelphia, Pa.; Robert Parker, Cincinnati, Ohio; Richard L. Woodard, Washington, D.C.

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(1) *United States Pharmacopeia* (1970) 18th Rev., Mack Publishing Co., Easton, Pa., p. 919
(2) *Official Methods of Analysis* (1970) 11th Ed., AOAC Washington, D.C., sec. 9.011
(3) *Official Methods of Analysis* (1970) 11th Ed., sec. 9.015

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COLOR ADDITIVES

Uncombined Intermediates in FD&C Red No. 40

DANIEL M. MARMION
Allied Chemical Corp., Specialty Chemicals Division, P.O. Box 1069, Buffalo, N.Y. 14240

The results of a collaborative study are reported. Nine collaborators from industry and government analyzed 3 samples each. The intermediates were separated from FD&C Red No. 40 and from each other on a cellulose column, using 40 and 20% aqueous ammonium sulfate as eluants, and were quantitated by UV spectrometry. Cresidinesulfonic acid, Schaeffer's salt, and 6,6'-oxybis(2-naphthalenesulfonic acid) were recovered at an average level of 99.7%. Between-laboratory standard deviations ranged from 0.007 to 0.072% when the intermediates were present at levels of 0.03 to 1.00%. The method has been adopted as official first action.

FD&C Red No. 40 (Allura[1] Red AC) is a new color additive listed in 1971 by the Food and Drug Administration for use in foods and drugs. The colorant is a monoazo dye formed by coupling diazotized 5-amino-4-methoxy-2-toluenesulfonic acid (cresidinesulfonic acid; CSA) with 6-hydroxy-2-naphthalenesulfonic acid (Schaeffer's salt).

Allura Red AC, like most color additives, frequently contains small amounts of the intermediates used to prepare it. In addition, the colorant may contain traces of 6,6'-oxybis(2-naphthalenesulfonic acid) (DONS), an impurity shown by Marmion *et al.* (1, 2) to be present in commercial Schaeffer's salt and in color additives made from it. In 1971 Marmion (3) reported a method for the determination of each of these colorless impurities in FD&C Red No. 40. This method has since been collaboratively studied and the results of that study are reported here.

The samples used for the study consisted of a sample which had been analyzed many times in the Associate Referee's laboratory by the proposed procedure, and 2 portions of the same material spiked with the impurities to be determined. (A purified sample of FD&C Red No. 40 was not used as a base sample since we felt that any steps taken to purify the colorant would render the sample nonrepresentative of commercial material by removing from it subsidiary dyes, inorganic salts, etc. normally found in certified colors.) The spiked samples were prepared by dissolving portions of the base sample and the impurities in distilled water, drying them at 50°C under vacuum, and grinding the products. The CSA, Schaeffer's salt, and DONS added to the base sample were laboratory-purified materials assayed by elemental analysis, spectrophotometry, and, in the case of CSA, nitrite titration.

The base sample had an historical average of 0.03% CSA (as free acid), 0.08% Schaeffer's salt (as Na salt), and 0.47% DONS (as di-Na salt). The spiked samples were prepared to contain: 0.203% CSA, 0.290% Schaeffer's salt, and 1.00% DONS; 0.164% CSA, 0.231% Schaeffer's salt, and 0.806% DONS. CSA was added as the free acid while Schaeffer's salt and DONS were added as the mono- and di-Na salts, respectively. A portion of each sample and a copy of the proposed method were distributed to each collaborator, with a request that he provide one result for each unknown.

The method provided was as follows:

METHOD

Intermediates in FD&C Red No. 40—Official First Action

34.C01 ***Principle***

FD&C Red No. 40 (C.I. No. 16035) is more strongly adsorbed on cellulose from concd $(NH_4)_2SO_4$ soln than are dye intermediates. 5-Amino-4-methoxy-2-toluenesulfonic acid (CSA) and Schaeffer's salt elute sep. with 40% $(NH_4)_2SO_4$; 6,6'-oxybis(2-naphthalenesulfonic acid) (DONS) elutes with 20% $(NH_4)_2SO_4$. Each intermediate is detd by UV spectrophotometry.

34.C02 ***Apparatus***

See 34.B02.

34.C03 ***Reagents***

See 34.B03.

[1] Allura is a trademark of Allied Chemical Corp.

34.C04 *Preparation of Chromatographic Column*

See 34.B04. Prep. and elute similar column as blank. Blank need be repeated only when new reagents are introduced.

34.C05 *Separation of Intermediates*

Weigh 0.100 g sample into 50 ml beaker. Add 5 ml H_2O and stir to dissolve. Proceed as in 34.B05, beginning "Add 2 g cellulose powder . . .", except elute with ca 1.5 L 20% $(NH_4)_2SO_4$.

34.C06 *Calculations*

See 34.B06. CSA normally elutes in fractions 4–6, Schaeffer's salt in fractions 8–10, and DONS in fractions 16–30.

% CSA (as free acid, mol. wt 217.2)

$= 0.186 \times \Sigma[(A_1 - A_2)_{252} - (A_3 - A_4)_{360}]$

% Schaeffer's salt (as Na salt, mol. wt = 246.2)

$= 0.395 \times \Sigma[(A_1 - A_2)_{282} - (A_3 - A_4)_{360}]$

% DONS (as di-Na salt, mol. wt 474.4)

$= 0.0585 \times \Sigma[(A_1 - A_2)_{240} - (A_3 - A_4)_{360}]$

Effective sample concn is 2 g/L; 53.8, 25.3, and 171 = a (L/g-cm) of CSA, Schaeffer's salt, and DONS, resp; *see* 34.B06 for calcn of constants.

Table 1. Collaborative results for uncombined intermediates in FD&C Red No. 40

Coll.	CSA, %	Schaeffer's salt, %	DONS, %
		Sample 1	
1	0.03	0.09	0.46
2	0.02	0.08 (0.04)[a]	0.44
3	0.02	0.09	0.46
4	0.02	0.03	0.46
5	0.04	0.11[b] (0.04)[a]	0.46
6	0.05	0.11 (0.04)[a]	0.45
7	0.02	0.05	0.46
8	0.02	0.04 (0.03)[a]	0.45
9	0.03	0.05	0.45
Mean	0.028	0.072	0.454
		Sample 2[c]	
1	0.20	0.30	1.05
2	0.16	0.24 (0.20)[a]	0.96
3	0.21	0.35	0.99
4	0.22	0.30	0.87
5	0.21	0.26[b] (0.19)[a]	0.93
6	0.21	0.29 (0.21)[a]	1.00
7	0.17	0.27	0.94
8	0.19	0.24	0.95
9	0.19	0.30	1.00
Mean	0.196	0.283	0.966
		Sample 3[d]	
1	0.16	0.26	0.89
2	0.14	0.22 (0.17)[a]	0.77
3	0.16	0.23	0.80
4	0.17	0.29	0.74
5	0.17	0.28[b] (0.19)[a]	0.83
6	0.17	0.21 (0.15)[a]	0.84
7	0.15	0.20	0.79
8	0.16	0.22	0.84
9	0.15	0.19	0.64
Mean	0.159	0.233	0.793

[a] Result reported by collaborator using 232 nm as analytical wavelength.

[b] Result calculated by Associate Referee from spectra submitted by collaborator.

[c] Sample 2 = Sample 1 + 0.173% CSA, 0.210% Schaeffer's salt, and 0.533% DONS.

[d] Sample 3 = Sample 1 + 0.134% CSA, 0.151% Schaeffer's salt, and 0.336% DONS.

Results and Recommendation

Results are tabulated in Tables 1–3. Collaborator 5 submitted values for Schaeffer's salt based on its absorption at 232 nm only, but since the collaborator submitted his spectra with his results, the Associate Referee calculated the Schaeffer's salt content at 282 nm.

The proposed procedure stated that, for greater sensitivity, Schaeffer's salt may be calculated at the absorption maximum near 232 nm rather than at the weaker absorption band at 282 nm (*see* Fig. 1). Several collaborators reported Schaeffer's salt based on calculations at both wavelengths. In all cases the results reported using the 232 nm absorption peak were erroneously low and were not considered further. The reason for the low results is not clear, but it is probably related to the fact that the spectral reference does not truly represent the eluant composition during the time

Table 2. Per cent recovered[a] for intermediates and impurities

Compound	Sample 2	Sample 3
CSA	97.1	97.8
Schaeffer's salt	100.5	106.0
DONS	96.1	100.9

[a] % Recovered = (mean % found − mean % found in sample 1) × 100/% added.

Table 3. Summary of between-laboratory variations in collaborative study[a]

	CSA		Schaeffer's salt		DONS	
Sample	Std dev.	% Error	Std dev.	% Error	Std dev.	% Error
1	0.011	39.3	0.030	42.0	0.007	1.60
2	0.020	10.3	0.035	12.4	0.052	5.36
3	0.011	6.63	0.035	15.2	0.072	9.13

[a] % Error = (std dev./mean) × 100% = coefficient of variation.

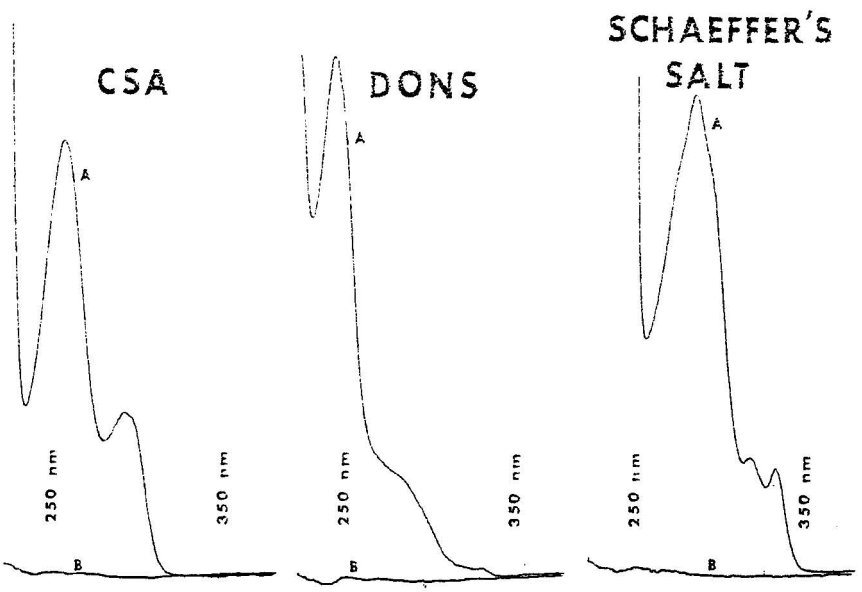

FIG. 1—Absorption spectra of cresidinesulfonic acid (ca 12 ppm), 6,6'-oxybis(2-naphthalenesulfonic acid (ca 5 ppm), and Schaeffer's salt (ca 35 ppm) in 1 cm fused quartz cell. 40% aqueous $(NH_4)_2SO_4$ used as solvent and reference, except for DONS (20%). A and B refer to spectra of sample and blank, respectively.

Schaeffer's salt elutes, since elution is based on a stepwise gradient.

One collaborator reported that the calculations were not clear enough and should be explained in more detail. A third collaborator pointed out that the factor for calculating Schaffer's salt should be 0.395, not 0.396 (as indicated in the collaborated method).

Based on the data obtained in this study, it is recommended that the method for uncombined intermediates in FD&C Red No. 40, modified as indicated according to collaborators' comments, be adopted as official first action.

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Special thanks are due to the following collaborators and their associates for their cooperation in this study:

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(3) Marmion, D. M. (1971) *JAOAC* 54, 131–136

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee G and was adopted by the Association; *see* (1973) *JAOAC* 56, 410.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Separation and Determination of FD&C Red No. 4 and FD&C Red No. 40 in Maraschino Cherries by Column Chromatography

RONALD E. DRAPER
Food and Drug Administration, 1560 E. Jefferson Ave., Detroit, Mich. 48207

FD&C Red No. 4 and FD&C Red No. 40 are extracted from a sample-Celite mixture in a chromatographic column by elution with a 5% solution of Amberlite LA-2 liquid anion exchange resin in *n*-butanol. The colors are extracted from the resin with dilute ammonia and then extracted into *n*-butanol. After evaporation to dryness, the colors are separated by Celite chromatography, using pH 1.5 HCl buffer as immobile phase. Red No. 4 is eluted with *n*-butanol-CCl_4 (1+1) and Red No. 40 is eluted with a 5% solution of Amberlite LA-2 resin in hexane. The compounds are measured spectrophotometrically. Results on a commercial sample (5 trials) ranged from 74 to 76 ppm Red No. 4 and 72 to 81 ppm Red No. 40. Spike recoveries at various levels averaged 98% for FD&C Red No. 4 and 95% for FD&C Red No. 40.

The use of FD&C Red No. 4 in foods in this country is limited to Maraschino cherries at a level not exceeding 150 ppm. The lack of an official method for this product prompted an investigation to find a suitable means of analysis.

Graichen and Molitor (1) have determined color additives in various foods, including Maraschino cherries, using Amberlite LA-2, a liquid anion exchange resin. Dolinsky and Stein (2) initially investigated the use of this resin for the extraction of colors from foods. The Graichen and Molitor method, with slight modification, was found suitable for determining FD&C Red No. 4 in Maraschino cherries when present as the only color additive.

However, with the recent approval of FD&C Red No. 40 as a new color additive, some manufacturers began using a mixture of Red No. 4 and Red No. 40 to color their cherries. This entailed developing a means of separation to determine the Red No. 4 content.

Various methods have been reported for separating FD&C colors (3–5), including chromatography on cellulose and alumina. Silk (6) separated D&C colors, FD&C Yellow No. 5, and FD&C Red No. 3 in lipsticks by Celite chromatography. No other reference to the separation of FD&C colors by Celite chromatography was found.

In the proposed procedure, Red No. 4 and Red No. 40 are extracted from a sample-Celite mixture in a chromatographic column with a 5% solution of Amberlite LA-2 resin in *n*-butanol. The colors are extracted from the resin with dilute ammonia and then extracted into *n*-butanol. After the solvent is evaporated to dryness, the colors are separated by Celite chromatography, using pH 1.5 HCl buffer as the immobile phase. Red No. 4 is eluted with *n*-butanol-CCl_4 (1+1) and Red No. 40 is eluted with a 5% solution of Amberlite LA-2 resin in hexane. The compounds are measured spectrophotometrically.

Results are reported for one commercial sample and fortified samples at various levels.

METHOD

Apparatus

(a) *Chromatographic columns.*—300 × 23 mm id.

(b) *Glass wool.*—Silanized, available from Applied Science Laboratories, State College, Pa.

Reagents

(a) *Solvents A and B.*—Add 500 ml 5% Amberlite LA-2 resin (Rohm & Haas Co., Philadelphia, Pa.) in *n*-butanol, 200 ml water containing 7.5 ml acetic acid, and 12.5 ml saturated $(NH_4)_2SO_4$ solution to separatory funnel. Shake vigorously 1 min and let phases separate. Lower layer is solvent A; upper layer is solvent B.

(b) *Hydrochloric acid.*—0.75% (1+49).

(c) *Buffer solution.*—pH 1.5. Mix 50 ml 0.2*M* KCl (14.911 g KCl/1 L water) and 41.4 ml 0.2*N* HCl in 200 ml volumetric flask and dilute to volume with water. Check with previously calibrated pH meter to ensure pH of 1.50±0.02.

(d) *Solvents C and D.*—Add 400 ml *n*-butanol-CCl_4 (1+1) and 200 ml buffer solution (c) to separatory funnel. Shake vigorously 2 min and let phases separate. Lower layer is solvent C; upper layer is solvent D. Prepare fresh daily.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

(e) *Resin-n-hexane.*—Add 500 ml 5% Amberlite LA-2 resin in *n*-hexane and 100 ml HCl (1+49) to separatory funnel. Shake 1 min. Discard lower phase.

(f) *Adsorbent.*—Celite 545, acid-washed, rinsed to neutrality, and dried.

Determination of Purity of Standards

(a) *FD&C Red No. 4.*—Finely powder ca 500 mg standard to pass No. 60 sieve. Accurately weigh ca 100 mg into 1 L volumetric flask. Dissolve in 0.01*N* $NH_4C_2H_3O_2$ and dilute to volume. Dilute 10 ml aliquot of this solution to 100 ml with 0.01*N* $NH_4C_2H_3O_2$. Determine *A* from 650 to 480 nm, maximum ca 502 nm, using 1 cm cells and 0.01*N* $NH_4C_2H_3O_2$ as blank. Calculate purity of standard, using absorptivity value of 54.0 L/g-cm for pure FD&C Red No. 4 (7).

(b) *FD&C Red No. 40.*—Proceed as for FD&C Red No. 4 above except dissolve standard in water and determine *A* from 650 to 480 nm, maximum ca 500 nm, using water as blank. Calculate purity of standard, using absorptivity value of 53.6 L/g-cm for pure FD&C Red No. 40 (8).

Preparation of Standard Solutions

(a) *FD&C Red No. 4.*—(*1*) *Stock solution.*—1 mg/ml. Dissolve 100 mg FD&C Red No. 4 in 100 ml water. (*2*) *Working solution.*—4 μg/ml. Transfer 1 ml stock solution to 100 ml beaker and evaporate to dryness on steam bath. Dissolve residue in 1 ml HCl (1+49), add 2 g adsorbent, and mix. Transfer to chromatographic column containing small plug of glass wool and packed with mixture of 3 ml solvent D and 5 g adsorbent. Dry-wash beaker with 0.5 g adsorbent and add wash to column. Wipe beaker with piece of glass wool and add wipe to column. Rinse beaker with two 5 ml portions solvent C and add rinses to column. After rinsings have entered column, elute column with 240 ml solvent C, collecting eluate in 250 ml volumetric flask. Dilute to volume with solvent C. Prepare fresh daily.

(b) *FD&C Red No. 40.*—(*1*) *Stock solution.*—1 mg/ml. Proceed as in (a)(*1*). (*2*) *Working solution.*—10 μg/ml. Proceed as in (a)(*2*), except rinse beaker with two 5 ml portions resin-*n*-hexane, and elute with 40 ml resin-*n*-hexane, collecting eluate in 100 ml volumetric flask. Dilute to volume with resin-*n*-hexane (solution is stable).

Preparation of Sample

Drain packing liquid as completely as possible from cherries and chop cherries 15 min in Hobart 84141 food cutter, or equivalent. Mix thoroughly while chopping. Transfer to Mason jar with tight-fitting lid.

Procedure

Weigh 5.0 g sample into 8 oz glass mortar, add 3 ml solvent A, and carefully grind with pestle 2 min. Add 15 g adsorbent and carefully grind additional 2 min. Scrape off pestle and thoroughly mix sample with spatula. Transfer mixture to chromatographic column containing small plug of glass wool and firmly pack with tamping rod. Wipe off mortar, pestle, and spatula with piece of glass wool and add wipe to column. Rinse mortar with 10 ml solvent B and add rinse to column. After rinse has entered column, elute column with 90 ml solvent B, collecting eluate in 125 ml separatory funnel containing 1 ml water. Add 30 ml hexane, shake, and let separate. Discard lower layer. Add 10 ml water (carefully rinsing around stopper and neck of separatory funnel) and 2 ml NH_4OH. Extract color by shaking 2 min. Let separate and drain lower layer into second 125 ml separatory funnel, rinsing stem with small portion water. Completely extract color from first separatory funnel with additional 10 ml water and 1 ml NH_4OH and add lower layer to second separatory funnel. Rinse first funnel with 5 ml water and add rinse to second separatory funnel. Wash combined aqueous extracts with two 25 ml portions $CHCl_3$, discarding $CHCl_3$ completely each time. Make acid with 2 ml HOAc and extract color with 50 ml *n*-butanol. Continue extraction with 10 ml portions *n*-butanol until color is visually completely extracted (3–6× are usually sufficient). Combine butanol extracts in 150 ml beaker, rinsing each separator with 2 ml butanol. Add 15–25 ml ethanol, mix with stirring rod, and evaporate just to dryness on steam bath under current of air.

Mix 5 g adsorbent and 3 ml solvent D in 100 ml beaker and transfer to chromatographic column containing small plug of glass wool. Pack with tamping rod. Dissolve color residue in 1 ml HCl (1+49), being sure to dissolve color on sides of beaker. Add 2 g adsorbent, thoroughly mix, and transfer to prepared column. Pack with tamping rod. Dry-wash beaker with 0.5 g absorbent and add wash to column. Wipe beaker with piece of glass wool and add wipe to column. Rinse beaker with three 5 ml portions solvent C and add rinses to column, allowing each to enter column before next one is added. Completely elute FD&C Red No. 4 with additional 180–235 ml solvent C, depending on amount of color in sample, but not exceeding 250 ml total. Collect in either 200 or 250 ml volumetric flask. Dilute to volume with solvent C. If necessary, dilute sample and/or working standard solution with solvent C to about the same concentration. Determine *A* of sample and standard solutions from 650 to 480 nm, ca 502 nm maximum, using solvent C as blank.

FD&C Red No. 40 can also be determined as follows: After complete elution of FD&C Red No. 4, pass 20 ml *n*-hexane through column and discard. Elute FD&C Red No. 40 with 50 ml resin-*n*-hexane,

collecting eluate in 100 ml volumetric flask. Dilute to volume with resin-*n*-hexane. If necessary, dilute sample and/or working standard solution with resin-*n*-hexane to about the same concentration. If sample or standard solution is cloudy, filter through small piece of glass wool. Determine A of sample and standard solutions from 650 to 480 nm, ca 500 nm maximum, using resin-*n*-hexane as blank.

Calculate amount of FD&C Red No. 4 or FD&C Red No. 40 in sample as:

$$\text{ppm Color} = (A/A') \times C \times (d/SW)$$

where A and A' = absorbances of sample and standard solution, respectively, C = concentration of standard in μg/ml (corrected for purity), d = dilution factor, and SW = sample wt in g.

Results and Discussion

Five trials on the same composite of a commercial Maraschino cherry sample gave the following results: 75, 76, 76, 75, and 74 ppm for Red No. 4; and 72, 75, 77, 81, and 78 ppm for Red No. 40.

Table 1 shows recoveries of various fortification levels of Red No. 4 and Red No. 40 added to a commercial Maraschino cherry sample. Recoveries range from 90 to 103% for FD&C Red No. 4 and from 88 to 103% for FD&C Red No. 40.

The initial extraction of the color additives from the cherries with resin-*n*-butanol solution proceeds slowly, but it appears to be the most efficient means of extraction available. Elution with a resin-*n*-hexane solution proceeds quickly, but the colors are not completely extracted.

Extraction of the colors is also enhanced by using HOAc as the immobile phase, as opposed to HCl or water, and by equilibrating the HOAc with the resin-*n*-butanol. However, the use of HOAc poses a problem. If the ammoniacal extracts are evaporated to dryness, a viscous, crystalline residue is obtained, probably caused by formation of ammonium acetate. When this residue is dissolved and put on the pH 1.5 buffer column, the separation is poor. Extraction of the color from the acidified ammoniacal extracts with *n*-butanol alleviates this problem, apparently by leaving the inorganic salts in the aqueous phase. Depending on the concentration of color in the sample, more than 3 extractions with 10 ml butanol may be necessary to completely remove the color. A total of six 10 ml butanol extractions was needed to completely remove the color at the higher fortification levels.

Table 1. Recoveries of various fortification levels of FD&C Red No. 4 and FD&C Red No. 40 added to Maraschino cherries

Level	Recovery,[a] % FD&C Red No. 4	Recovery,[a] % FD&C Red No. 40
200 ppm Red 4 + 200 ppm Red 40	100	100
200 ppm Red 4 + 100 ppm Red 40	101	103
100 ppm Red 4 + 200 ppm Red 40	103	94
100 ppm Red 4 + 100 ppm Red 40	94	91
50 ppm Red 4 + 50 ppm Red 40	90	88

[a] Amount of color from sample based on average of results as listed in text

The separation of the colors on the pH 1.5 buffer column is good. Red No. 4 elutes first in a wide band while Red No. 40 moves slowly down the column in a fairly tight band. There is a small amount of diffusion from the Red No. 40 band but by limiting the elution to a maximum of 250 ml *n*-butanol-CCl_4 (1+1), all of the Red No. 40 remains on the column.

Combinations of Red No. 4 with FD&C Red No. 2 and FD&C Red No. 3 have also been encountered in Maraschino cherries. We are presently studying methods to separate these combinations by Celite chromatography.

It is recommended that the separation and determination of FD&C Red No. 4 and Red No. 40 be studied collaboratively.

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The recommendation of the Associate Referee was approved by the Referee and Subcommittee G and accepted by the Association; *see* (1973) *JAOAC* 56, 410.

ANTIBIOTICS

Oxytetracycline Residues in Milk Following Intramammary Infusion

STANLEY E. KATZ, CAROL A. FASSBENDER, ARTHUR J. HACKETT, and RALPH G. MITCHELL

College of Agriculture and Environmental Science,[1] Rutgers University—The State University of New Jersey, New Brunswick, N.J. 08903

An intramammary infusion of oxytetracycline was used to study the potential of residues occurring in milk from a recommended usage program. Residues from the treated quarters persisted as long as 5.5 days. Residues in milk from the rear quarters disappeared in 3.5–4.5 days; residues in the milk from the front quarters persisted 5.0–5.5 days. In any case, residues persisted longer than an interval consistent with the recommended 4 day withdrawal period. Residues in the milk of the untreated quarters appeared to arise from transfer via the circulatory system and not from septal transfer.

It is recognized that the prime source of antibiotic residues occurring in milk is a result of treatment of lactating cows for mastitis (1, 2). Antibiotic residues occur from mastitis therapy in 2 general ways: (*1*) failure to withold the milk from marketing for the prescribed time interval after intramammary infusion or parenteral injection; (*2*) residues resulting from accepted use even though withholding periods were adhered to strictly. Milk from cows treated for mastitis is unsatisfactory for marketing for 2 basic reasons: (*1*) The milk comes from infected animals, and (*2*) the milk contains antibiotic residues which are unwanted and illegal.

Oxytetracycline is one of the drugs commonly used to treat mammary infections of lactating cows. It is supplied for intramammary application in self-contained applicator tubes to be infused into the infected quarter(s) after milking. The milk from the animal is not to be marketed for 96 hr after treatment of the infected quarter(s).

We report the results of a study, using oxytetracycline as a pilot antibiotic, to examine the premise that antibiotic residues in milk can occur after prescribed usage. The study utilized methods of a greater degree of sensitivity (3) than those recommended for use in the FDA methods and protocols (4).

Experimental

Animals

Two Holstein Friesian cows were used in this study. Cow A was 5 years old, weighed 1395 lb, and milked between 25 and 40 lb milk per day during the experiments. Cow B was 6 years old, weighed 1673 lb, and milked 25 lb per day. Both cows were kept in loose housing and were fed corn silage *ad libitum* and grain according to milk production. During trial periods, they were kept in box stalls. In all trials, a control sample was taken from the evening milking; then the appropriate quarter was infused by using a tube holding 14.2 g of a formulation containing 30 mg oxytetracycline hydrochloride per gram, a total treatment of 426 mg oxytetracycline. To obtain individual samples from each quarter of the udder, a quarter milking machine was used, with the milk from each quarter being collected in individual canisters. To prepare the cow for milking, the udder was washed with a warm solution of a disinfectant and a few streams of milk were taken from each teat. The quarter milker was then put on and removed when milk ceased to flow.

Methods of Analysis

(a) *Milk.*—Milk from each quarter was placed immediately in bottles and stored in a refrigerator until analyzed. Milk was analyzed daily (it was never stored for more than 18 hr before analysis) for oxytetracycline activity, using procedures developed for chlortetracycline (5) and adapted for oxytetracycline (3). The following dilutions were used for the treated quarter:

Days	*Dilution*
0.0	Standard
0.5	1:250
1.0	5:150
1.5	10:150
2.0	50:150
2.5	Standard

[1] Authors Katz and Fassbender are with the Department of Biochemistry and Microbiology; authors Hackett and Mitchell are with the Department of Animal Sciences.

The standard 100 ml sample was used for the analyses after 2 days and for all the milk samples from the untreated quarters.

(b) *Blood.*—The procedure was based upon those previously mentioned (3). Blood was sampled from the appropriate vein, mammary or jugular: 25 ml samples were taken and sodium citrate was added to prevent coagulation. A 20 ml aliquot of blood was diluted with 40 ml pH 4.5 buffer, blended, centrifuged, and assayed for oxytetracycline activity.

Results and Discussion

The analytical parameters are defined as follows: The procedure (3) utilized is capable of detecting 0.02–0.03 μg oxytetracycline/ml milk in laboratory "spiking" studies. Analytical measurement is routinely possible above 0.02 μg/ml milk. In these studies, measurable residues are defined as those greater than 9.5 mm in diameter; trace zones are those 9.0 to 9.5 mm in diameter; zones 9.0 mm or less are not considered as indicating the presence of oxytetracycline.

To illustrate, Fig. 1a shows a typical negative plate where there is no zone of inhibition or where inhibition is slightly above the diameter of the cylinder, 8.5 mm; Fig. 1b shows slight inhibition around the cylinder but less than 9.5 mm in diameter; Fig. 1c shows analytically measurable zones greater than 9.5 mm in diameter and ranging as high as 25 mm, depending on the dilution taken.

A 9.0–9.5 mm zone is used as a trace designation because blank fresh milk from cows generally gives slight inhibitory zones approximately 8.5 mm in diameter. The trace designations were established to eliminate possible errors in defining measurable zones. Spiking studies of 0.02 and 0.03 μg oxytetracycline/ml milk yielded zones of inhibition 9.0–9.5 mm in diameter. Zones of 9.5 mm and greater are in the analytically measurable category because the 0.025 μg/ml standard consistently yielded zones 9.5 to 10.0 mm in diameter.

By comparison, the procedures suggested in the FDA methods, reports, and protocols (4) were able to detect and measure consistently 0.10 to 0.12 μg oxytetracycline/ml milk. Although Myers (6), summarizing the tentative procedures of Kirshbaum *et al.* (7), reported a sensitivity of 0.025 μg oxytetracycline/ml milk, less than 10 μg oxytetracycline/100 ml milk could never be detected in the authors' laboratories when the recommended dilutions were used.

Oxytetracycline residues disappeared from the treated quarters of cow A in accordance with first-order-type kinetics for approximately 2.5 days at which point there was a significant change in slope. Disappearance of residues again continued to follow first-order-type kinetics for an additional 2.5 to 3.0 days. Measurable residues were found after as long as 5.5 days; the shortest interval was 3.5 days (Fig. 2). During the same studies blood was taken from the mammary and jugular veins at 4 hr intervals to examine the contribution of the circulatory system to removal of the antibiotic. Blood levels in the mammary vein were found within 4 hr; they reached 0.09 μg oxytetracycline/ml at 8 hr and remained relatively constant for 20 hr, when the antibiotic was no longer detected. Blood levels in the jugular vein appeared after 8 hr and could only be measured for a total of 16 hr. The levels in the blood taken from the jugular vein remained constant at 0.088 μg/ml.

The first part of the disappearance curve appears to show both the physical removal of the oxytetracycline from the infused quarter via the milk and physiological removal by the circulatory system. The second part of the curve shows the removal of mechanically occluded drug from the quarter itself and a lessened contribution from the circulatory system because of the lower concentrations of the drug in the udder.

The levels of oxytetracycline found in milk from the front quarters were much higher than those found in the rear quarters (Table 1). This is not unexpected because production of milk from the rear quarters accounts for some 60% of the total versus 40% for the front quarters. Assuming that the drug could be applied in absolutely the same concentrations to each quarter which had exactly the same internal geometry and relationship to the circulatory system and that milk production followed precisely the 60:40 ratio, the concentration in the milk of the front quarter should be 1.5 times that of the rear quarters. The actual ratio here was 1.8 at the first milking after treatment without consideration of the actual milk yield from the individual quarters.

Figures 3a and b show the disappearance from the front and rear quarters separately. The only differences are quantitative. The changes in slope in the front quarter appear at 2.5 days versus 2.1 days for the rear quarters. Measurable residues were found in the front quarters from 4.5 to 5.5 days and in the rear quarters from 3.5 to 4.5 days.

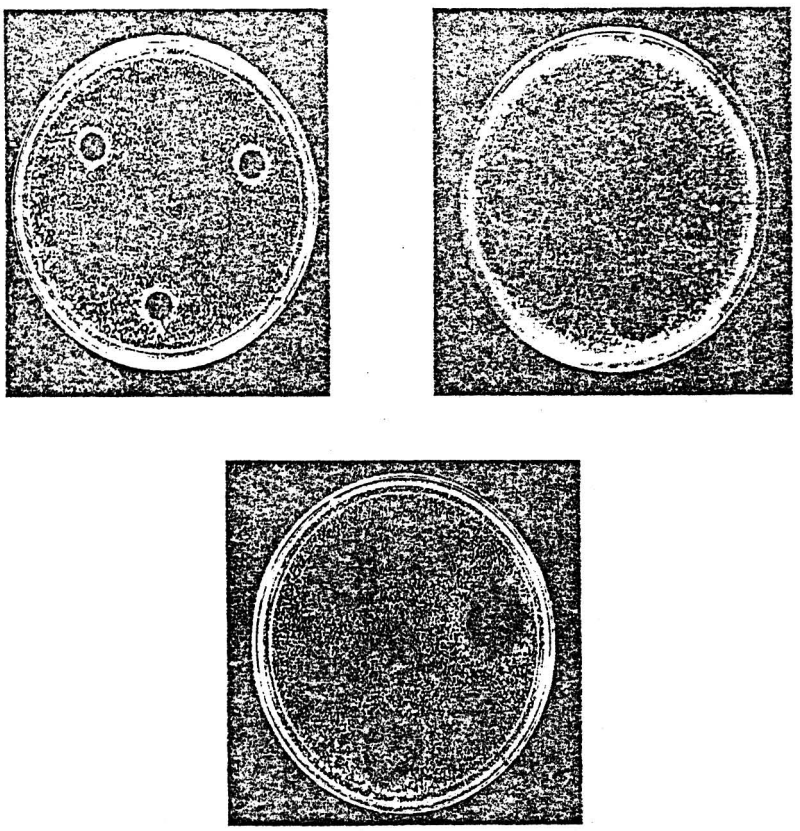

FIG. 1—Plates showing zones of inhibition produced by oxytetracycline: a (top left), typical negative plate; b (top right), slight inhibition (<9.5 mm in diameter); and c (bottom), analytically measurable zones (>9.5 mm to 25 mm in diameter, depending on dilution taken).

Table 1, in addition to showing a decrease in antibiotic residues in the milk from the treated quarter, shows the appearance of residues in the milk from the untreated quarters. The question as to whether these residues are a result of septal transfer cannot be answered unequivocally, but it would appear that the major mechanism of transfer is via the circulatory system. This was shown when 20 ml of a 50 mg oxytetracycline hydrochloride/ml preparation was injected intravenously and the relationship between blood levels and milk residues was investigated (Fig. 4). As can be seen, blood levels drop within 28 hr to a level below the analytical ability (3) to detect antibiotic presence while measurable residues appear in milk up to 48 hr after injection. The additional 20 hr in which residues occur reflect the fact that blood levels are indeed present but not measurable and the antibiotic can be concentrated by the alveoli.

The appearance of antibiotic residues in the milk of untreated quarters after mastitis treatment has been previously noted. Siddique and co-workers (8) reported the phenomenon of transfer of antibiotic residues to untreated quarters with penicillin, neomycin, and dihydrostreptomycin and felt that much of the variability in such data was a result of the inadequate sensitivity in the analytical methods utilized. Schipper and Petersen (9) postulated that such transfers occurred via the blood stream, although they felt that there was some degree of direct diffusion

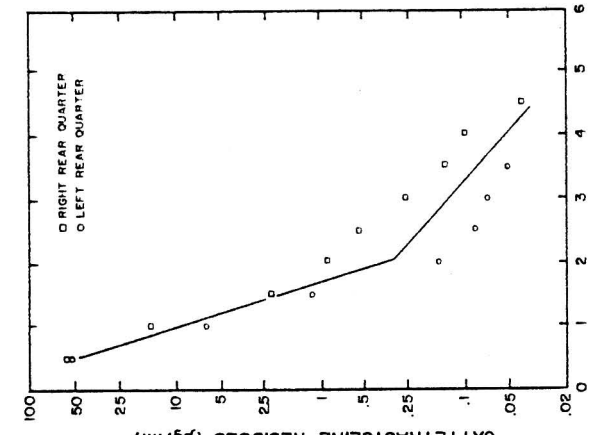

FIG. 2—Disappearance of oxytetracycline from milk of all quarters after intramammary infusion.

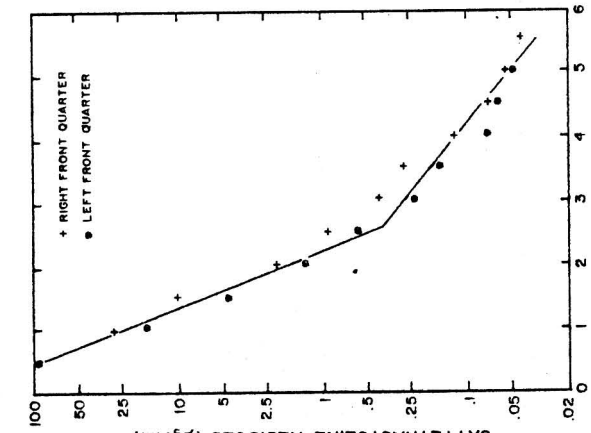

FIG. 3a—Disappearance of oxytetracycline from milk of front quarters after intramammary infusion.

FIG. 3b—Disappearance of oxytetracycline from milk of rear quarters after intramammary infusion.

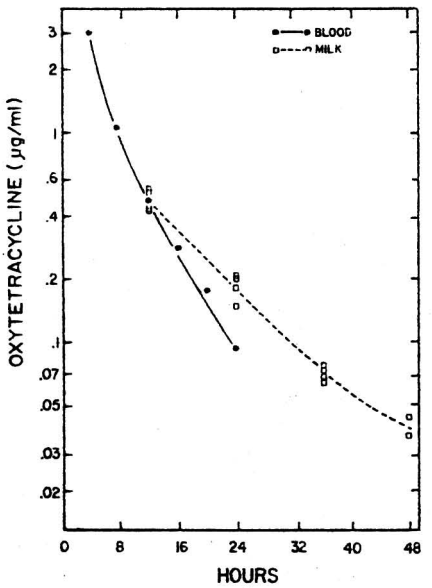

FIG. 4—Disappearance of oxytetracycline from blood and milk after intravenous injection.

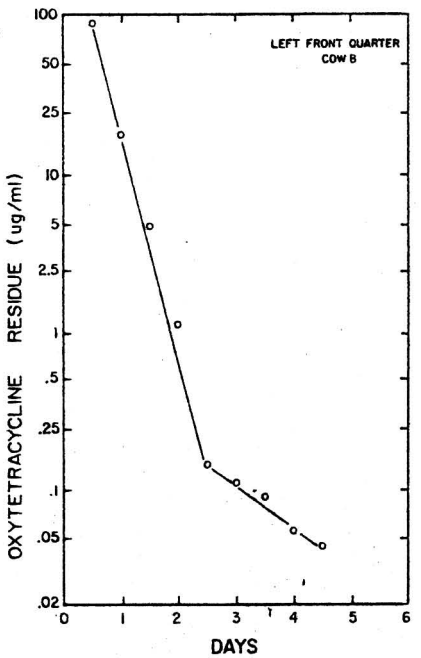

FIG. 5—Disappearance of oxytetracycline from milk of left front quarter after intramammary infusion of cow B.

across the membranes. Randall *et al.* (10) reported that chlortetracycline could be transferred to the milk of untreated quarters but that oxytetracycline transfer could not be detected. This observation is undoubtedly a case where analytical sensitivity was the major factor, since chlortetracycline is more easily detected and measured than oxytetracycline by at least a factor of 3. Baumgartner and Mueller (11) reported the presence of tetracycline residues in milk after intramuscular injection. This report indicated that the circulatory system contributed significantly to the distribution of antibiotics in the milk.

To insure that the observed phenomena (antibiotic residues persisting longer than expected in the milk after mammary infusion and low level residues appearing in the milk from untreated quarters) was not unique to this test animal, the experiment was repeated, using an additional lactating cow. Figure 5 and Table 2 show a profile similar to the results obtained with cow A. Measurable residues persisted in the treated quarter for 4.5 days with the trace designation continuing 1 additional day. Measurable residues in the untreated quarters appeared for 3.5 days.

From these experiments, it should be noted that residues of antibiotics can occur from a prescribed usage. If these data are reviewed in light of the sensitivity of 0.10–0.12-μg oxytetracycline/ml that these investigators could achieve with the FDA suggested procedure (4), residues would have disappeared in about 3.5 days. Although this does not allow for a full additional 24 hr safety period, it is in reasonable agreement with the 4 day withdrawal period. In light of the sensitivity of the FDA suggested procedure, the original requirements related to the intramammary infusion are not incorrect. However, based on the data from these studies and accepting a 5 day average for residues to disappear, a 6 day withdrawal interval is in order; based on the longest time measurable residues were found (5.5 days) a 6.5 day withdrawal period is in order. These withdrawal time suggestions are based upon the results found from within these studies and should be interpreted in light of the very limited number of animals used. Obviously, a larger number of animals should be studied and recommendations made from these larger studies.

Table 1. Disappearance of oxytetracycline residues from treated and untreated quarters[a]

Days after treatment	Oxytetracycline in milk from treated rear quarters, μg/ml							
	R.R.[b]	L.R.	R.F.	L.F.	R.R.	L.R.[b]	R.F.	L.F.
0.0	N.D.[c]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
0.5	58.20	0.054	0.067	0.058	0.054	54.00	0.054	0.125
1.0	15.12	0.047	0.040	0.040	0.054	6.10	0.057	0.079
1.5	2.20	T	T	T	T	1.13	T	0.065
2.0	0.90	T	T	T	N.D.	0.15	N.D.	N.D.
2.5	0.54	0.061	0.04	T	N.D.	0.083	N.D.	N.D.
3.0	0.25	T	T	T	N.D.	0.072	N.D.	N.D.
3.5	0.14	T	N.D.	N.D.	N.D.	0.050	N.D.	N.D.
4.0	0.10	T	N.D.	N.D.	N.D.	T	N.D.	N.D.
4.5	0.04	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5.0	T[d]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Oxytetracycline in milk from front quarters, μg/ml							
	R.R.	L.R.	R.F.[b]	L.F.	R.R.	L.R.	R.F.	L.F.[b]
0.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
0.5	0.061	0.050	115.00	0.050	0.072	T	0.065	97.00
1.0	0.046	0.048	28.80	T	T	0.054	0.054	15.30
1.5	T	T	9.90	N.D.	0.043	0.043	0.045	4.50
2.0	N.D.	N.D.	2.10	N.D.	T	T	T	1.30
2.5	N.D.	N.D.	0.90	N.D.	N.D.	N.D.	N.D.	0.54
3.0	N.D.	N.D.	0.40	N.D.	N.D.	N.D.	N.D.	0.23
3.5	N.D.	N.D.	0.27	N.D.	N.D.	N.D.	N.D.	0.15
4.0	N.D.	N.D.	0.12	N.D.	N.D.	N.D.	N.D.	0.070
4.5	N.D.	N.D.	0.068	N.D.	N.D.	N.D.	N.D.	0.060
5.0	N.D.	N.D.	0.052	N.D.	N.D.	N.D.	N.D.	0.046
5.5	N.D.	N.D.	0.040	N.D.	N.D.	N.D.	N.D.	T
6.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
6.5	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
7.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a] R.R. = Right Rear Quarter; L.R. = Left Rear Quarter; R.F. = Right Front Quarter; L.F. = Left Front Quarter.
[b] Treated quarter.
[c] N.D. = Not Detected; zone approximately 8.5 mm in diameter.
[d] T = Trace; zones 9.0–9.5 mm in diameter.

Table 2. Disappearance of oxytetracycline residues from treated and untreated quarters[a]

Days after treatment	Oxytetracycline in milk from front quarter, μg/ml			
	R.R.	R.F.	L.R.	L.F.[b]
0.0	N.D.[c]	N.D.	N.D.	N.D.
0.5	0.046	0.043	0.046	97.50
1.0	0.043	0.047	0.043	18.80
1.5	0.041	0.045	0.041	4.90
2.0	0.046	0.058	0.052	1.15
2.5	0.049	0.045	0.051	0.15
3.0	0.051	0.049	0.049	0.12
3.5	0.045	0.046	0.046	0.095
4.0	T[d]	T	0.045	0.057
4.5	N.D.	N.D.	T	0.046
5.0	N.D.	N.D.	N.D.	T
5.5	N.D.	N.D.	N.D.	T
6.0	N.D.	N.D.	N.D.	N.D.
6.5	N.D.	N.D.	N.D.	N.D.

[a–d] See footnotes, Table 1.

However, the original premise concerning the possibilities of unwanted residues from prescribed use is valid. Whether or not the residues of the magnitude found in these studies are a potentially serious health hazard is difficult to say unequivocally. Nonetheless, it is apparent that a review of current usages is reasonable to assure minimal exposure of the public to antibiotic residues.

In addition to the health ramifications, oxytetracycline residues in the range 0.05–0.10 μg/ml were shown by Feagan (12) to cause a 30% reduction in acid production in various cheese starters. Cogan (13) noted that residues in milk from mastitis therapy may be an important cause of delayed lactic acid production during cheese and yogurt manufacture.

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Acknowledgment

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This paper was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Pharmacognosy Meeting

The American Society of Pharmacognosy and the Pharmacognosy and Natural Products Section of the Academy of Pharmaceutical Sciences announce a joint meeting to be held July 15–20, 1973 at the Atlantic Carriage Inn, Jekyll Island, Ga. Three days will be devoted to a Symposium on Biotransformations and Fermentations.

The *deadline* for submission of abstracts of original research for presentation at the scientific sessions of this meeting is June 1, 1973. For further information write to Dr. S. J. Stohs, College of Pharmacy, University of Nebraska, Lincoln, Neb. 68508.

PESTICIDE RESIDUES

Gas Chromatographic Determination of Residues of Methylcarbamate Insecticides in Crops as Their 2,4-Dinitrophenyl Ether Derivatives

EDWARD R. HOLDEN
Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. 20705

A general procedure was developed for determining methylcarbamates in plant materials. The residue is extracted from crop material with acetonitrile, and the extractive is purified by partitioning with petroleum ether and coagulating with phosphoric acid-ammonium chloride solution. Phenolic impurities are largely eliminated by partitioning a methylene chloride extract with 0.1*N* potassium hydroxide. Carbamate residues are then treated with 1-fluoro-2,4-dinitrobenzene to form the ether derivative without a prior hydrolysis step. Efficiency in the conversion of the phenolic moieties to the phenyl ethers is essentially 100%, thus providing for good reproducibility. Residues may be determined at levels as low as 0.05 ppm. Recoveries generally range between 90 and 110%.

A multiresidue method for determining carbamate pesticides is needed that can be incorporated into the existing system for determining chlorinated and phosphated pesticides (1). With capability extended to the carbamates, a considerable number of all 3 general classes of organic pesticides might be determined by the use of aliquots of an acetonitrile extract from a single crop sample.

The present paper describes a multiresidue method based on the conversion of the carbamates to their 2,4-dinitrophenyl ether derivatives for determination by electron capture gas chromatography. The method has greater specificity than that of the earlier 2,4-dinitroaniline method (2). Moreover, less work is required, and no significant interference is introduced by impurities.

METHOD

Reagents

(a) *Borax.*—5% aqueous solution.

(b) *Celite 545.*—Johns-Manville Co. Wash thoroughly with acetone, and dry 2 hr at 110°C.

(c) *Coagulating solution.*—Dissolve 20 g NH_4Cl and 40 ml H_3PO_4 (85%) in 360 ml water to prepare stock solution. Dilute 100 ml stock solution to 1 L for coagulation.

(d) *1-Fluoro-2,4-dinitrobenzene.*—Eastman Kodak Co. Redistill at 128°C and 1 mm pressure. Dissolve 1.5 ml in 25 ml acetone.

(e) *Pesticides.*—Best quality obtainable from manufacturers, analytical grades when available. Compounds tested are identified by chemical name in Table 1.

(f) *Potassium hydroxide solution.*—0.5*N* aqueous solution.

(g) *Sodium chloride solution.*—Saturated aqueous solution.

(h) *Solvents.*—Acetone, CH_2Cl_2, and isooctane (all redistilled in glass); acetonitrile (pesticide grade, Matheson, Coleman & Bell); petroleum ether (technical grade, redistill over dispersed sodium); acetophenone and methanol (analytical grade).

Gas Chromatographic Apparatus

A Packard Model 802 gas chromatograph equipped with tritium electron capture detector was used. The 18″ × ¼″ od glass column contained 10% DC-200 (12,500 cst) on 60–70 mesh Anakrom ABS (Analabs, Hamden, Conn.). Porous Teflon end plugs for ¼″ od glass tubing (Chemical Research Services, Inc., Addison, Ill.) are preferable, but glass wool can be used at outlet and omitted at inlet if necessary. (Glass wool at inlet tends to adsorb derivatives gradually and to release them later, giving rise to "ghost images" of the compounds.) Equilibrate column 2 days at 250°C and 2 weeks at 212°C. Operating conditions: column 212°C, detector 218°C, standby temperatures 190 and 200°C, respectively; nitrogen carrier gas 60 ml/min; sensitivity setting 1×10^{-9} amp full scale; and detector potential either 25 or 50 v, depending on response level needed (⅓ to ⅔ full scale peak height with injections equivalent to 4 ng carbamate).

Extraction of Pesticides

Place 100 g sample and 200 ml acetonitrile in square screw-top jar, and macerate in blender operated 2 min at moderate speed. Filter with suction into 500 ml round-bottom flask through rapid paper in 11 cm Büchner funnel. Transfer aliquot equiva-

Table 1. Chemical identity of pesticides studied

Common name	Chemical name
Aminocarb	4-(dimethylamino)-*m*-tolyl methylcarbamate
Bay 32651	4-(methylthio)-*m*-tolyl methylcarbamate
Bay 78537	2,3-dihydro-2,2-dimethyl-7-benzofuranyl acetylmethylcarbamate
Bux®:	
Major isomer[a]	*m*-(1-methylbutyl)phenyl methylcarbamate
Minor isomer	*m*-(1-ethylpropyl)phenyl methylcarbamate
Carbanolate	6-chloro-3,4-xylyl methylcarbamate
Carbaryl	1-naphthyl methylcarbamate
Carbofuran	2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate
Ciba C-9643	o-(4-methyl-1,3-dioxolan-2-yl) phenyl methylcarbamate
Decarbofuran (Bay 62863)	2,3-dihydro-2-methyl-7-benzofuranyl methylcarbamate
Hercules 5727	*m*-cumenyl methylcarbamate
Hercules 9007	*m*-cumenyl (chloroacetyl)methylcarbamate
Landrin®:	
Major isomer[b]	3,4,5-trimethylphenyl methylcarbamate
Minor isomer	2,3,5-trimethylphenyl methylcarbamate
Mesurol® (Bay 37344)	4-(methylthio)-3,5-xylyl methylcarbamate
Mexacarbate (Zectran®)	4-(dimethylamino)-3,5-xylyl methylcarbamate
Mobam	benzo[*b*]thien-4-yl methylcarbamate
Promecarb	*m*-cym-5-yl methylcarbamate
Propoxur	o-isopropoxyphenyl methylcarbamate

[a] Ratio between major and minor active isomers in Bux is 3:1, which in total represent 65% of the product.
[b] Ratio between major and minor active isomers in Landrin is 4:1.

lent to 40 g crop, e.g., 112 ml filtrate of crop containing 85% water[1], to 250 ml separatory funnel, and shake sample 10 sec with 25 ml saturated NaCl solution. Drain and discard aqueous phase. Repeat once with fresh NaCl solution. Add 100 ml petroleum ether, and shake 30 sec. Drain acetonitrile phase into 1 L separatory funnel. Strip petroleum ether phase by shaking 20 sec with 50 and then with 10 ml acetonitrile, draining each into the 1 L separatory funnel. Add 300 ml water, 25 ml saturated NaCl solution, and 50 ml methanol. Extract mixture with 100 and with two 25 ml portions CH_2Cl_2, shaking each 20 sec, and drain lower layer into 500 ml round-bottom flask. Add 2 drops acetophenone, and evaporate in rotary evaporator connected to water aspirator pump. During evaporation, keep water bath within 40–50°C range and remove flask from water bath when extract volume has been reduced to few ml, so that final evaporation to dryness takes place at low temperature.

Add 5 ml acetone, and swirl flask to dissolve residue. Add 50 ml coagulating solution, mix by swirling, add 1–2 g Celite, and swirl again for few moments. Pour solution into 150 ml suction filter of medium porosity packed with ¼" Celite, and collect filtrate in 500 ml round-bottom flask. Break vacuum immediately after liquid is drawn into Celite layer. Rinse sides of flask with 5 ml acetone, swirl, and repeat coagulation procedure. Rinse flask with 20 ml coagulating solution, and add rinse to filter just after liquid of second coagulation is drawn into Celite layer. After filtration is complete (ca 5 min), transfer filtrate to 250 ml separatory funnel. Extract carbamates by shaking 20 sec with three 25 ml portions CH_2Cl_2, rinsing filter flask with each portion before adding to separatory funnel. Drain CH_2Cl_2 (lower) extract into another 250 ml separatory funnel. Add 40 ml water and 10 ml 0.5*N* KOH, mix briefly by gentle swirling, and shake 20 sec. Drain CH_2Cl_2 through granular anhydrous Na_2SO_4 supported by glass wool in filter funnel, and collect filtrate in 250 ml Erlenmeyer flask. Add 10 ml CH_2Cl_2 to separatory funnel, swirl gently, and drain organic phase. Repeat once. Rinse filter with two 10 ml portions CH_2Cl_2. Add 2 drops acetophenone, and evaporate with same technique used in first evaporation.

Determination

Add 100 ml water, 2 ml 0.5*N* KOH, and 1 ml 1-fluoro-2,4-dinitrobenzene solution. Stopper, and mix 20 min at high speed on mechanical agitator. Add 10 ml 5% borax, swirl to mix, and heat on steam bath 20 min. Cool to room temperature by placing flasks in shallow water bath for 10 min. Add 5 ml isooctane, stopper, shake at high speed 3 min, and pour into 250 ml separatory funnel. Drain aqueous phase, and rinse twice with water. Drain isooctane solution into glass-stoppered test tube, and inject 10 µl sample into gas chromatograph. If necessary, dilute sample and rechromatograph. Recovery, ppm = ng from standard curve × 0.0125 × dilution factor.

[1] g crop = g sample extracted × ml aliquot/total volume. Total volume = ml water in sample + acetonitrile added −5 ml correction for volume contraction (3).

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

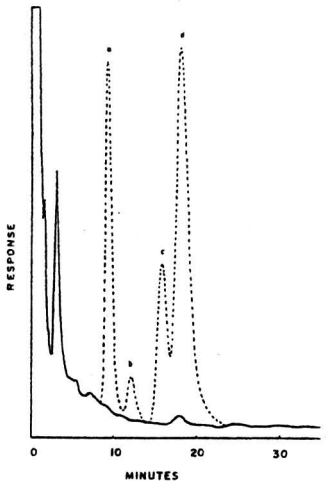

FIG. 1—Response to carbamates recovered from kale at 0.05 ppm (broken lines) superimposed on chromatogram from unfortified kale sample (solid lines). a, propoxur; b, Landrin, minor isomer; c, Landrin, major isomer; d, carbanolate.

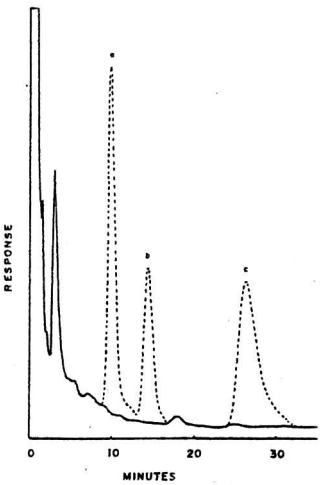

FIG. 2—See Fig. 1 caption. a, Hercules 5727; b, Bay 78537; c, mobam.

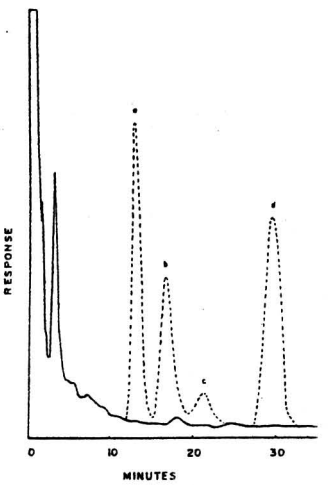

FIG. 3—See Fig. 1 caption. a, promecarb; b, Bux, major isomer; c, Bux, minor isomer; d, Mesurol.

Preparation of Standard Curves

Dissolve 50 mg of each carbamate in 100 ml benzene and store in brown bottles. Combine 5 ml aliquots from these solutions in groups as shown in Figs. 1–5, dilute to 50 ml with benzene, and store in brown bottles. Transfer 50 μl of each group to 250 ml Erlenmeyer flask, and derivatize as under *Determination*. After extraction of derivatives, solutions will contain the equivalent of 0.5 ng each carbamate/μl. Chromatograph 4, 6, 8, and 10 μl and plot mm response versus ng carbamate.

This paper reports the results of research only. Mention of an insecticide or proprietary product in this paper does not constitute a recommendation or an endorsement of this product by the U.S. Department of Agriculture.

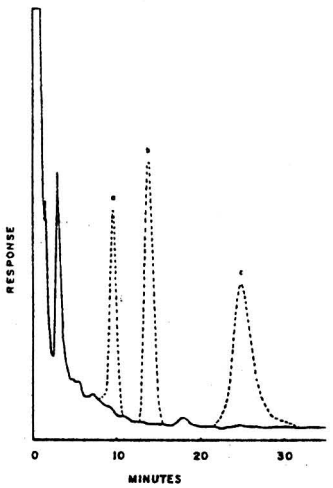

FIG. 4—See Fig. 1 caption. a, Hercules 9007; b, carbofuran; c, carbaryl.

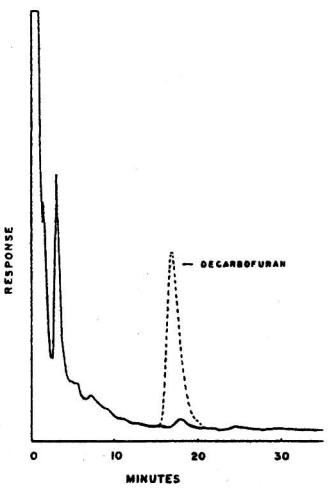

FIG. 5—See Fig. 1 caption.

Table 2. Recoveries of 13 carbamate insecticides from kale fortified at levels indicated

Insecticide	% Recovery at: 0.2 ppm[a]	0.05 ppm
Bay 78537	91	98
Bux:		
Minor isomer	70	80
Major isomer	89	88
Carbanolate	99	102
Carbaryl	97	107
Carbofuran	98	103
Decarbofuran	88	105
Hercules 5727	90	94
Hercules 9007	87	76
Landrin:		
Minor isomer	95	94
Major isomer	98	100
Mesurol	100	103
Mobam	92	108
Promecarb	95	97
Propoxur	95	100

[a] In these determinations, the solvent was evaporated without the aid of a keeper to reduce pesticide losses.

Results

The response to carbamate residues is illustrated in Figs. 1–5. Residues were extracted from kale fortified at 0.05 ppm. As shown by the blank determination (solid lines), the background was negligible.

Recoveries of 13 carbamates (including those of each active isomer of Bux and Landrin) from kale are listed in Table 2. The values at the 0.2 ppm level, although generally acceptable, were probably lowered somewhat by small losses in the evaporative steps. Such losses became more evident at the 0.05 ppm level, and the use of a keeper (acetophenone) was instituted to minimize volatilization. Recoveries of 4 carbamate pesticides (including those of both isomers of Landrin) from 9 vegetable crops fortified at 0.05 ppm are given in Table 3. Average recovery for the 45 values was 102.5%, range, 88 to 115%.

Discussion

The method described is generally suitable for the determination of aromatic methylcarbamates that do not contain an amino group. The only exception found in this category was Ciba C-9643, which was not recovered in the cleanup procedure. The phenol of Bay 32651 was found to form the phenyl ether derivative, but the parent carbamate was not available for recovery tests. Carbamates containing a *p*-dimethylamino group, notably aminocarb and mexacarbate, were not recovered in cleanup. Dimetilan, dimetan, and Pyrolan (dimethylcarbamates) and aldicarb (an oxime) did not form satisfactory derivatives.

The 2,4-dinitrophenyl ether derivatives of carbamates are stable compounds with good chromatographic characteristics. At the relatively high column temperature required in gas chromatography, impurities tend to elute well in

Table 3. Recoveries of 4 insecticides from various vegetable crops fortified at 0.05 ppm

Crop	Recovery, %				
	Propoxur	Landrin, minor isomer	Landrin, major isomer	Carbanolate	Carbaryl
Asparagus	102	103	103	95	97
Carrots	102	88	107	101	100
Cucumbers	108	104	104	104	106
Eggplant	104	92	102	99	102
Green beans	108	96	115	115	95
Lettuce	94	96	93	95	100
Spinach	108	108	108	105	109
Squash	108	100	103	104	106
Tomatoes	106	98	104	105	112

advance of the carbamate range, thus minimizing interference. Solvents and reagents, when available in analytical grades, required no further purification. At levels as low as 0.05 ppm, background was negligible in all recovery tests, and no blank determinations were needed for data correction.

Recommendation

The method described has been constructed within the framework of the AOAC objectives for multiresidue determinations, and it appears to be well suited to such purpose. It is therefore recommended that the method be studied collaboratively.

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee E and was accepted by the Association; *see* (1973) *JAOAC* 56, 404–405.

Factors Relating to Completeness of Solvent Extraction of Dieldrin from Milk

CATHARINA Y. W. ANG[1] and LEROY R. DUGAN, JR.
Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Mich. 48823

Milk containing added dieldrin was separated into 2 lipid fractions by differential solvent extraction. Fraction 1 was extracted with nonpolar hydrocarbon solvents and Fraction 2 with the same solvent system after mixing with sodium oxalate and ethanol. Three solvent systems were compared. Dieldrin concentrations in Fraction 1 of all 3 solvent systems were much higher than in Fraction 2 on a fat basis. Less than 50% of the dieldrin was recovered in Fraction 1, probably because of the low recovery of hydrocarbon solvents from fluid milk or factors that depressed the separation of solvents from milk. Factors possibly involved were the adsorption of solvents and pesticide to the hydrophobic groups of membrane proteins and/or serum proteins or the penetration of solvents and pesticide into the fat globules of milk.

The extraction from milk of chlorinated hydrocarbon pesticide residues and lipids usually requires the use of alcohol or other agents to disrupt the fat globule membrane before extraction with hydrocarbon solvents. Beroza and Bowman (1) reported that several nonpolar pesticides added to milk were also poorly recovered by a solvent mixture of hexane and ethyl ether. In the present study the problem of solvent extraction of dieldrin from milk was investigated further.

Milk fortified with a known amount of dieldrin was extracted with nonpolar hydrocarbon solvents. The residual portion was mixed with ethanol and sodium oxalate before further extraction with the same solvent system. The amounts of lipids and the concentrations of dieldrin in these fractions were determined. Three solvent systems were compared.

Experimental

Reagents

(a) *Solvents.*—Ethyl ether, hexane, and petroleum ether, nanograde; acetone, spectrophotometric grade; ethanol, 200 proof, USP grade.

(b) *Dieldrin.*—99% HEOD (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo* - *exo* - 5,8 - dimethanonaphthalene); Applied Science Laboratories.

Apparatus

Gas chromatograph.—A Beckman GC-4 gas chromatograph equipped with electron capture detector was used. *Column:* 4′ × ¼″ od packed with 11% of a mixture of QF-1 and OV-17 (3+1) on 60–80 mesh Gas-Chrom Q. Column temperature, 220°C; carrier gas, helium; flow rate, 30 ml/min. The peak heights of dieldrin in samples were compared standards for quantitative evaluations.

Procedure

One ml acetone solution containing 500 mg dieldrin was added dropwise to 100 ml commercial pasteurized homogenized milk with stirring (at a 5 ppm level on a weight basis). The mixture was allowed to equilibrate for 45 min at room temperature with occasional stirring.

Three solvent systems were compared for extraction. System 1 was hexane-ethyl ether (1+1, v/v) (1). One hundred ml milk was mixed thoroughly with 100 ml of this solvent mixture in a separatory funnel. The milk and solvent mixture was then transferred to a centrifuge bottle and centrifuged at 2000 × *g* for 15 min. The separated solvent layer was removed with a pipet and the remaining portion was re-extracted 2 more times with the same solvent system. The solvent extracts were combined, the volume was measured, the extract was concentrated under a stream of nitrogen gas on a steam bath, and the resulting lipids were weighed.

The second lipid fraction was obtained from the residual portion of milk by adding 100 ml ethanol and 1 g sodium oxalate and then extracting 3 times with the same solvent mixture. The combined extracts were washed with 100 ml 5% NaCl solution and dried over a column of anhydrous Na_2SO_4 (2.5 cm). The volume of this combined solution was measured before concentration.

Solvent systems 2 and 3 were petroleum ether and ethyl ether-petroleum ether (1+1, v/v), respectively. The amounts of solvents used and the techniques involved were those referred to in the FDA *Pesticide Analytical Manual* (2), except that lipid Fraction 1 was extracted with nonpolar hydrocarbon

[1] Present address: Food Science Associates, Inc., Dobbs Ferry, N.Y. 10522.

solvents only, and Fraction 2 was extracted from the residual portion of milk as described above.

The extracted lipids of each fraction were cleaned up by a Florisil-Celite column technique described by the Shell Chemical Company (3) and dieldrin was determined by gas chromatography.

Results and Discussion

Results in Table 1 show that only a small percentage of the milk lipids was obtained in Fractions 1 by any of the 3 solvent systems used. Slightly higher recovery of lipids was provided by solvent system 3. Fractions 1 contained relatively greater concentrations of dieldrin on a lipid basis than Fractions 2 did. It is thus apparent that added dieldrin was more readily extracted with hydrocarbon solvents than were the milk lipids. However, all residue was not extracted unless all lipids were extracted, as indicated in Table 1, since the total recoveries of dieldrin in Fractions 1 were only 40.6–47.4%.

The present results on the recoveries of lipid Fractions 1 from milk were somewhat lower than those reported by Beroza and Bowman (1) with solvent system 1. However, the recoveries of dieldrin with the nonpolar hydrocarbon solvents were higher than Beroza and Bowman indicated. The concentration of dieldrin in the total extracted lipids was higher with solvent system 1 than with the other 2 systems. The total amounts of lipids extracted by solvent system 1 were very low; therefore the total recovery of dieldrin was still lower by system 1 than by the other 2 solvent systems. Lawrence and Burke (4) also observed that the residue levels tended to be higher on a fat basis when only a portion of the total fat was extracted from milk and analyzed.

Table 1. Distribution of lipid and dieldrin in solvent-separated milk fractions[a]

Solv. system[b]	Fraction no.[c]	% Lipid of milk	Dieldrin concn in lipid, ppm	% Rec. of dieldrin	% Approx. rec. of solv.
1	1	0.11	1878.0	40.6	20
	2	2.56	97.5	47.6	100
	Total	2.67	168.3	88.2	
2	1	0.09	2353.0	43.4	20
	2	3.17	87.7	52.0	100
	Total	3.26	151.8	95.4	
3	1	0.84	285.5	47.4	43
	2	2.45	91.8	45.0	100
	Total	3.29	141.3	92.4	

[a] Commercial pasteurized homogenized milk fortified with 5 ppm dieldrin on a weight basis.

[b] Solvent system 1, hexane-ethyl ether; system 2, petroleum ether; system 3, ethyl ether-petroleum ether.

[c] Ether-extractable lipids and alcoholic ether-extractable lipids for the first and second fractions, respectively.

The less than 100% total recovery of dieldrin might be due to the cleanup and evaporation, and only a small percentage, if any, of the loss would be due to inefficient extraction. This study supports the concept that the distribution of dieldrin in lipid fractions extracted differently would be variable and it is very important to extract all of the lipids from milk if the subsequent residue analysis is to be based on the isolated lipid fraction.

Since butterfat is enclosed within the fat globule membrane, milk fat as well as physiologically incorporated lipophilic pesticide cannot be totally extracted by hydrocarbon solvents unless the fat globule membrane is disrupted with ethanol or other agents. Beroza and Bowman (1) found that the recovery of added nonpolar pesticides with the solvent mixture of hexane-ethyl ether was very low unless alcohol was used. They thought that the low recovery was due to the adsorption of pesticides onto the fat globules rather than to their penetration into the fat body. They also stated that the binding of nonpolar residues with the aqueous phase appeared to be important when the cream was removed. However, they did not report the recovery of the nonpolar solvents used.

It was observed during the course of this study that the nonpolar hydrocarbon solvents or the combined solvents were very difficult to separate completely from the fluid milk once they were mixed. Approximate recoveries of the solvents were 20–43% as shown in Table 1. Separations of the solvents from the skim milk phase were also poor. On the other hand, when ethanol and oxalate were added, recoveries of added solvents improved significantly. The solvent volume collected this time accounted for approximately all the solvent added for the second extraction and the remaining solvent from the first extraction. This finding implied that the low recovery of dieldrin, and possibly milk fat as well, might be due in part to low recovery of solvents, or to

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factors which depressed the recovery of solvents from milk, e.g., adsorption of solvents and pesticide onto the hydrophobic groups of the membrane proteins and/or serum proteins. Alcohol is needed to break up the emulsion between the solvents and the milk components, as well as to disrupt the fat globule membrane.

It is also possible that the added nonpolar hydrocarbon pesticide and nonpolar solvents have penetrated into the fat globules, as suggested by the concept of Swope (5) that the milk fat globule membrane appeared to be composed of aggregates of particles instead of intact layers. Another study conducted in this laboratory showed that most of the added dieldrin was recovered in the butteroil fraction after separation (6); this recovery could be due to either the penetration of pesticide through the fat globule membrane or an altered deposition during the separation processes.

Numerous factors may be involved in the distribution and recovery of pesticides in the milk system. The roles of adsorption, mutual solubility, solvent release, and other factors are yet to be fully assessed.

Acknowledgments

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Determination of Several Chlorinated Pesticides by the AOAC Multiresidue Method with Additional Quantitation of Perthane after Dehydrochlorination: Collaborative Study

RICHARD T. KRAUSE[1]
Food and Drug Administration, 240 Hennepin Ave., Minneapolis, Minn. 55401

The AOAC multiresidue method for chlorinated pesticides, 29.001–29.017, was studied collaboratively by 7 laboratories. Perthane, heptachlor epoxide, mirex, and dieldrin were determined in apple and cauliflower samples fortified at 2 levels, and Perthane was determined in butterfat samples fortified at 2 levels. Perthane was quantitatively measured before and after a simplified dehydrochlorination procedure. Average recoveries and coefficients of variations for pesticides in the apple samples for both levels were: Perthane 100±10%, heptachlor epoxide 102±14%, mirex 96±9%, dieldrin 101±14%, and Perthane (after dehydrochlorination and recalculation to the parent compound) 100±9%. Average recoveries and coefficients of variation for pesticides in the cauliflower samples for both levels were: Perthane 105±9%, heptachlor epoxide 103±15%, mirex 103±7%, dieldrin 98±11%, and Perthane (after dehydrochlorination and recalculation) 100±10%. The average recovery for both levels of Perthane (after dehydrochlorination and recalculation) in the butterfat samples was 91±10%. For a spinach sample with field-incurred residues the collaborators found an average of 4.47 ppm Perthane before dehydrochlorination and 5.09 ppm Perthane as the olefin after dehydrochlorination. In most instances, the identities of all the residues were confirmed by TLC. The dehydrochlorination technique has been adopted as official first action.

A collaborative study for Perthane, heptachlor epoxide, mirex, and dieldrin residues was conducted, using the AOAC method for multiple pesticide residues, 29.001–29.017 (1). The study also included the quantitative measurement of Perthane as the olefin after dehydrochlorination by the method previously described (2). The AOAC method utilizes the extraction and Florisil cleanup procedures reported by Mills *et al.* (3), with quantitative determination of the residues by gas-liquid chromatography (GLC) (4) and qualitative confirmation by thin layer chromatography (TLC) (5). The AOAC multiresidue method has previously been collaboratively studied with a number of pesticides (1). This paper extends collaborative data on the method to include Perthane, heptachlor epoxide, mirex, and dieldrin in nonfatty foods and Perthane in fatty foods. The 6% eluates of the various samples were also subjected to the dehydrochlorination procedure (2) to enable quantitation of Perthane as the olefin to which the electron capture GLC detector is more sensitive.

Instructions to Collaborators

The collaborators were instructed to subject 17 μg Perthane to the dehydrochlorination procedure (2) and to calculate the per cent of theoretical yield of the Perthane olefin which they obtained. This familiarized the collaborators with the dehydrochlorination procedure and provided data on the conversion of Perthane to its olefin. The collaborators were next instructed to analyze the various products which were provided. Each collaborator was sent one homogenized sample each of apples and cauliflower, a sample of canned spinach, and 3 samples of butterfat. Four ampoules containing acetonitrile solutions of the 4 pesticides were provided for fortifying the apple and cauliflower samples. The canned spinach contained field-incurred residues of Perthane and 2 of the butterfat samples had been previously fortified with 3.00 and 0.54 ppm Perthane. The collaborators were instructed to analyze a portion of both the apple and the cauliflower samples "as is" for a crop blank and to fortify two 100 g portions of each with the designated solution. The levels of pesticides in the fortified samples are given in Table 1. The tolerance levels for Perthane, heptachlor epoxide, mirex, and dieldrin in or on apples or cauliflower are 15, 0,

[1] Present address: Division of Chemistry and Physics, Food and Drug Administration, Washington, D.C. 20204.

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Table 1. Pesticide levels (ppm) in fortified collaborative samples

Sample	Perthane	Hept. Epoxide	Mirex	Dieldrin
Apple, A	17.0	0.150	0.121	0.150
Apple, B	1.00	0.0540	0.0452	0.0722
Cauliflower, A	15.0	0.120	0.0905	0.120
Cauliflower, B	0.700	0.0540	0.0452	0.0540

0.01, and 0.1 ppm, respectively. The tolerance level for Perthane in meat and milk is 0. The 3 samples of butterfat and the canned spinach were to be analyzed "as is."

The collaborators were instructed to use the appropriate extraction and cleanup procedures specified in 29.009 for each product analyzed. Pesticides were to be quantitatively measured by electron capture GLC, using a 10% DC-200 column and conditions described in 29.017. If separation of pesticides found in the sample was inadequate, a 10% DC-200/15% QF-1 (6) and/or 3% DEGS column (2) could be used as previously described (2). Retention times of 46 pesticides with the DC-200, QF-1/DC-200, and DEGS liquid phases are given in reference 2.

Collaborators were to quantitate the pesticides found in the 6 and 15% mixed ether eluates, and then subject the 6% eluate to the dehydrochlorination procedure (2). They were then to reinject the sample into a gas chromatograph with a 10% DC-200 column and electron capture detector and, from the amount of Perthane olefin found, calculate the ppm Perthane originally in the sample.

Standard solutions were to be freshly prepared, using EPA/FDA Pesticide Reference Standards. The Perthane olefin standard used in this study was prepared and purified by the author. The final purity of the Perthane olefin standard was determined to be 99+% by GLC.

Collaborators were instructed to use TLC procedure 29.018 or 29.024 for identity confirmation of pesticides found in the fortified samples. They were given the option of not purifying the alumina when high purity grade alumina was available. The collaborators were also furnished with an adaptation of the TLC method of Fehringer and Westfall (7) for identity confirmation of the pesticide residues found in the canned spinach. The original procedure employed 2-dimensional TLC on silica gel with *n*-heptane as the mobile solvent for the first dimension, and 2% acetone in *n*-heptane for the second dimension. The procedure provided the collaborators is given below; chromatography is in one dimension only.

METHOD

Reagents

(a) *MN-Kieselgel G-HR.*—Adjusted for aflatoxin separation (Machery, Nagel & Co., distributed by Brinkmann Instrument Co., Cantiague Rd., Westbury, N.Y. 11590).

(b) *Chromogenic agent for chlorinated pesticides.*—*See* 29.003(c).

(c) *Developing solvents.*—(*1*) *n*-Heptane and (*2*) 1% acetone in *n*-heptane. Both solvents are "distilled in glass" (Burdick and Jackson Laboratories, Inc., Muskegon, Mich. 49442).

Apparatus

See 29.006.

Preparation of TLC Plates

Before coating, wash plates in hot soapy water and thoroughly rinse with distilled water. Prepare slurry of 30 g MN-kieselgel G-HR and 50 ml 0.2% silver nitrate, freshly prepared in water, and shake $\geq$30 sec to insure proper mixing. (Slurry is enough to spread 5 plates at thickness of 250 μm.) Dry plates 20 min at 80°C and store in desiccator cabinet. *Note:* Wash spreader immediately after use to prevent silver nitrate from reacting with metal spreader.

Spotting

Proceed as in 29.020. Spot 0.2 μg pesticide to be identified in sample as well as 0.2 μg standard and 0.2 μg *p,p'*-TDE to be used to calculate R_{TDE}. Proceed as in 29.021. Use 1% acetone in *n*-heptane for 6% eluate not subjected to dehydrochlorination. For exposure of plates, proceed as in 29.026.

Results and Discussion

Table 2 gives the results for the conversion of standard Perthane to its olefin by the dehydrochlorination procedure. The per cent of theoretical yield varied from 96 to 105, with an average of 100%.

Florisil chromatography used by Collaborators 1, 2, and 4–6 resulted in separation of Perthane, heptachlor epoxide, and mirex into the 6% eluate and dieldrin into the 15% eluate. Collaborator 3 did not achieve separation of the pesticides into the proper eluate. By using 150 ml 3% mixed ethers and 240 ml 15% mixed ethers he separated Perthane and mirex into the 3% eluate and dieldrin into the 15% eluate. Approximately 95% of the heptachlor epoxide was recovered in the

Table 2. Conversion of standard Perthane to its olefin by the dehydrochlorination procedure

Coll.	Trial	Per cent of theoretical yield
1	A	103
	B	100
2	A	100
	B	100
3	A	103
	B	105
4	A	96
	B	102
5	A	99
	B	100
6	A	99
	B	100
7	A	98
	B	96
Av.		100

3% eluate. The remainder was eluted with the 15% ethers. No separation of the pesticides was obtained by Collaborator 7. All pesticides eluted in the 6% eluate. This collaborator reported that the adsorptive capacity of his Florisil, based on the lauric acid value as described by Mills (8), was very low.

Collaborators 3 and 6 operated their gas chromatographs at 2 and 5 times, respectively, the sensitivity specified in 29.017(b), "at which 1 ng heptachlor epoxide produces recorder response of 40–50% full scale deflection." All other collaborators operated their instruments at the sensitivity directed.

All collaborators except Collaborator 7 used the 10% DC-200 column to determine those pesticides added to the apple, cauliflower, and butterfat samples. Collaborator 7 used a 10% DC-200/15% QF-1 column for all heptachlor epoxide and mirex determinations and a 10% QF-1 column for all dieldrin recoveries. He used a 3% DEGS column for the measurement of Perthane as its olefin. The parent Perthane could not be calculated by this collaborator for any of the GLC columns because of the interferences of heptachlor epoxide or dieldrin.

Collaborator 7 calculated the amount of pesticide found from a linearity curve determined on the day of the analysis. Collaborator 2 reported 4 results that were not used in the statistical analyses of this study. These results were probably affected by use in quantitation of sample and standard GLC peak heights which were widely different and which probably exceeded the linear range of response. All other collaborators had "residue and std peaks . . . of similar size" as stated in 29.017 "for most accurate measurement. . . ."

Four collaborators reported from 0.09 to 0.177 ppm dicalfal (Kelthane) in the apple crop blank. The other 3 collaborators also detected the dicalfal peak but did not identify it. Three collaborators reported a trace of DDE, 2 collaborators reported a trace of DDT, and one collaborator reported traces of dieldrin and the Perthane olefin. Another collaborator also reported a trace of heptachlor.

Fortification levels and recoveries of the pesticides added to the apple samples are given in Table 3. The average per cent recoveries for the 2 levels A and B, respectively, were Perthane 99 and 101, heptachlor epoxide 99 and 104, mirex 94 and 97, and dieldrin 101 and 101. The amount of Perthane olefin found after dehydrochlorination was calculated to the parent compound with average per cent recoveries of 101 and 100, respectively, obtained for the 2 levels.

Some low level residues were reported in the cauliflower crop blank. Three collaborators reported trace to 0.006 ppm dieldrin, 2 collaborators reported a trace of endrin, one collaborator reported traces of DDE and TDE, and one collaborator reported a trace of TCNB. The dieldrin found in the crop blank was subtracted from the amount found in the fortified cauliflower samples.

The 2 fortification levels and the recoveries obtained for those pesticides added to the cauliflower samples are given in Table 4. The average per cent recoveries for the 2 levels were Perthane 106 and 104, heptachlor epoxide 100 and 106, mirex 99 and 107, and dieldrin 92 and 105. The amount of Perthane olefin found after dehydrochlorination was recalculated to the parent compound with average per cent recoveries of 99 and 101.

Two collaborators reported residues in the butterfat blank. One collaborator reported traces of heptachlor epoxide, *p,p'*-DDE, BHC, and dieldrin. A second collaborator reported 0.01 ppm heptachlor epoxide, 0.02 ppm *p,p'*-DDE, 0.018 ppm dieldrin, and traces of *p,p'*-DDT and Perthane (as the olefin).

Recoveries of Perthane added to the butterfat samples are given in Table 5, along with the 2

Table 3. Recovery (ppm) of pesticides added to apples, using the AOAC multiresidue method and additional Perthane quantitation as the olefin

	Perthane before DHC[a]		Perthane as olefin after DHC		Heptachlor epoxide		Mirex		Dieldrin	
Coll.	A	B	A	B	A	B	A	B	A	B
1	17.3	1.17	18.0	1.13	0.166	0.0663	0.114	0.0508	0.169	0.0795
2	15.4	0.897	15.2	0.808	0.121	0.0491	0.106	0.0368[b, c]	0.140	0.0716
3	16.1	0.943	16.8	0.972	0.136	0.0552	0.113	0.0420	0.135	0.0711
4	18.4	1.10	17.6	1.00	0.140	0.0555	0.118	0.0469	0.162	0.0620
5	19.0	0.951	17.7	1.03	0.174	0.0588	0.122	0.0367	0.155	0.0797
6	15.1	1.00	18.0	1.11	0.160	0.0636	0.110	0.0390	0.112	0.0641
7	[d]	[d]	17.0	0.977	0.137	0.0448	0.118	0.0480	0.183	0.0843
Added, ppm	17.0	1.00	17.0	1.00	0.150	0.0540	0.121	0.0452	0.150	0.0722
Av. rec., ppm	16.9	1.01	17.2	1.00	0.148	0.0562	0.114	0.0439	0.151	0.0732
Std dev.	1.6	0.10	1.0	0.11	0.019	0.0076	0.005	0.0055	0.024	0.0084
Av. rec., %	99	101	101	100	99	104	94	97	101	101
Coeff. of var., %	10	10	6	11	13	14	5	13	16	11

[a] DHC = dehydrochlorination.
[b] Results not included in statistical analysis; validity of quantitation was in question because of dissimilar sample and standard peak heights.
[c] Peak heights of 18 and 106 mm, respectively.
[d] Could not be calculated. Perthane and dieldrin eluted in 6% eluate.

Table 4. Recovery (ppm) of pesticides added to cauliflower, using the AOAC multiresidue method and additional Perthane quantitation as the olefin

	Perthane before DHC[a]		Perthane as olefin after DHC		Heptachlor epoxide		Mirex		Dieldrin	
Coll.	A	B	A	B	A	B	A	B	A	B
1	17.5	0.655	15.8	0.747	0.143	0.0597	0.0966	0.0496	0.127	0.0533
2	13.2	0.894[b, c]	12.8	0.633	0.0879	0.0439	0.0800	0.0380[b, d]	0.0981	0.0568
3	14.6	0.710	15.0	0.792	0.117	0.0579	0.0919	0.0459	0.110	0.0560
4	18.4	0.812	13.5	0.632	0.114	0.0537	0.0880	0.0544	0.100	0.0550
5	16.9	0.790	16.6	0.696	0.122	0.0629	0.0976	0.0497	0.0895	0.0597
6	15.0	0.678	16.0	0.795	0.135	0.0702	0.0876	0.0467	0.113	0.0360[e]
7	[f]	[f]	14.7	0.642	0.118	0.0531	0.0852	0.0453	0.141	0.0598
Added, ppm	15.0	0.700	15.0	0.700	0.120	0.0540	0.0905	0.0452	0.120	0.0542
Av. rec., ppm	15.9	0.729	14.9	0.705	0.120	0.0573	0.0896	0.0482	0.111	0.0568
Std dev.	2.0	0.069	1.4	0.073	0.018	0.0083	0.0063	0.0034	0.018	0.0026
Av. rec., %	106	104	99	101	100	106	99	107	92	105
Coeff. of var., %	12	9	9	10	15	14	7	7	16	5

[a, b] See footnotes, Table 3.
[c] Peak heights of 12 and 156 mm, respectively.
[d] Peak heights of 9 and 55 mm, respectively.
[e] Result is an outlier on the basis of the Q-test (9) and is not included in statistical analysis.
[f] Perthane could not be calculated because of interference from dieldrin or heptachlor epoxide.

fortification levels. Of those collaborators who made an initial GLC injection of butterfat representing 3 mg sample, only one reported a trace of Perthane at the high spiking level prior to dehydrochlorination. At a 5 mg injection level and a higher GLC sensitivity, Collaborator 3 was able to quantitate the unreacted Perthane. Perthane olefin found after dehydrochlorination, calculated as the parent compound, averaged 88 and 94% of the fortified 2 levels.

Collaborators 1–6 used TLC to confirm the identity of the pesticides reported in the spiked samples. Collaborator 7 did not attempt TLC confirmation. Collaborators 1–4 and 6 correctly identified Perthane, heptachlor epoxide, mirex, and dieldrin in each of the apple and cauliflower samples. They also identified the Perthane olefin after dehydrochlorination in the apple, cauliflower, and butterfat samples. Collaborators 3, 4, and 6 identified Perthane in the butterfat sample

before dehydrochlorination. Collaborators 1 and 4 used Camag D-5 alumina for TLC without prior purification. Collaborators 3 and 6 used alumina which they purified prior to coating their TLC plates. Collaborator 5 used commercially prepared alumina TLC plates for the fortified samples. Sensitivity with the commercial plates was not as good as with those prepared in the laboratories. Collaborator 5 was unable to identify Perthane in the fortified samples due to the poorer TLC sensitivity. However, he did identify heptachlor epoxide, mirex, dieldrin, and the Perthane olefin in the fortified apple and cauliflower samples and the Perthane olefin in the fortified butterfat samples. Collaborator 2 used silica gel adsorbent for the TLC confirmation of the pesticides in the fortified samples.

Table 5. Recovery (ppm) of Perthane from butterfat, using the AOAC multiresidue method and additional Perthane quantitation as the olefin

	Perthane before DHC[a]		Perthane as olefin after DHC	
Coll.	A	B	A	B
1	Trace[b]	None	2.87	0.530
2	None	None	2.39	0.342[c]
3	[d]	0.560[e]	[d]	0.527
4	None	None	3.13	0.485
5	None	None	2.18	0.466
6	2.40	Trace	2.84	0.310[f]
7	None	None	2.39	0.524
Added, ppm	3.00	0.540	3.00	0.540
Av. rec., ppm			2.63	0.506
Std dev.			0.37	0.029
Av. rec., %			88	94
Coeff. of var., %			14	6

[a] DHC = dehydrochlorination.
[b] Trace = peaks <3% full scale deflection.
[c] Result not included in statistical analysis; validity of quantitation was in question because of dissimilar sample and standard peak heights (30 and 163 mm, respectively.
[d] Sample lost.
[e] Larger amount of sample injected at a higher GLC sensitivity that than used by the other collaborators.
[f] Result is an outlier on the basis of the Q-test (9) and is not included in the statistical analysis.

The spinach sample with field-incurred residues was included in the collaborative study as representative of the type of sample that residue analysts may encounter in their daily work. This sample was chosen to provide information on the collaborators' utilization of the 3 GLC columns (10% DC-200, 10% DC-200/15% QF-1, and/or 3% DEGS) for determining qualitatively and quantitatively the residues in the sample. The sample was also used to obtain information on the practical use of the TLC procedure adapted from that of Fehringer and Westfall (7).

The GLC results obtained from the spinach sample are given in Table 6. All 7 collaborators found Perthane in the sample prior to dehydrochlorination. Collaborators 1, 3, 4, and 6 used the 3% DEGS column to measure Perthane. The average ppm Perthane found by Collaborators 1, 3, and 4 was 4.74 (range of 4.42 to 4.92). The results of Collaborator 6 were not used in deter-

Table 6. Pesticides (ppm) extracted from canned spinach containing field-incurred residues

	Residues found before dehydrochlorination					Residues found after dehydrochlorination		
Coll.	Perthane	*p,p'*-DDE	*p,p'*-TDE	*p,p'*-DDT	HCB	Perthane olefin[a]	*p,p'*-DDE	*p,p'*-TDE olefin[a]
1	4.42	0.026	0.039	Trace	Trace	5.45	0.028	0.047
2	6.05[b]	0.031	0.053			4.69	Not detd	Not detd
3	4.92	0.033	0.042	Trace		5.00	0.030	Not detd
4	4.89	0.025	0.035			4.89	Not detd	Not detd
5	[c]	0.032				5.66	0.026	
6[d]	6.80	0.042	0.070		Trace	7.48	Not detd	Not detd
7	[e]	0.024[f]	0.047			4.83	Not detd	Not detd
Av., ppm	4.74	0.029	0.043			5.09	0.028	
Std dev.[g]		0.0037	0.0070			0.38		

[a] Calculated back to parent compound.
[b] Result not used to calculate average because of interference from *p,p'*-DDE with use of 10% DC-200/15% QF-1 column.
[c] 10% DC-200 column used. Perthane was found but could not be measured quantitatively because of interference.
[d] Results not used to calculate averages because of possible loss of moisture from samples.
[e] 10% DC-200/15% QF-1 column used. Perthane was found but could not be measured quantitatively because of interference from *p,p'*-DDE.
[f] Result not used to calculate average because of interference from Perthane with use of 10% DC-200/15% QF-1 column.
[g] Calculated only for those pesticides with ≥5 valid values.

mining the average levels of Perthane or other residues reported. This collaborator lost the first 100 g portion of sample, but analyzed the remaining 35 g approximately 2 weeks later; the high results obtained are probably due to a loss of moisture from the second portion of sample. Collaborator 5 used the 10% DC-200 column and did not quantitate the Perthane in the sample due to electron capture response interference from *p,p'*-TDE. Collaborator 7 used the 10% DC-200/15% QF-1 column and did not quantitate Perthane in the sample due to interference from *p,p'*-DDE. Collaborator 2 also used the 10% DC-200/15% QF-1 column for Perthane and he reported 6.05 ppm Perthane, which includes some interference from *p,p'*-DDE.

After dehydrochlorination of the 6% eluate, all collaborators reported and quantitated the Perthane olefin found. The amount of Perthane olefin found was calculated back to ppm parent compound. Collaborators 1, 3, 6, and 7 used the 3% DEGS column and Collaborators 2, 4, and 5 used the 10% DC-200 column for the quantitative measurement of the Perthane olefin as the parent. Based on the results of Collaborators 1–5 and 7, the average amount of Perthane as the olefin was 5.09 ppm with a standard deviation of 0.38.

All 7 collaborators quantitated *p,p'*-DDE before dehydrochlorination. Collaborators 1, 2, and 5 used the 10% DC-200 column, and Collaborators 3, 4, and 6 used the 3% DEGS column. Collaborator 7 used the 10% DC-200/15% QF-1 column and encountered interference from Perthane. Based on the results obtained from Collaborators 1–5, the average *p,p'*-DDE found prior to dehydrochlorination was 0.029 ppm with a standard deviation of 0.0037. The data obtained for *p,p'*-DDE with the 10% DC-200 and 3% DEGS columns are comparable.

Only Collaborators 1, 3, and 5 quantitatively measured *p,p'*-DDE after dehydrochlorination. Collaborator 1 used the 10% DC-200/15% QF-1 column, Collaborator 3 used the 3% DEGS column, and Collaborator 5 used the 10% DC-200 column. The average *p,p'*-TDE was 0.028 ppm with a range of 0.026 to 0.030 ppm.

All collaborators except Collaborator 5 identified and quantitated *p,p'*-TDE in the spinach. Collaborators 1, 3, 4, and 6 used the 3% DEGS column, while Collaborators 2 and 7 used the 10% DC-200/15% QF-1 column. From the results obtained from Collaborators 1–4 and 7, the average amount of *p,p'*-TDE found was 0.043±0.0070 ppm. The data obtained for *p,p'*-TDE with the 3% DEGS and 10% DC-200/15% QF-1 columns are in fair agreement.

Only Collaborator 1 quantitated *p,p'*-TDE as the olefin after dehydrochlorination. He used the 10% DC-200/15% QF-1 column and reported 0.047 ppm *p,p'*-TDE.

Collaborators 1 and 3 reported a trace of *p,p'*-DDT, and Collaborators 1 and 6 reported a trace of hexachlorobenzene prior to dehydrochlorination.

Collaborators 1–4 and 6 identified Perthane and *p,p'*-DDE in the spinach sample, using the TLC procedure described. Collaborators 4 and 6 also identified *p,p'*-TDE by this procedure. Collaborator 5 used commercial silica gel plates but obtained poorer sensitivity. Perthane was not detected; however the Perthane olefin and *p,p'*-DDE were detected.

Comments of Collaborators

Collaborator 1 stated that the method showed the value of the dehydrochlorination reaction in confirming the initial result as well as increasing the sensitivity for Perthane.

Collaborator 2 used a 400 ml beaker containing a small amount of paraffin oil for the oil bath for the dehydrochlorination reaction. The reaction tube was placed in the beaker and, because of insufficient cooling of the upper part of the tube, there was some loss of the Perthane olefin due to volatility. Use of a 100 ml beaker filled to within ¾″ of the top with paraffin oil eliminated the problem.

Collaborator 4 suggested the use of a Kuderna-Danish type collection vessel and a micro-Snyder column as an alternative to the centrifuge tube for the dehydrochlorination reaction. This arrangement has been used by others (10) and would probably be satisfactory. This collaborator thought that the TLC procedure with a silica gel adsorbent was faster and superior in separations than 29.018 and 29.024, which employ alumina.

Collaborator 5 commented that he found the dehydrochlorination procedure to be fast, convenient, and reliable.

Conclusions and Recommendations

Satisfactory interlaboratory recoveries were obtained for Perthane, heptachlor epoxide, mirex, and dieldrin with the AOAC multiresidue method

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Comparative Extraction of Chlorinated Hydrocarbon Insecticides from Soils 20 Years After Treatment

RALPH G. NASH, WILLIAM G. HARRIS, PETER D. ENSOR, and EDWIN A. WOOLSON

Agricultural Environmental Quality Institute, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. 20705

Three methods, shake, Soxhlet, and column, were compared for efficiency of extraction of certain chlorinated hydrocarbon insecticides from a Congaree sandy loam soil which had been treated 20 years earlier. Column extraction had a tendency to be less efficient than the other 2 methods, but statistically there were no differences in extraction efficiencies among the 3 methods for aldrin, dieldrin, heptachlor, chlordane, isodrin, endrin, toxaphene, or Dilan and their residue products. However, shake extraction was significantly more efficient for the BHC isomers than Soxhlet extraction and the latter was significantly more efficient than column extraction.

Numerous experiments have been reported on the extraction of chlorinated hydrocarbon insecticides from soils (1–10), and the literature has been reviewed (11, 12). Some of the results reported are based on extraction immediately or within a few months after treatment (3, 6, 7). A few extraction experiments have been conducted on soils that were exposed to considerable weathering after treatment (1, 8, 9).

Two common extraction methods are Soxhlet (1, 3–10) and shake or blend (1, 3, 5–8, 10). Both have proved satisfactory in short-term experiments, using a variety of solvents, provided that the soils were moistened before extraction (1–3, 6, 7, 9, 10).

Nash and Harris (3) developed a column extraction procedure to limit conversion of DDT to DDE during extraction of highly alkaline soils. Column extraction was more rapid than Soxhlet or shake and did not require heat as does Soxhlet extraction. However, column extraction was less efficient than Soxhlet extraction for DDT residues.

This is a report on the comparative efficiency of Soxhlet, shake, and column extraction techniques for aldrin, dieldrin, heptachlor, chlordane, isodrin, endrin, BHC, toxaphene, Dilan, and their residue products from a Congaree sandy loam 20 years after treatment (Table 1). Gas-liquid chromatography (GLC) is used as the determinative step.

Experimental

The soil was a Congaree sandy loam with a pH of 6.2, organic matter content of 2.2%, and a 0.33 bar moisture content of 14.5%. Plots were treated in 1951 (13) at the extremely high rates of 56, 112, or 224 kg insecticide/hectare (14). In 1971, 5 cores (38 cm deep) were taken per plot and composited. The composite was stored at −5°C at the field moisture condition. The soils were screened through a 2 mm sieve immediately before sampling for extraction and moisture content determination.

METHOD

Apparatus

(a) *Soxhlet extraction.*—Use fritted glass thimbles.

(b) *Shake extraction.*—Reciprocal shaker.

(c) *Column extraction.*—Chromatographic tubes, 400 × 20 mm id, with fritted glass disk.

(d) *Kuderna-Danish concentrator.*—500 ml capacity.

(e) *Gas-liquid chromatograph.*—Electron capture ^{63}Ni detector; 1.8 m by 4 mm id glass columns packed with (*1*) 5% SE-30 on 60–80 mesh Chromosorb W (acid-washed, dimethylchlorosilane treated; AW, DMCS), nitrogen flow 40 ml/min, and injection, column, and detector temperatures of 210, 200, and 250°C, respectively; (*2*) 15% QF-1 + 10% DC-200 (1+1) on 80–100 mesh Chromosorb W (AW, DMCS), nitrogen flow 60 ml/min, temperatures of 210, 200, and 250°C, respectively; (*3*) 5% OV-225 on 60–80 mesh Chromosorb W, 5% methane/argon flow 60 ml/min, temperatures of 250, 250, and 330°C, respectively; and (*4*) 1.5% OV-17 + 2% QF-1 (1+1) on 80–100 mesh Chromosorb W (AW, DMCS), nitrogen flow 40 ml/min, temperatures of 190, 180, and 250°C, respectively.

Mention of proprietary products does not imply endorsement or approval by the U.S. Department of Agriculture to the exclusion of other suitable products.

This report of the Associate Referee, E. A. Woolson, was presented at the Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

Table 1. Common and chemical names of insecticide residues

Common	Chemical
Aldrin	1,8,9,10,11,11-hexachloro-2,3-7,6-*endo*-2,1-7,8-*exo*-tetracyclo[6.2.1.1^{3,6}.0^{2,7}]dodeca-4,9-diene
Dieldrin	1,8,9,10,11,11-hexachloro-4,5-*exo*-epoxy-2,3-7,6-*endo*-2,1-7,8-*exo*-tetracyclo-[6.2.1.1^{3,6}.0^{2,7}]dodec-9-ene
Isodrin	1,8,9,10,11,11-hexachloro-2,1-7,8-*endo*-2,3-7,6-*endo*-tetracyclo[6.2.1.1^{3,6}.0^{2,7}]-dodeca-4,9-diene
Endrin	1,8,9,10,11,11-hexachloro-4,5-*exo*-epoxy-2,1-7,8-*endo*-2,3-7,6-*endo*-tetracyclo-[6.2.1.1^{3,6}.0^{2,7}]dodec-9-ene
Heptachlor	1,3-*exo*-7,8,9,10,10-heptachloro-2,3-6,5-*endo*-tricyclo[5.2.1.0^{2,6}]deca-4,8-diene
Chlordane	1,3-*exo*-4-*exo*(or *endo*)-7,8,9,10,10-octachloro-2,3-6,5-*endo*-tricyclo[5.2.1.0^{2,6}]-dec-8-ene (cis-*exo*,*exo* and trans-*exo*,*endo*)
Toxaphene	67–69% chlorinated camphene
BHC	$\alpha,\beta,\gamma,\delta$, and ϵ isomers of 1,2,3,4,5,6-hexachlorocyclohexane
Dilan	1,1-bis(*p*-chlorophenyl)-2-nitrobutane (Bulan®) and 1,1-bis(*p*-chlorophenyl)-2-nitropropane (Prolan®)
Endrin ketone	1,8,9,9,10,11-hexachloropentacyclo-[6.3.0.1^{2,5}.0^{3,7}.0^{6,10}]dodecan-12-one
Endrin alcohol	1,8,9,9,10,11-hexachlorohexacyclo-[6.3.0.1^{2,5}.0^{3,7}.0^{6,10}.0^{11,12}]dodecan-12-ol
Endrin aldehyde	1,8,9,9,10,11-hexachloropentacyclo-[6.3.0.0^{2,11}.0^{3,7}.0^{6,10}]undecan-12-carboxaldehyde
Heptachlor epoxide	1,3-*exo*-7,8,9,10,10-heptachloro-4,5-*exo*-epoxy-2,3-6,5-*endo*-tricyclo[5.2.10^{2,6}]-dec-8-ene
Nonachlor	1,3-*exo*-4-*endo*-5-*exo*-7,8,9,10,10-nonachloro-2,3-6,5-*endo*-tricyclo[5.2.1.0^{2,6}]dec-8-ene
DDT	1,1,1-trichloro-2,2-bis[*p*-chlorophenyl]ethane

Reagents

(a) *Solvents.*—Hexane, acetone, methanol, and petroleum ether (bp 30–60°C), all redistilled in glass.

(b) *Florisil.*—60–100 mesh PR grade activated 3.5 hr at 1250°F (Floridin Co., 3 Penn Center, Pittsburgh, Pa. 15235).

(c) *Sodium sulfate.*—Anhydrous granular.

(d) *Sand.*—20–30 mesh, acid-washed and water- and acetone-rinsed.

(e) *Ammonium chloride.*—0.2*M* solution.

Extraction

Soxhlet extraction.—Place 5 g Florisil in Soxhlet thimble, add 5 g moist soil, and cover with 1.5–2 cm sand (3). Extract sample 12 hr with hexane-acetone-methanol (HAM) (8+1+1). Transfer extract to Kuderna-Danish concentrator and reduce volume to ca 10 ml. Concentrate successively again with two 25 ml portions of hexane, reducing volume to ca 10 ml after each addition. Dilute to appropriate volume with hexane for GLC analysis.

Shake extraction.—Place 10 g moist soil in 250 ml glass-stoppered Erlenmeyer flask. Add 7 ml 0.2*M* NH_4Cl solution and let mixture stand 15 min to assure thorough saturation of soil. Then add 100 ml hexane-acetone (HA) (1+1), stopper flask, and shake 17 hr (overnight is convenient). Decant supernate into separatory funnel, add 500 ml distilled water, and shake 1 min. Let layers separate and draw off lower layer into second separatory funnel. Add 100 ml hexane to second funnel, shake, let layers separate, and discard lower layer. Combine the 2 hexane layers in first funnel. Add 500 ml water to funnel, shake, let layers separate, and again discard lower layer. Dry extract by adding 5 g anhydrous Na_2SO_4 to separatory funnel. Dilute dry extract to appropriate volume with hexane for GLC analysis.

Column extraction.—Place 8 g Florisil in column, add 5 g moist soil, and cover with 2 cm sand (3). Extract with 200 ml HAM (4+3+3) and collect eluate in separatory funnel. Add 500 ml water and 20 ml saturated NaCl to funnel. Shake funnel 1 min, let layers separate, and draw off and discard lower layer. Add 500 ml water to funnel, shake, let layers separate, and discard lower layer. Dry extract by passing through 1–2 cm column of anhydrous Na_2SO_4, Dilute to appropriate volume with hexane for GLC analysis.

Moisture

Take sample of soil (10–15 g) for moisture determination during subsampling for extraction. Weigh moist soil, heat 24 hr in 104°C oven, and reweigh to determine moisture content.

Determination

GLC peak heights were used for quantitative analysis of all pesticides except toxaphene, for which peak areas were also used. Thin layer chromatography (TLC) on alumina plates was also used for analysis of chlordane residues. The heptachlor epoxide area on the TLC plate was scraped off and eluted for mass spectrometric determination.

Results and Recommendation

Soil colloids may be expanded or collapsed, depending upon the cations in the soil solution. When solutions of water, 0.2*M* $CaCl_2$, NH_4Cl, KCl, and 0.5*M* NH_4Cl were used as saturation agents prior to extraction of heptachlor and heptachlor epoxide by shaking, the relative recoveries were 75, 91, 100, 85, and 82%, respectively. The 0.2*M* NH_4Cl gave the highest recoveries for a single extraction. Water alone improved the extraction efficiency of the shake

method by 12% for total DDT residues. Typical recovery values for the Soxhlet and column methods have been reported previously (3).

The shake procedure was subjected to spiking and recovery on Lakeland sandy loam and Christiana clay loam. Recovery, standard deviation, and coefficient of variation are given in Table 2. Results from the Christiana soil were more variable than those from the Lakeland soil; however, the values are satisfactory.

The data from the comparative extractions were subjected to an analysis of variance. The statistical analyses are available from the authors. Results from the aldrin- and dieldrin-treated plots were analyzed together, as were those from isodrin and endrin and heptachlor and chlordane treatments because of common residues in these plots.

The results of Soxhlet, shake, and column extraction of aldrin, dieldrin, isodrin, endrin, heptachlor, chlordane, BHC, toxaphene, and Dilan from Congaree sandy loam 20 years after treatment are given in Tables 3–8. Soxhlet and shake extraction appeared to be more efficient than column extraction although, statistically, there were no differences among the 3 methods except for BHC. The less than quantitative recoveries with the column method were reported previously (3).

Shake extraction was significantly more efficient than Soxhlet or column extraction of the BHC isomers. Consequently, soil from the BHC-treated plots was extracted again. Data from both extractions were combined for statistical analysis. The greater number of degrees of freedom (119 vs. 59) for the 2 extractions provided a more sensitive test. When this was done, the shake extraction was still the most efficient method and column extraction was the least efficient method. The differences were significant at the 99% level. Differences between the first and second assays were not significant.

The Soxhlet and shake extraction methods used herein recovered nearly identical quantities of 9 chlorinated hydrocarbon insecticides. Column extraction, although less efficient, required less expensive apparatus and was less time-consuming, but total labor was about equal to that for the other 2 methods.

In the shake method 7 ml 0.2*M* NH_4Cl is used. This is equivalent to nearly a 1:1 soil-water ratio when the soil moisture content is near 0.33 bar tension for the Congaree soil. The excess moisture presented no problems on this soil. With a high clay soil, a significant amount of clay-water sus-

Table 2. Recovery of insecticides from fortified soil by the shake flask method

Insecticide	Added, μg/10 g soil	Recovered, μg/10 g soil	Std dev.	Coeff. of var.
Lakeland Sandy Loam				
Heptachlor	47.3	47.2	1.4	3.0
Aldrin	43.0	43.1	1.0	2.3
Dieldrin	41.0	42.4	2.0	4.7
Endrin	43.0	43.6	1.8	4.1
DDT	55.0	51.0	2.1	4.1
Mean	45.8	45.5	1.7	3.6
Christiana Clay Loam				
Heptachlor	47.3	46.5	1.5	3.6
Aldrin	43.0	42.5	1.2	2.8
Dieldrin	41.0	41.4	2.3	5.6
Endrin	43.0	46.6	2.8	6.0
DDT	55.0	51.0	2.4	4.7
Mean	45.8	45.6	2.0	4.5

Table 3. Comparative extraction of aldrin and dieldrin from Congaree sandy loam

Compound	Treatment rate (1951), kg/hectare	Residues (1952),[a] ppm	Residues (1971), ppm		
			Soxhlet	Shake	Column
Aldrin (Pure)					
Aldrin	56	19.5[b]	0.09	0.09	0.07
Dieldrin			2.66	2.56	2.44
Total			2.75	2.65	2.51
Aldrin	224	96.5	0.21	0.18	0.15
Dieldrin			12.70	11.22	9.41
Total			12.91	11.40	9.56
Aldrin (Technical)					
Aldrin	56	30.3	0.02	0.03	0.02
Dieldrin			5.07	5.28	4.53
Total			5.09	5.31	4.55
Aldrin	224	143.5	0.12	0.11	0.09
Dieldrin			21.16	16.38	19.84
Total			21.28	16.49	19.93
Dieldrin					
Aldrin	56	23.5	0.10	0.07	0.09
Dieldrin			3.01	2.41	2.14
Total			3.11	2.48	2.23
Aldrin	224	105.5	0.28	0.22	0.19
Dieldrin			15.16	13.44	12.10
Total			15.44	13.66	12.29

[a] Determined by total chlorine content of treated plots less control plots (14).
[b] All values are the average of duplicate plots.

Table 4. Comparative extraction of isodrin and endrin residues from Congaree sandy loam

Compound	Treatment rate (1951), kg/hectare	Residues (1952),[a] ppm	Residues (1971), ppm: Soxhlet	Shake	Column
Isodrin					
Isodrin	50	33.5[b]	0.09	0.09	0.08
Dieldrin			0.31	0.28	0.26
Endrin			1.93	1.84	1.75
Endrin aldehyde$_1$[c]			0.28	0.49	0.52
Endrin aldehyde$_3$[c]			0.14	0.19	0.13
Endrin alcohol			0.52	0.34	0.24
Endrin ketone			4.24	4.22	3.44
Total			7.51	7.45	6.42
Isodrin	224	154.7	0.71	0.68	0.55
Dieldrin			1.07	1.11	0.97
Endrin			6.22	4.52	4.82
Endrin aldehyde$_1$			0.55	0.80	1.01
Endrin aldehyde$_3$			0.24	0.23	0.18
Endrin alcohol			1.60	1.43	1.27
Endrin ketone			28.45	21.06	25.66
Total			38.84	29.83	34.46
Endrin					
Isodrin	50	33.5	0.01	0.01	0.01
Dieldrin			0.35	0.38	0.33
Endrin			5.96	5.81	5.67
Endrin aldehyde$_1$			0.59	0.30	0.31
Endrin aldehyde$_3$			0.23	0.23	0.16
Endrin alcohol			0.28	0.14	0.24
Endrin ketone			2.34	2.04	1.91
Total			9.76	8.91	8.63
Isodrin	224	109.0	0.18	0.13	0.14
Dieldrin			0.68	0.68	0.63
Endrin			21.70	24.06	20.65
Endrin aldehyde$_1$			1.21	1.37	0.90
Endrin aldehyde$_3$			0.90	1.04	0.74
Endrin alcohol			1.17	0.90	0.87
Endrin ketone			20.57	19.41	19.33
Total			46.41	47.59	43.26

[a, b] See Table 3.

[c] Endrin aldehyde$_1$ standard (14). Endrin aldehyde$_3$ is believed to be the true endrin aldehyde (13). Both compounds are estimated amounts.

pension may be present. If this occurs, the organic layer should be decanted carefully and passed through a 2–3 cm column of Florisil. Both the flask and the column should be rinsed with the extracting solvent.

Occasionally a soil extract requires cleanup prior to GLC analysis, especially high organic matter soils. In this case, the official AOAC Florisil cleanup method for pesticide residues, 29.014, should be used.

Only heptachlor, heptachlor epoxide, and *cis*- and *trans*-chlordane were measured in the hepta-

Table 5. Comparative extraction of heptachlor and chlordane residues from Congaree sandy loam

Compound	Treatment rate (1951), kg/hectare	Residues (1952),[a] ppm	Residues (1971), ppm: Soxhlet	Shake	Column
Heptachlor					
Heptachlor	112	80.3[b]	0.38	0.35	0.38
Heptachlor epoxide			3.19	3.77	3.02
trans-Chlordane			4.77	6.54	4.67
Nonachlor			1.02	1.04	1.58
Total			9.36	11.70	9.65
Heptachlor	224	224.3	2.06	1.68	1.99
Heptachlor epoxide			6.64	6.55	6.50
trans-Chlordane			16.66	16.72	16.48
Nonachlor			4.57	7.25	4.59
Total			29.93	32.20	29.56
Chlordane					
Heptachlor	112	37.0	0.07	0.08	0.07
Heptachlor epoxide[c]			0.34	0.31	0.31
trans-Chlordane			1.26	1.29	1.29
cis-Chlordane			1.23	1.28	1.27
Total			2.90	2.96	2.94
Heptachlor	224	81.5	0.12	0.13	0.11
Heptachlor epoxide			0.12	0.13	0.12
trans-Chlordane			3.18	3.67	3.32
cis-Chlordane			2.19	2.51	2.23
Total			5.61	6.44	5.78

[a, b] See Table 3.

[c] Verified by injecting onto a 15.24 m SE-30 SCOT column in a gas-liquid chromatograph/mass spectrometer combination. At 80 eV, the fragmentation pattern in the extract spectrum was identical to that of standard heptachlor epoxide. Both had molecular ion at m/e 386.

Table 6. Comparative extraction of BHC residues from Congaree sandy loam

Isomer	Treatment rate (1951), kg/hectare	Residues (1952),[a] ppm	Residues (1971), ppm[c]: Soxhlet	Shake	Column
Alpha	112	46.3[b]	0.14	0.14	0.10
Gamma			0.82	0.88	0.68
Beta			0.69	0.68	0.60
Delta			0.72	0.90	0.76
Epsilon			0.10	0.12	0.11
Total			2.47	2.72	2.25
Alpha	224	92.8	0.57	0.55	0.45
Gamma			2.17	2.25	1.89
Beta			1.37	1.43	1.25
Delta			2.38	2.69	2.50
Epsilon			0.30	0.32	0.26
Total			6.79	7.24	6.35

[a, b] See Table 3.

[c] Average of 2 extractions. Shake extraction was significantly greater than Soxhlet extraction and Soxhlet was significantly greater than column extraction at the 99% statistical level.

Table 7. Comparative extraction of toxaphene residues from Congaree sandy loam

Treatment rate (1951), kg/hectare	Residues (1952),[a] ppm	Residues (1971), ppm		
		Soxhlet	Shake	Column
112	55.8[b]	24.24	28.08	21.61
224	137.0	57.29	61.19	50.44

[a, b] See Table 3.

chlor- and chlordane-treated plots. However, by both GLC and TLC, other halogen components were present in the chlordane-treated soils when high concentrations were analyzed. These appeared to be compounds typical of technical chlordane, but were present in much lower amounts than the 4 compounds measured.

To verify the heptachlor epoxide residue from the chlordane-treated plots, a large quantity of soil was extracted and cleaned up on Florisil columns; the chlordane components were separated on alumina TLC plates. The heptachlor epoxide area from the TLC plates was extracted and concentrated for mass spectrometry. The mass spectrum from the soil extract corresponded exactly to that from standard heptachlor epoxide.

The total amount of residues (%) remaining in the Congaree sandy loan after 20 years was as follows: technical aldrin—15.1, pure aldrin—13.2, dieldrin—12.8, isodrin—22.3, endrin—35.5, heptachlor—13.5, chlordane—7.7, BHC—6.6, toxaphene—45.1, and Dilan—7.3. These values are based on the pooled means of Soxhlet and shake extraction recoveries from both treatment rates.

It is recommended that the shake flask procedure be studied collaboratively.

Acknowledgment

The authors wish to express their appreciation to U. I. Klingebiel, of their organization, for the mass spectrometry analysis.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee E and was accepted by the Association; *see* (1973) *JAOAC* 56, 405.

Table 8. Comparative extraction of Dilan residues from Congaree sandy loam

Compound	Treatment rate (1951), kg/hectare	Residues (1952),[a] ppm	Residues (1971), ppm		
			Soxhlet	Shake	Column
Unknown[c]	112	44.5[b]	0.68	0.62	0.86
Bulan			4.45	3.25	2.10
Total			5.13	3.87	2.96
Unknown	224	173.5	1.51	1.64	1.21
Bulan			5.89	5.94	3.62
Total			7.40	7.58	4.83

[a, b] See Table 3.
[c] Estimated value; compound is not Prolan.

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An Isolation and Cleanup Procedure for Low Levels of Organochlorine Pesticide Residues in Fats and Oils

MILDRED L. PORTER and JERRY A. BURKE
Division of Chemistry and Physics, Food and Drug Administration, Washington, D.C. 20204

An isolation and cleanup is described for low levels of organochlorine residues (about 0.005 ppm) in fats and oils, prior to electron capture gas-liquid chromatography. The fat or oil is distributed on a column of unactivated Florisil and the residues are partitioned into an eluant of 10% water in acetonitrile. Florisil column chromatography with an elution solvent system comprised of mixtures of methylene chloride, acetonitrile, and hexane is used for the final cleanup. Extracts prepared in this manner are sufficiently free of fatty extractives to permit injection of the equivalent of 50–60 mg fat sample for GLC. The procedure should be especially useful in determination of low levels of organochlorine pesticide residues in the fat of certain dietary composites.

Multiresidue methodology (1, 2) normally employed by our laboratories to analyze fatty foods for organochlorine pesticides provides for cleanup of a maximum of 3 g fat. Restriction of sample size due to inefficiency of cleanup limits the lower level of residue detection attainable. In some analyses, particularly with certain dietary composites, quantitative determination is desired of residues present at 0.01 ppm and below in the isolated fat. With a sample size limited to 3 g and the degree of cleanup attainable with available procedures it is often difficult and time-consuming to prepare fatty samples for analyses at the desired level. Our objective was to develop a cleanup procedure capable of handling fat and oil samples large enough to permit electron capture gas chromatographic (GLC) determination of organochlorine pesticide residues at approximately 0.005 ppm in the fat or oil. Such a procedure is desirable for use in the analysis of fat-containing dietary composites in the Food and Drug Administration's "total diet" program (3). To attain this objective and to maintain the electron capture detector sensitivity previously employed (1, 2), it is necessary to inject approximately 50 mg equivalent of the fat or oil sample for GLC determination.

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METHOD

Apparatus

(a) *Gas chromatograph.*—Equipped with electron capture detector and 6′ × 4 mm id glass column packed with either (*1*) 10% DC-200 on 80–100 mesh Chromosorb W (HP) or (*2*) 15% QF-1 + 10% DC-200 (1+1) on 80–100 mesh Chromosorb W (HP). Operating conditions: nitrogen flow rate, 120 ml/min; column and detector temperature, 200°C; concentric design electron capture detector operated at dc voltage to produce ½ full scale recorder deflection for 1 ng heptachlor epoxide when full scale deflection is 1×10^{-9} amp.

(b) *Chromatographic column.*—Plain, 22 mm id × 250 mm (Kontes Glass Co., Vineland, N.J., No. K-420300, size 21, or equivalent).

(c) *Chromatographic column.*—For Florisil column cleanup (4); 22 mm id × 300 mm, with Teflon stopcock and coarse fritted disk (Kontes Glass Co., No. K-420140, size 233, or equivalent).

(d) *Kuderna-Danish concentrator.*—500 ml capacity with Snyder column and volumetric or graduated receiving tube (Kontes Glass Co., Nos. K-621400 and K-570050, or equivalent).

(e) *Micro evaporative concentrator column.*—To fit Kuderna-Danish receiving tube (Kontes Glass Co., No. K-569251, or equivalent).

(f) *Spatula.*—5/16″ width (Arthur H. Thomas, Philadelphia, Pa., No. 8339-M 10, or equivalent).

Reagents

(a) *Florisil.*—60/100 PR grade (Floridin Co., 3 Penn Center Blvd., Pittsburgh, Pa. 15235). Immediately transfer from bulk container to glass containers with glass-stoppered or foil-lined screw top lids and store in dark. (*1*) *Unactivated for partition chromatography.*—Use as received from manufacturer. Do *not* heat. (*2*) *Activated for Florisil column cleanup* (*5*).—Heat ≥5 hr but preferably overnight at 130°C before use. Store at 130°C in glass-stoppered or foil-covered bottles. Alternatively, store stoppered container in desiccator at room temperature and reheat at 130°C after 2 days.

(b) *Solvents.*—Hexane, CH_2Cl_2, petroleum ether, and CH_3CN; distilled in glass; free from electron capturing substances. (Suitable products available from Burdick and Jackson Laboratories, Muskegon, Mich.)

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

(d) *Water in acetonitrile.*—10%. Add CH_3CN to 100 ml water, let reach room temperature, and adjust to 1 L with CH_3CN.

(e) *Eluant mixtures.*—For Florisil column cleanup (5). (*1*) *Eluant A.*—20% CH_2Cl_2-hexane (v/v). Add 200 ml CH_2Cl_2 to hexane, let mixture reach room temperature, and adjust volume to 1 L with hexane. (*2*) *Eluant B.*—50% CH_2Cl_2-0.35% CH_3CN-49.65% hexane (v/v/v). Pipet 3.5 ml CH_3CN into 500 ml CH_2Cl_2 and dilute with hexane. Let reach room temperature and adjust to 1 L with hexane. (*3*) *Eluant C.*—50% CH_2Cl_2-1.5% CH_3CN-48.5% hexane (v/v/v). Pipet 15 ml CH_3CN into 500 ml CH_2Cl_2 and dilute with hexane. Let reach room temperature and adjust to 1 L with hexane.

Cleanup

(Fat or oil sample *must* be free of all traces of organic solvents.)

Weigh 8 g fat or oil into 250 ml beaker. Add 25 g *unactivated* Florisil (a)(*1*) to sample and stir with spatula until all lumps are eliminated and thoroughly mixed, free flowing powder remains. Place glass wool plug in bottom of 22 mm id × 250 mm chromatographic tube and add 3 g *unactivated* Florisil (a)(*1*). Completely transfer oil-Florisil mixture to chromatographic tube while settling column bed by tapping tube sharply after addition of increments of Florisil. Place glass wool plug ca 15 mm thick on top of Florisil to hold it in place. Place 1 L separatory funnel under column to collect eluate. Elute column with 150 ml 10% water-acetonitrile, letting eluant pass through column at its own rate. Before eluting, rinse beaker used for mixing sample and Florisil with few ml eluant and add rinse to column. When elution is complete, add exactly 100 ml petroleum ether to separatory funnel and shake vigorously 2 min. Add 10 ml saturated NaCl solution and ca 600 ml water to funnel and mix vigorously 1 min. Let layers separate and discard aqueous layer. Wash petroleum ether layer with 2 successive 100 ml portions of water. Discard washings and transfer petroleum ether to 100 ml stoppered graduate and record volume (*P*). Add ca 15 g anhydrous Na_2SO_4 and shake vigorously.

Determine weight of sample represented in eluate as follows:

Wt sample in eluate = wt oil or fat sample × [*P* (ml petroleum ether recovered)/100 (ml petroleum ether added)]

Complete cleanup on a column of activated Florisil (a)(*2*), using methylene chloride-acetonitrile-hexane eluants as described by Mills *et al.* (5). Concentrate eluates from Florisil column chromatography to suitable definite volumes for determination of organochlorine residues by electron capture GLC. Use micro evaporative concentrator column for concentration to volumes <5 ml. (*Note:* Concentration of eluate to 1 ml for analysis by electron capture GLC with sensitivity described under *Apparatus* permits limit of detection of ca 0.005 ppm.)

Results and Discussion

Experimentation with the partition chromatography residue isolation procedure of Giuffrida *et al.* (4), in combination with the improved Florisil cleanup column chromatography eluant system of Mills *et al.* (5), showed a marked improvement in cleanup of lipid extractives. Further investigation of the partition chromatography procedure indicated the possibility of increasing the capacity for sample size above the 2 g fat or oil previously used (4).

This procedure utilizes a partitioning column chromatographic process in which the oil or fat sample is distributed along a column of inert support and the nonpolar organochlorine residues are removed by a moving liquid phase. Unactivated Florisil is used as the support and 10% water-acetonitrile is the moving liquid. Elution of the pesticides is a function of solubility rather than chromatography. The relative distribution of pesticides between the sample fat or oil and the 10% water-acetonitrile phase follows the *p*-values reported by Bowman and Beroza (6). The Florisil cleanup column chromatographic system recently reported by Mills *et al.* (5) uses an eluant system

Table 1. Weight of fatty residues after partition chromatography and after Florisil column adsorption chromatography: 8 g original sample

Sample	mg Eluted		
	10% water/ CH_3CN[a]	Eluant A[b, c]	Eluant B[b, d]
Corn oil	131	0.3	1.3
Butterfat	272	0.5	19.5
Cod liver oil	187	0.1	0.7
Chicken fat	73	0.0	0.5
Dietary composite (dairy products)	244	0.6	15.0
Dietary composite (meat, fish, poultry)	83	0.9	5.2
Dietary composite (oils, fats, shortening)	131	0.0	1.5

[a] Residue from 8 g fat after partition chromatography.
[b] Residue after Florisil column adsorption chromatography: cleanup of fat weight remaining after partition chromatography.
[c] Eluant A—20% CH_2Cl_2-nexane.
[d] Eluant B—50% CH_2Cl_2—0.35% CH_3CN—49.65% hexane.

Table 2. Elution of pesticides from partition chromatography column[a]

Pesticide	Vol. 10% water/CH_3CN added to column, ml								
	0–50	50–75	75–100	100–125	125–135	135–145	145–155	155–165	165–175
Lindane	+	+	0	0	0	0	0	0	0
p,p'-DDE	+	+	+	+	+	+	+[b]	+[b]	0
p,p'-TDE	+	+	+[b]	0	0	0	0	0	0
p,p'-DDT	+	+	+	+	+[b]	0	0	0	0
Mirex	+	+	+	+	+	+	+	+	+

[a] 28 g unactivated Florisil, 8 g corn oil; see *Method*.
[b] Amount <1%.

of mixtures of methylene chloride-acetonitrile/hexane instead of petroleum ether-ethyl ether mixtures to significantly improve the cleanup of lipid extracts.

Our investigations were aimed at scaling up the procedure of Giuffrida *et al.* to accommodate significantly larger samples. Many experiments were conducted with varying amounts of unactivated Florisil and volumes of 10% water-acetonitrile, weights of oil and fat samples, and representative organochlorine pesticides in order to arrive at optimum parameters for good recovery of pesticides with minimum elution of lipids. No attempt was made to change the basis of the earlier approach, i.e., unactivated Florisil column material and 10% water-acetonitrile eluant. Instead of adding the sample to the column in petroleum ether solution (4), the fat or oil sample was thoroughly mixed with Florisil to produce a free-flowing powder prior to addition to the column (7). One partition with 100 ml petroleum ether was used to extract the pesticides from the water-acetonitrile eluate; the sample weight represented in the petroleum ether phase was calculated using the approximation, volume of petroleum ether recovered divided by 100, as has been done previously (2). The Florisil adsorption column chromatography cleanup was used as previously described (5). Fat was extracted from dietary composites (dairy products; fish, meat, and poultry; and oils, fats, and shortening) by procedures previously described (2, 8).

The removal of fatty substances from 8 g samples of representative fats and oils by the method described above is shown in Table 1. Approximately 96.5% (butterfat) to 99% (chicken fat) of the original sample was retained during the partition chromatography residue isolation step (column 2 in Table 1). The fats and oils extracted from dietary composites were handled as effectively as the individual fats and oils.

Table 3. Recoveries of pesticides and Arocior 1254 added to 8 g corn oil[a]

Chemical[b]	Sample	Added, ppm	Rec., %
Lindane	1	0.05	97, 100
	2	0.005	106
	3	0.0025	107
	4	0.0005[c]	91
p,p'-DDE	1	0.1	100, 98
	2	0.010	92
	3	0.005	101
	4	0.001[c]	93
p,p'-TDE	1	0.13	98, 96
	2	0.013	104
	3	0.0065	112
	4	0.0013[c]	97
p,p'-DDT	1	0.4	96, 102
	2	0.04	100
	3	0.02	107
	4	0.004[c]	105
Mirex	1	0.5	58, 56
	2	0.05	49
	3	0.025	54
	4	0.005[c]	93
Heptachlor epoxide	1	0.1	105, 104
	2	0.010	103
	3	0.005	113
	4	0.001[c]	105
Endosulfan I	1	0.2	98, 103
	2	0.02	93
	3	0.01	109
	4	0.002[c]	93
Dieldrin	1	0.13	102, 102
	2	0.013	106
	3	0.065	102
	4	0.0013[c]	103
Endosulfan II	1	0.08	88, 102
	2	0.008	103
	3	0.004	99
	4	0.0008[c]	86
Endosulfan sulfate	1	0.5	105, 106
	2	0.05	108
	3	0.025	97
	4	0.005[c]	89
Arocior 1254		0.1	85

[a] Isolation and cleanup described under *Method*.
[b] All pesticides added in admixture.
[c] Reagents fortified; no oil present.

The partition chromatography procedure provides a more effective cleanup of 8 g fat than petroleum ether-acetonitrile partitioning (1, 2) achieves with a 3 g sample. In experiments conducted with 3 g portions of butterfat, the weight of fatty substances partitioned from petroleum ether to acetonitrile, 29.011 (2), ranged from 373 to 541 mg and averaged 433 mg for 15 samples. Fatty residues from 8 g butterfat of 244 to 272 mg were eluted from the partition chromatography column. Less than 1 mg residue from fatty samples appeared in the first eluate from the Florisil adsorption cleanup column. Fatty residues in the second eluate from this column were generally of the order of 2 mg, but up to 20 mg was eluted from butterfat. Butterfat was the most difficult of the fats studied to clean up through the complete method. The per cent retention of fatty substances by Florisil adsorption chromatography was essentially the same with butterfat, the dairy products dietary composite, and the fish, meat, and poultry dietary composite.

Butterfat and corn oil behaved differently on the partition column. The volume of aqueous acetonitrile eluant seemed to be the determining factor in the amount of butterfat eluted from the column. Increase of eluant volume from 150 to 300 ml resulted in an increase in fatty residue from about 280 to 550 mg, regardless of the amount of Florisil used for the column (15–45 g) or the amount of butterfat (5–10 g). The primary factor in the cleanup of corn oil was the ratio of weight of oil to weight of Florisil. Additional Florisil resulted in improved cleanup of corn oil. Rice oil was not cleaned up effectively by the described method.

Table 2 shows the elution behavior of 5 organochlorine pesticides on the partition chromatographic column. The fractional elutions were made with an 8 g sample of corn oil on the column. Lindane and mirex were chosen to represent divergent behavior as judged by their partitioning values between hexane and acetonitrile. An eluant volume of 150 ml was chosen as the

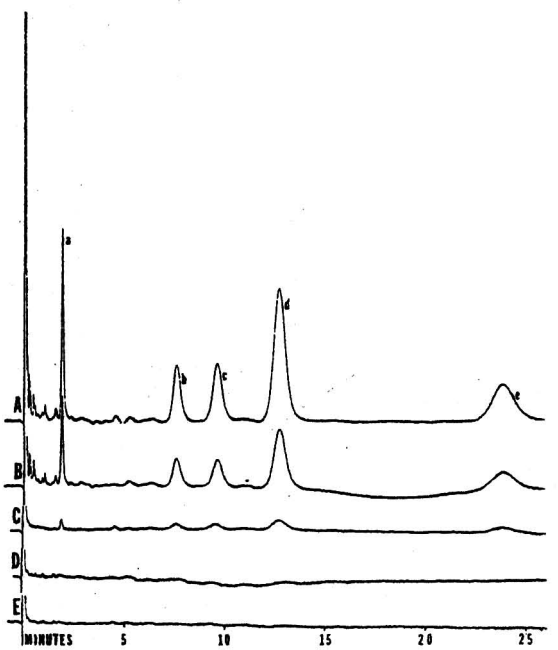

FIG. 1—Electron capture GLC chromatograms from eluate A. GLC conditions given under Apparatus; injection equivalent to 57 mg sample for all chromatograms. A, 8 g corn oil fortified at following ppm: a. lindane 0.005, b. *p,p'*-DDE 0.01, c. *p,p'*-TDE 0.013, d. *p,p'*-DDT 0.04, e. mirex 0.05; B, 8 g corn oil fortified at one-half levels in A; C, reagents (no oil) fortified equivalent to one-tenth levels in A; D, 8 g corn oil (not fortified); E, reagent blank.

most favorable for recovery of representative organochlorine pesticides with minimum elution of fat. We wish to reiterate the usefulness of partitioning values for hexane-acetonitrile (6) in estimating compound behavior on this column.

Table 3 gives recoveries of 10 representative organochlorine pesticides and Aroclor® 1254 from corn oil. The overall fortification levels ranged from 0.0005 ppm lindane to 0.5 ppm endosulfan sulfate and mirex. Corn oil was chosen for recovery experiments because of the general presence of low levels of organochlorine pesticide and/or polychlorinated biphenyl (PCB) residues in animal fats and oils. With the exception of mirex, recoveries of all compounds were good. The low recovery of mirex was predictable from partitioning values (6). Likewise the 85% recovery for Aroclor 1254 would be predicted to be lower for higher chlorinated PCB and higher for PCB of less chlorination (9).

Figures 1–3 show the electron capture GLC chromatograms obtained from samples carried through the complete method. Chromatograms of the 10 representative organochlorine pesticides and metabolites (lindane, *p,p'*-DDE, *p,p'*-TDE, *p,p'*-DDT, heptachlor epoxide, dieldrin, endosulfan I and II, endosulfan sulfate, and mirex) and PCB (Aroclor 1254) chosen for this study are included in the figures. The lowest fortification levels, around 0.001 ppm (Figs. 1 and 2), were carried through the method in the absence of oil sample in order to avoid any exaggeration of the response due to low residue levels in the oil. A chromatogram of the unfortified corn oil is shown for comparison. These chromatograms show the suitability for electron capture GLC of extracts prepared as described in this paper.

Dietary composites (dairy products; meat, fish, and poultry; and oils, fats, and shortenings) (3) have been analyzed as described under *Method* (J. Underwood and Kansas City Total Diet Analytical Laboratory, Food and Drug Administration (1973) private communication). Levels of the organochlorine pesticide residues determined compared well with those found when petroleum ether-acetonitrile partitioning and Florisil column chromatography with ethyl ether-petroleum ether eluants (1, 2) were used for cleanup. The improved cleanup obtained with the described method permitted the recognition and/or measurement of residues sometimes not determinable after cleanup by the usual procedure.

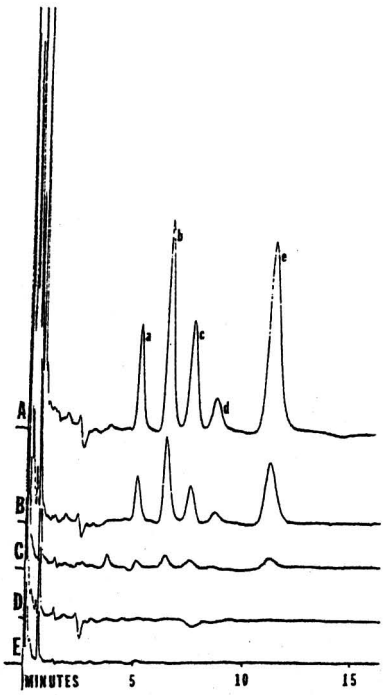

FIG. 2—Electron capture GLC chromatograms from eluate B. GLC conditions given under *Apparatus*; injection equivalent to 57 mg sample for all chromatograms. A, 8 g corn oil fortified at following ppm: a. heptachlor epoxide 0.01, b. endosulfan I 0.02, c. dieldrin 0.013, d. endosulfan II 0.008, e. endosulfan sulfate 0.05; B, 8 g corn oil fortified at one-half levels in A; C, reagents (no oil) fortified equivalent to one-tenth levels of A; D, 8 g corn oil (not fortified); E, reagent blank.

Acknowledgment

The authors wish to acknowledge the suggestion of Paul A. Mills for increasing the capacity for sample size of the Giuffrida *et al.* partition chromatography procedure.

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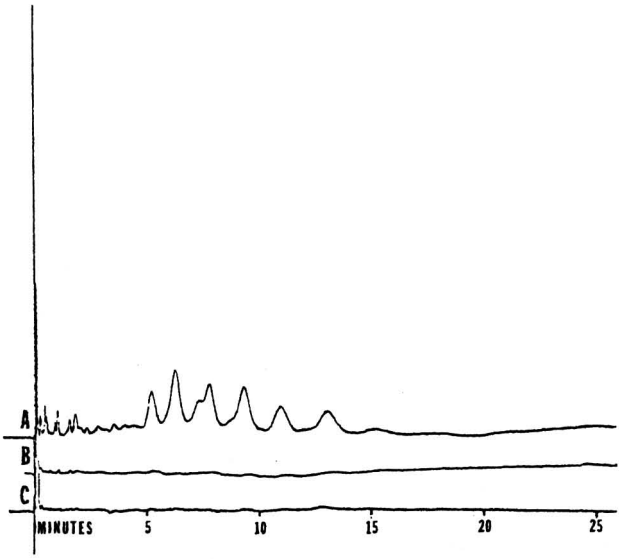

FIG. 3—Electron capture GLC chromatograms from eluate A. GLC conditions given under *Apparatus*; injection equivalent to 57 mg sample for all chromatograms: A, 8 g corn oil fortified with 0.1 ppm Aroclor 1254: B, 8 g corn oil (not fortified); C, reagent blank.

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FRUITS AND FRUIT PRODUCTS

Control of Authenticity of Fruit Juices by Isotopic Analysis

JACQUES BRICOUT
Institut de Recherches Appliquées aux Boissons, 87 rue de Paris, 93 Montreuil, France

In order to differentiate a natural fruit juice from a dilute concentrate, we have investigated the isotopic composition of their water. We have chosen the stable isotopes deuterium and ^{18}O. The water of orange juices is strongly enriched in heavy isotopes, as compared to rain water. This enrichment is much lower for French apple juices and seems to be due to evapotranspiration. The relationship between deuterium and ^{18}O observed for meteoric water is modified in the fruits. If a fruit juice is concentrated and then rediluted with natural water, the isotopic composition of its water would be very similar to the original deuterium and ^{18}O content of the dilution water. Isotopic analysis allows a very confident distinction between natural fruit juices and reconstituted juices.

The fruit juices, particularly citrus, in the Common Market are mostly imported from southern areas, and in order to reduce the transport cost they are usually imported as concentrates. The French law distinguishes a natural fruit juice from a dilute concentrate; therefore it is necessary to have an analytical method to detect the less expensive reconstituted juices.

It is known that vacuum concentration with aroma recovery does not affect the chemical composition of fruit juices (1); therefore we have only considered the water content. In order to differentiate the water of the fruit from tap water, we analyzed the stable isotopes of hydrogen and oxygen. The different stable isotopic species found in natural water are as follows (2): H, 99.985; D, 0.015; ^{16}O, 99.759; ^{17}O, 0.037; ^{18}O, 0.204 atom per cent.

Experimental

The relative abundances of stable isotopes were measured by mass spectrometry. For the determination of deuterium, the water was reduced in a 600°C uranium furnace directly connected to the inlet system of a mass spectrometer specially designed for the isotopic analysis of hydrogen (3). The ratio of the intensity of the peak at m/e = 3 (HD^+) to that at m/e = 2 (H_2^+) was determined and the atom per cent deuterium in the hydrogen sample was calculated.

For oxygen we considered only the ^{18}O isotope, which was determined indirectly (4). The water was equilibrated with carbon dioxide in a closed vessel at constant temperature and pressure:

$$H_2{}^{18}O + C^{16}O_2 \rightleftarrows H_2{}^{16}O + C^{16}O^{18}O$$

The same operation was performed with standard water of known ^{18}O content. The ^{18}O in carbon dioxide was analyzed by mass spectrometry (Varian Mat CH7 with a double collector and 2 inlet systems). The relative difference between the ratio of the peaks at m/e = 46 ($C^{16}O^{18}O^+$) and m/e = 44 ($C^{16}O_2^+$) for the standard and for the sample was measured. The ^{18}O content of this standard water was previously determined against the international standard SMOW (Standard Mean Ocean Water) as defined by Craig (5).

The results are expressed as the difference per mil ($\delta\,^{18}O$) between the $^{18}O/^{16}O$ ratio in the sample and in the standard SMOW.

$$\delta\,^{18}O = 1000 \times \frac{[^{18}O]/[^{16}O]\ (\text{sample}) - [^{18}O]/[^{16}O]\ (\text{SMOW})}{[^{18}O]/[^{16}O]\ (\text{SMOW})}$$

It is then convenient to express the results of the deuterium analysis in the same unit with [D]/[H] (SMOW) = 155.76×10^{-6}.

$$\delta\,D = 1000 \times \frac{[D]/[H]\ (\text{sample}) - [D]/[H]\ (\text{SMOW})}{[D]/[H]\ (\text{SMOW})}$$

Results and Discussion

The analysis of ^{18}O can be performed directly on the fruit juice, or on the water extracted by total distillation under vacuum in a closed vessel, as in the deuterium analysis. The results of 4 analyses of the same apple juice are given in

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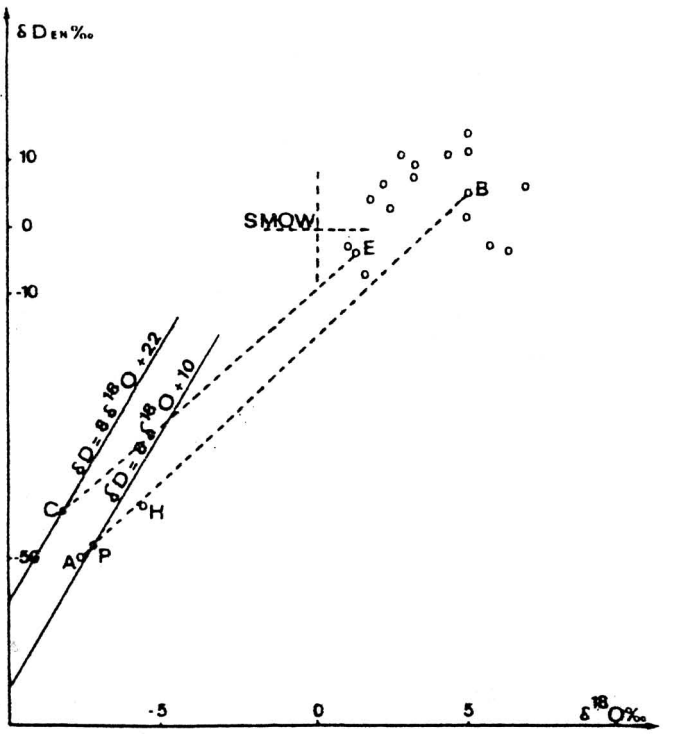

FIG. 1—Deuterium and ^{18}O content in rain water and in orange juice water. Rain water: Corsica (C); Brazil (A); Paris (P). Orange juice water: Corsica (E); Brazil (B); other origins (O). Reconstituted orange juice (H).

Table 1. The results show that the distillation does not introduce any isotopic fractionation, within the limits of the analytical accuracy.

We have analyzed the isotopic composition of different tap waters and different orange and apple juices. The natural variations of the isotopic composition of water have been extensively studied by different authors and particularly by Craig (6). Rain water is lighter than ocean water but there is a correlation between D and ^{18}O content for oceanic rain water: $\delta D = 8\delta\,^{18}O + 10$.

Table 1. Isotopic composition of water from apple juice sample

Sample	δD, °/₀₀	$\delta^{18}O$, °/₀₀
1st distn	−28.0	−3.8
2nd distn	−28.0	−3.6
3rd distn	−28.0	−3.7
No distn	—	−3.6

For Mediterranean rain water the correlation is slightly modified (7): $\delta D = 8\delta\,^{18}O + 22$.

Figure 1 shows the results of the isotopic analysis of rain waters and orange juices of different origins. It appears that orange juices are enriched in heavier isotopes, as compared to rain water. We have estimated this enrichment in 2 cases: (*a*) For Corsica, we observed an enrichment between the irrigation water (C) and the orange juice (E) of 9.5°/₀₀[1] in ^{18}O and 40°/₀₀ in deuterium; (*b*) for Brazil, the enrichment between irrigation water (B) and orange juice (A) is 12.6°/₀₀ for ^{18}O and 55°/₀₀ for deuterium.

If an orange juice is concentrated and then reconstituted, its isotopic content is close to the original content of the water used for dilution. For example, a concentrate (65 Brix) was diluted

[1] °/₀₀ = parts per thousand.

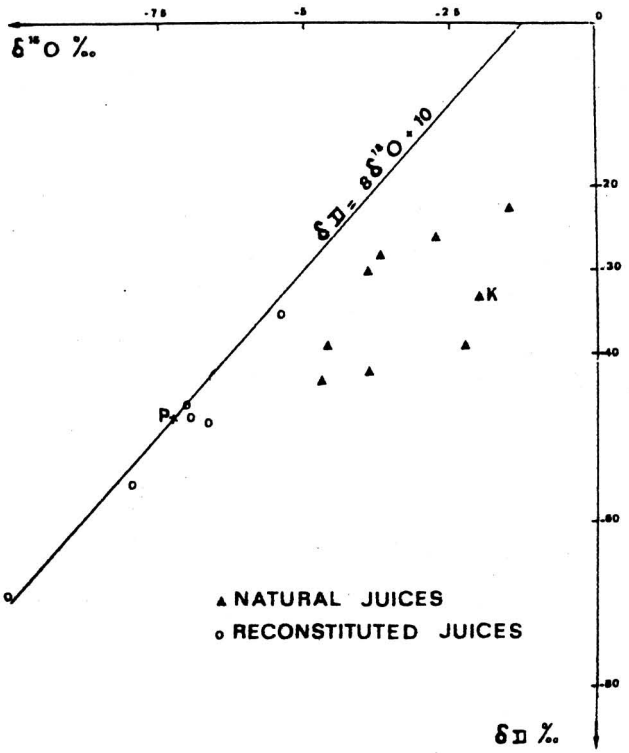

FIG. 2—Deuterium and ^{18}O content in apple juices.

with tap water (P, Fig. 1) up to 12 Brix. The water of this reconstituted juice was determined at point H. This method of analysis allows a very good distinction between natural juices and reconstituted juices and even of their mixtures in equal parts. It also seems possible to detect the addition of water to orange juice of known origin by this method.

We have also investigated the isotopic composition of the water extracted from French apple juices from different parts of the country. The results are represented in Fig. 2. We have observed that all these waters are enriched in heavy isotopes, as compared to rain water. The enrichment factor is much lower than for orange juice. For example, we have compared the main rain water of Paris (P) with an apple juice of the same origin (K). The enrichment factor is 5‰ for ^{18}O and 14‰ for deuterium. The heavy isotopes enrichment in fruit is probably due to evapotranspiration, and this phenomenon is not so intense in France as in citrus-growing areas.

When an apple juice is obtained by dilution of a concentrate, its isotopic composition is very similar to the deuterium and ^{18}O content of the dilution water. On Fig. 2 the positions are indicated of the points representative of the isotopic composition of different apple concentrates diluted with different waters. The origin of the concentrate has no effect and these reconstituted juices show no heavy isotopes enrichment in their water, as compared to the oceanic rain water used for the dilution.

In the control of fruit juices we must consider separately citrus fruit juices and apple juices. The isotopic content of citrus fruit juices is much higher than the isotopic content of reconstituted juices, while no rain water in Europe has an isotope value near SMOW. So it is generally sufficient to measure the relative abundance of

either deuterium or ^{18}O. We preferably measure deuterium because this analysis is performed with an entirely automated mass spectrometer (3) which allows 32 determinations per day. For apple juices it is necessary to measure the relative abundance of deuterium and ^{18}O in order to calculate the deviation of this isotopic composition from the regression equation of rain water.

Acknowledgments

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Symposium on Forensic Toxicology

The European Symposium of the International Association of Forensic Toxicologists will be held at the State University of Ghent, Belgium on August 31–September 1, 1973. The language of the Symposium, as at previous meetings, will be English.

For further information, contact Prof. A. Heyndrickx, Department of Toxicology, State University of Ghent, Hospitaalstraat 13, 9000 Ghent, Belgium.

Analytical Constituents of Strawberry and Raspberry and Their Change in Jam Production

JOHN J. RYAN and JO ANNE DUPONT
Research Laboratories (Foods), Health Protection Branch, Ottawa, Ontario, Canada K1A 0L2

The constituents of strawberry and raspberry have been studied in order to regulate the fruit content of jams made from these fruits. Four constituents, potassium, insoluble solids, amino acids, and phosphorus, were examined to ascertain the variation, means, and minimum acceptable values. From data taken after about 1950 from these laboratories and from the literature, the variation of the first 3 constituents was less than 30% and acceptable, while that of phosphorus was too large and rejected. Strawberry and raspberry fruit samples of known constituent content were processed into jams and the amount of fruit in jam was estimated by using the constituent values. There was little or no difference between the per cent fruit in jams found by using potassium and insoluble solids and the true values determined by weighing. The percentage of fruit using total amino acid values was unreliable and could not be used. Minimum and mean values determined for strawberry jam containing 52% fruit are, for potassium, 45 and 77 mg/100 g and, for insoluble solids, 0.49 and 0.87 g/100 g.

One of the issues which has confronted food chemists over a number of years is the estimation of the amount of fruit in processed fruit products. To date there has been no satisfactory solution to this problem, even though considerable effort has been expended to this end. The most successful method in estimating ingredients in fruit products, e.g., the amount of fruit in jam, has been to analyze a large number of samples for one or more constituents which are intrinsically present in the fruit. If there is no change of the constituents on processing, a statistical treatment of the data as first explained by Steiner (1), combined with the percentage used in the processed food, enables an assignment to be made on the probable limits of the value of these constituents in the food. These limits, calculated so that there is only a small probability that a value will lie outside, are the basis for judging the acceptability of a sample under test for that constituent. This approach is necessary as it is impossible to assign a precise value to most food constituents due to the natural variation which exists from sample to sample. This variation, caused by multiple factors such as cultural practices, varieties, and countries usually comprises a normal distribution.

In order to define the particular constituents which characterize a fruit, it is imperative that the relative variation of constituent values be less than about 30% of the mean. Otherwise the interval of the limits chosen will be so large that many adulterated samples will be included within the chosen limits which are, in fact, not acceptable, as illustrated in Table 1. This table shows that, if there exists a population of constituent values of relative standard deviation of 20% which are then all decreased by, for instance, 30% (as would be the case in dilution), 58% of these new values would overlap with the old. That is to say—58% of new diluted values would be passed as satisfactory, using the old limits, when in fact they are not. Hence a compromise must be chosen between narrow limits and the per cent dilution which can be readily detected. The disadvantages of this approach are that (*1*) a considerable amount of data must be amassed to give a rigorous statistical treatment, (*2*) many constituents show such wide natural variation that they cannot be used, and (*3*) the constituent must not be readily altered by simple addition. For instance, the total acidity of strawberries is not a useful criterion, since organic acids are cheap and regularly used in jam production; a jam deficient in this constituent due to low fruit content could be brought up to standard by simple addition.

Food and drug regulations in Canada state the amount of both strawberry and raspberry which are to be contained in jam made from these fruits. Using the approach outlined, we have studied various constituents of fruit with a view to using them for regulatory purposes. This paper gives constituent values of strawberry and raspberry fruits taken from our recent investigations and from our unpublished data accumulated over the years plus values compiled from the literature. The effect of processing conditions on the selected

Table 1. Proportion of deficient fruit products likely to be passed as satisfactory, using a tolerance level of 95%

% Deficient	Rel. std dev. 10%	20%	30%
10	76	90	93
20	33	79	89
30	3	58	82
40	0	17	70
50	0	4	48

Table 2. Reproducibility of determinations on single samples of strawberry

Component	Mean[a]	Std dev.	Rel. std dev., %
Potassium, mg/100 g	153.1	4.8	3.1
Insoluble solids, g/100 g	1.28	0.06	4.4
Amino acids, mequiv./100 g	6.18	0.48	7.8

[a] Six determinations.

constituent is also assessed. From these data, the best estimate of the lowest acceptable value for constituents of these 2 fruits in jams is then calculated.

Experimental

Material and Methods

Fruit samples were purchased at local markets either as fresh fruit or as unsweetened frozen packs and kept frozen (−10°C) until used. The country of origin was known for each sample.

Jam was made by an open kettle method to a desired soluble solids content reading by refractometer (usually 66%). The exact percentage of fruit in jam was determined by measuring the weight of fruit and finished jam.

Analysis

(a) *Potassium.*—Fruit (3–4 g) or jam (6–7 g) was blended, boiled 1 hr in 50–75 ml distilled water, cooled, and diluted to 100 ml with distilled water. The solution was filtered and 50 ml was collected. Potassium values were then determined by using flame emission photometry at 767 nm.

(b) *Insoluble solids.*—These values were determined by Official Methods of the Food and Drug Laboratories (FO-36, 1956), Ottawa, using 25 g aliquots for jams and 12.5 g aliquots for fruit.

(c) *Amino acids.*—Blended jams and fruits were extracted as in 22.003 (2). The water extract (250 ml), corresponding to 100 g jam or 50 g fruit, was clarified by filtering through Celite and titrated to pH 8.4 with NaOH as in 22.114. Then 100 ml 37% formaldehyde was added so that the ratio of extract to formaldehyde was 2.5:1. The resulting solution was retitrated to pH 8.4 with 0.1*N* NaOH.

(d) *Phosphorus.*—This element was determined by ashing blended samples and using the molybdenum blue colorimetric method described by Vandercook and Guerrero (3).

Results and Discussion

Of the many chemical constituents which could be measured in fruit, only potassium, insoluble solids, amino acids, and phosphorus were considered likely to fulfill the desired criteria as outlined. However, from earlier data, it appeared that the natural variation of phosphorus would be large and therefore this element was studied less. The precisions of the methods used to determine values for the other 3 constituents were satisfactory (Table 2). Two of the constituents, potassium and insoluble solids, can be measured with less than 5% error while the amino acid variation is less than 10%. This experimental error is included in the total variation as measured on the various samples of diverse origin. Of these 4 methods, considerable difficulty was experienced in obtaining reproducible values for the amino acid content by formol titration (22.114). In this method, a water extract of the fruit is titrated to pH 8.4, formaldehyde is added, and the solution is retitrated to the same pH. We found that the water extract of fruit had to contain a certain percentage of fruit, the ratio of added formaldehyde to water extract had to be kept constant, and the titration had to be carried out without delay in order to obtain reproducible results.

In selecting analytical values of constituents from the literature it was decided that only individual values and not averages would be used as these lead to a more rigorous statistical treatment. In addition, there is evidence available that most analytical values for strawberry have decreased in the last 20 years due to a greater water content in berries. Because of this change (associated with cultural practices) all literature values and our own values prior to approximately 1950 were discarded. The data in Table 3 are compiled from our laboratories and the published work of Boland *et al.* (4) and Zubeckis (5) and include fruit from Canada, United States, and Mexico. These results show that potassium has the lowest relative deviation from the mean and phosphorus

Table 3. Compilation of constituent values in strawberry and raspberry

Constituent	$\bar{x}$	Rel. std dev., %	n[a]	Range	Tolerance[b]
Strawberry					
Potassium, mg/100 g	149	20	39	100–198	87
Amino acids, mequiv./100 g	1.05	31	77	0.43– 2.37	0.40
Insoluble solids, g/100 g	1.68	22	117	0.76– 2.74	0.94
Phosphorus, mg/100 g	13.1	34	36	7.4 –23.1	3.3
Raspberry					
Potassium, mg/100 g	166	16	37	121–236	110
Amino acids, mequiv./100 g	2.19	27	34	1.13– 4.00	0.90
Insoluble solids, g/100 g	4.93	28	51	2.52– 9.37	2.05
Phosphorus, mg/100 g	15.0	30	21	10.2 –26.9	4.1

[a] n = number of samples.
[b] 95% of population greater than or equal to this value with P = 0.05.

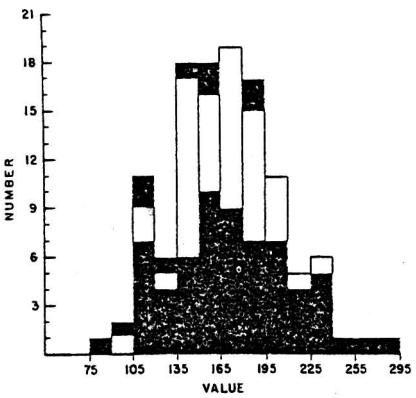

FIG. 1—Histogram for insoluble solids in individual strawberry samples (mg/100 g); n = 117.

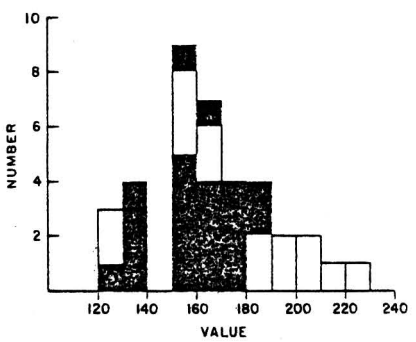

FIG. 2—Histogram for potassium in individual raspberry samples (mg/100 g); n = 37.

the highest. The tolerance value states that, in the case of potassium, 95% of the population will have a value equal to or greater than 87 with a certainty of 95%. Because of the high scatter of the individual values for phosphorus this constituent was not studied further, since its tolerance value would include too many unacceptable values, e.g., the samples could be diluted 50% and over half of these would still fall within acceptable limits. Histograms for insoluble solids in strawberry and potassium in raspberry (Figs. 1 and 2) show that these values approximate a normal population with most values near the mean.

With these data, both the correlation and linear combination of values were studied. If 2 constituents are highly positively related (correlation coefficient $r \sim 1$) then nothing is to be gained by measuring both, since they are related by a constant factor. There was also the possibility that the correlation could be used to construct a linear combination of components, $X_c = X_1 + KX_2$, such that the variation of this new variable was less than either of its components according to the method of Steiner (6). The results in Table 4 show that potassium and insoluble solids are positively correlated and potassium and amino acids less so. Linear combination of any of these constituents gives only a slight reduction in the relative standard deviation in some cases and, in others, none at all. Due to the high positive correlation this statistical treatment of linear combination of variables could not be used in setting narrower tolerance limits.

The change of the chosen constituents, potassium, insoluble solids, and amino acids, on

Table 4. Correlation and combination of analytical values

Constituent, x/y	V_x/V_y[a]	n[b]	r[c]	V_c[a]
Strawberry				
Potassium Insoluble solids	20.0 25.1	39	+0.77[c]	19.4
Potassium Amino acids	15.3 22.0	17	+0.26	14.4
Raspberry				
Potassium Insoluble solids	11.3 17.9	22	+0.67[c]	11.5
Potassium Ash	11.3 11.4	16	+0.90[c]	11.4

[a] Relative standard variation of variables x, y, and c.
[b] n = number of samples.
[c] Significant at P > 0.05.

Table 5. Differences between experimental and actual values for per cent fruit in jam

	Strawberry			Raspberry		
Constituent	d[a]	Std dev.	n[b]	d[a]	Std dev.	n[b]
Potassium	+1.8	3.6	6	−4.5	3.7	4
Insoluble solids	+4.7	3.6	9	−1.0	8.5	4
Amino acids	+7.7	11.3	9	−4.3	3.5	4
Phosphorus	−10.1	9.7	5	−5.4	0.7	3

[a] Difference between experimental and actual fruit content.
[b] n = number of samples.

Table 6. Minimum and mean values for potassium and insoluble solids in jams

	Strawberry		Raspberry	
Component	Mean	Min.[a]	Mean	Min.[a]
Potassium, mg/100 g	77	45	75	50
Insoluble solids, g/100 g	0.87	0.49	2.22	0.92

[a] Based on 95% tolerance and strawberry and raspberry jams of 52 and 45% fruit, respectively.

processing into jams was then studied. Nine independent samples of strawberry and 4 of raspberry, whose analytical constituents had previously been measured, were processed into pure fruit jams by standard methods. These jams, containing a known percentage of fruit determined by weighing, were then analyzed for the 3 constituents of interest. The small amounts of both pectin and acid used in the jam process were shown not to affect the constituent values. The differences between the per cent fruit in jam determined from the constituent values and the per cent fruit in jam calculated by weighing were determined (Table 5). The fruit content calculated from the potassium values of strawberry differ the least from the actual values and the scatter of differences is not large even though, in the case of some Canadian fruit with low total amounts of all constituents, the differences were high. With the insoluble solids the differences were again on the positive side, although the deviation, particularly with Canadian fruit, is larger. This difference in percentage of fruit of the jam calculated by using the insoluble solids was not caused by interference from pectin, precipitation of a substance from the juice, use of a small aliquot, or the amount of extracting liquid.

The fruit content calculated from the amino acid values in Table 5 for strawberry is higher than the preceding 2 constituents and shows a larger deviation. In addition, the method used in this determination is suspect, particularly when the titer values are small. This poor reproducibility was not due to anything inherent in the jam making, such as the size of aliquot taken, pectins, acids, or heat treatment. A mixture of standard amino acids similar in kinds and levels to those which occur naturally in these fruits (7) was prepared and titrated as in the formol method. Erratic and non-reproducible values were obtained which did not correspond to the known amino acid content. Part of the discrepancy was traced to the dilution of water with the formaldehyde (2.5:1 ratio) which produced an acidity which was definite but not consistent. Subtraction of a blank value did not give true results. Because of the erratic effects of the method and large deviations from true values the total amino acid value could not be used for estimating percentage of fruit in jam. Moreover, these results cast doubt on use of this method in berry fruits. Some representative values for phosphorus are included in Table 5 but the differences are markedly low. Much the same trend of results was shown with raspberry fruit although the number of samples was small and the results are not as reliable.

The only work available for comparison of these values is that of Osborn (8) who reported analytical values for potassium and phosphorus in a series of fruits and jams. The actual per cent

composition of jam is not stated but the values found by using potassium were close to or equal to those found by other criteria while the results obtained by using phosphorus were low; both are in agreement with the results here. Because the changes in processing fruit into jam for both potassium and insoluble solids are small and within experimental error, it is believed that the minimum amount of fruit in a jam can be estimated by direct comparison of the 2 constituent values found in a jam with those minimum values known to occur in a series of fruit without any correction for processing. In Canada, where the minimum amount of fruit in strawberry and raspberry jam is 52 and 45%, respectively, the minimum and mean values for potassium and insoluble solids so calculated are shown in Table 6. Thus it can be said that, from the available data, strawberry jam prepared to contain 52% fruit will contain a mean potassium value of 77 mg/100 g for a series of samples and the minimum value with 95% confidence is 45 mg/100 g. A very small fraction of authentic samples will fall below this minimum value but a large portion of genuine samples can be substantially diluted and still have constituent values greater than the minimum. It is believed that these minimum values represent the best that are available with the present techniques and data. It is realized that a greater number of samples with a wider selection of years, countries, and seasons would be desirable as well as constituents with less natural variation but these 2 requirements can not be met at the present time.

This paper was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

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VITAMINS AND OTHER NUTRIENTS

Use of Heat to Saponify Xanthophyll Esters and Speed Analysis for Carotenoids in Feed Materials: Collaborative Study

FORREST W. QUACKENBUSH
Department of Biochemistry, Purdue University, W. Lafayette, Ind. 49707

Twelve collaborators analyzed 4 pairs of samples to compare a 20 min hot (56°C) saponification technique with the overnight extraction technique at room temperature in the official first action method for analysis of carotenoids in feed materials, 39.018–39.023, with a view to (a) make the method applicable to products which contain xanthophyll esters and (b) speed up the analysis. In general, the collaborators were in good agreement, with saponification of esters essentially complete after the 20 min heating of samples that contained marigold meal. While the sum of extracted pigments from corn gluten and alfalfa meal tended to be slightly higher with the longer (overnight) contact with the solvent than with the hot extraction, the differences were not statistically significant. Significant differences between hot and cold treatments were absent also in alfalfa pigment fractions, but hot saponification significantly lowered monohydroxy pigments and increased dihydroxy pigments in extracts from corn gluten. The results showed good precision with either extraction technique. The method, modified to include hot saponification as an alternative step, is now applicable to all dried feed materials, including those which contain xanthophyll esters (e.g., marigold meal); the modification has been incorporated into the official first action method, 39.018–39.023.

The current official first action method for carotenoid analysis in feed materials, 39.018–39.023, has now been in use with good satisfaction for a 3 year period. The chief advantages of the method over others are (*a*) its applicability to both corn gluten and alfalfa meal and to mixed feeds which contain these products and (*b*) exclusion of non-pigmenting polyoxy pigments. Its measurement of dihydroxy pigment (DHP) equivalents has shown good correlation with biological effectiveness in skin pigmentation of broilers (1). Disadvantages of the method are that (*a*) the overnight extraction technique precludes its use in some industrial laboratories where a quick result is desired, and (*b*) while the cold extraction technique saponifies chlorophylls it does not effect saponification of xanthophyll esters which are present in such materials as marigold meal and citrus by-products. Esterified xanthophylls behave like monohydroxy pigments (MHP) or carotenes on chromatography. A study in the Associate Referee's laboratory (2) indicated that a 20 min treatment at 56°C could effect saponification of the xanthophyll esters and eliminate the necessity of overnight extraction, without significant reduction in pigment yield. This modification, which should extend the usefulness of the method substantially, now has been subjected to collaborative study.

Collaborative Study

Eight test samples and a sample of the dye for instrument standardization were delivered to each of 13 collaborators. A ninth sample which contained a mixture of feed materials, including marigold meal, was included as a practice sample. Sample I was corn gluten from a lot currently in use by a local feed manufacturer. Sample II was the same fresh corn gluten blended with 10% of an older sample. Sample IV was a commercial dehydrated alfalfa meal, received April 24, 1972. Sample III was the same sample of alfalfa meal blended with 30% of an older sample of commercial dehydrated meal. Sample V was a commercial marigold meal, received May 8, 1972. Sample VI was the same marigold meal blended with 20% of an older sample. Sample VII was a commercial poultry supplement blended with 10% of Sample IX. Sample VIII was the same commercial poultry supplement with 20% of Sample IX. Sample IX, the "practice sample," was a blend of Samples I, IV, and V. Instructions to collaborators are summarized as follows:

Refrigerate the 9 samples immediately. All samples were ground to pass a No. 40 sieve and are ready for analysis. After checking spectrophotometer with dye, analyze Sample IX to assure methodology is well in hand; the mixture

should yield (mg/lb) approximately 55 carotenes, 20 MHP, and 275 DHP. Then make one analysis of each of the 8 other samples, using the hot saponification technique. Using silica gel G column, analyze for total carotenes, MHP, and DHP. Repeat analysis of samples I–IV, with overnight saponification as the only change. To minimize variations due to carotenoid losses during storage, complete analyses on June 1 or as close to that date as possible. Specific procedural instructions are enclosed (see *Recommendations*).

Results and Discussion

Twelve collaborators reported. All completed the analyses as requested (Tables 1 and 2), within approximately a 2 week period prior to and after June 1, 1972.

In general, the results from the different collaborators agreed quite well, and no collaborator was sufficiently divergent from the others to require that his data be discarded as a whole. Some outliers were clearly evident, however. Techniques applied in the decision to discard data as unreliable were (*a*) the ranking test of laboratories (3), (*b*) the 2-sample plot (3), and (*c*) the Dixon test (4). For apparent outliers as observed from these approaches, first scrutiny was on the sum of the 3 fractions (carotenes, MHP, and DHP) which should be a measure of pigment deterioration and efficiency of extraction but should not reflect effectiveness of saponification of xanthophyll esters. A preliminary test, using all data, showed no significant differences between the sum of reported values for hot saponification and those for overnight extraction.

Rank test results (range limits, 12 to 144) showed that, while the results of Collaborator 3, with a score of 22, were usually higher and those of Collaborator 12, with a score of 130, were usually lower than the others, neither was sufficiently biased to be classed as a consistent outlier on total pigments reported. However, 2-sample plots indicated results of the following to be out of acceptable range: Collaborator 1 for Sample IV; Collaborator 3 for Sample I; Collaborator 9 for Sample VIII; and Collaborator 12 for Sample I. All data for these specific cases (underlined values in Table 1) were finally discarded on the basis of the Dixon test. Suspected values for individual pigment fractions were then subjected to the Dixon test and those which failed ($P < 0.05$) were underlined and excluded from subsequent statistical evaluation. A special case was the results of Collaborators 2 and 3 for Samples VII and VIII in which the carotene values proved to be outliers. It was reasoned that, since the sum of their reported pigments was within acceptable range, the fractionation was inadequate and that the MHP and DHP values therefore were also unreliable. The values were discarded accordingly.

When a value was discarded for any reason the collaborator's result for the other sample of the same material was automatically excluded from statistical treatment, since all results were calculated on a paired-sample basis. This experimental design, using sample pairs, provides a more reliable evaluation of precision than is obtainable with duplicate analyses of the same sample (3).

The standard deviations (Tables 1 and 2), although relatively high when compared to values for more stable components, are considered acceptable for labile substances such as the carotenoids. They reflect primarily collaborator bias rather than lack of precision as shown in Table 3. Coefficients of variation of AAFCO alfalfa meal check samples are usually much larger than those reported in Table 1.

In this study of alfalfa meals, collaborators showed less bias (S_b^2 and F-ratio, Table 3) than in the earlier study with the official method (5). For the corn gluten data they showed slightly more bias than in the earlier study. There is no apparent pattern of differences in either precision or systematic error between hot and cold treatments.

Mean values for overnight extraction tended to be slightly higher than those for hot saponification. This tendency toward higher results with a longer extraction period had been observed earlier (6) and may reflect a more exhaustive solubilization of pigments during extended contact with the solvent mixture. However, the differences in sums of pigments reported were not statistically significant for either the corn gluten or the alfalfa meal pair. Furthermore, in the alfalfa pair, none of the pigment fractions differed statistically. However, in the corn gluten pair, the hot saponification treatment gave lower MHP values ($P < 0.01$) and higher DHP values ($P < 0.05$) than the overnight treatment. This evidence that a small percentage of corn xanthophylls occurs in ester form is consistent with an earlier observation (7) in which the mean value as ester in 5

Table 1. Collaborative test results comparing hot and cold saponification techniques for 4 samples[a]

	Corn gluten — sample I								Alfalfa meal — sample III							
	Hot saponification				Cold saponification				Hot saponification				Cold saponification			
Coll.[b]	Car.	MHP	DHP	Sum	Car.	MHP	DHP	Sum	Car.	MHP	DHP	Sum	Car.	MHP	DHP	Sum
1	26	21.1	125	172	31.8	24.2	126	182	78.7	9.2	126	214	70.2	8.0	145	223
2	30	24	125	179	33	28	122	183	70	7	106	183	76	8	118	202
3	29	27	127	183	40	36	163	239	73	10	123	206	64	7	133	204
4	30.4	21.6	127.4	179	31.8	30.1	113.0	175	77.6	11.1	113.0	202	75.3	8.4	119.0	203
6	26	20	124	170	28	27	125	180	73	6	113	192	72	8	121	201
7	26.4	20.0	124.8	171	30.1	27.8	121.0	179	73.0	5.9	116.3	195	76.2	7.1	119.6	203
8	23.6	17.3	125.3	166	31.6	26.3	128.2	186	68.0	6.5	117.8	192	74.8	9.0	125.7	210
9	25.1	20.8	130	176	28.1	20.8	130.0	179	69.5	8.0	123	200	66.5	8.0	113	188
10	26.7	21.4	124	172	30.9	25.6	120	176	67.1	6.5	108	182	70.4	7.7	118	196
11	28.5	20.3	125.9	175	31.4	29.5	124.2	185	71.8	5.7	110.6	188	73.8	10.7	121.3	206
12	38.5	22.3	93.4	154	31.9	29.3	90.6	152	68.0	6.5	98.2	173	79.4	7.6	89.1	176
13	22.7	20.8	125.5	169	26.7	26.4	119.9	173	69.5	6.2	116.0	192	68.0	7.4	113.5	189
Mean	26.7	21.3	126	174	30.4	26.6	123	180	72	7.4	114	193	72	8.1	120	200
	Corn gluten — sample II								Alfalfa meal — sample IV							
1	28.2	20.8	123	172	29.5	25.8	117	172	115	9.5	164	288	106	8.5	189	304
2	26	23	120	169	31	27	116	174	96	8	143	247	101	9	151	261
3	28	22	131	181	35	30	119	184	101	13	163	277	106	14	158	278
4	28.4	22.1	115.4	166	28.9	26.5	108.2	164	98.4	9.6	140.7	249	101.3	9.6	151.5	262
6	26	20	120	166	27	25	115	167	98	7	142	247	101	10	150	261
7	25.5	17.7	121.5	165	28.4	25.7	114.0	168	100.2	7.3	146.0	254	103.4	8.2	153.0	265
8	23.3	17.6	121.0	162	29.8	24.8	120.3	175	97.5	8.9	150.0	256	98.0	9.5	155.0	262
9	25.1	19.0	128	172	25.1	19.0	115	159	97.6	8.6	155	261	91.8	9.2	152.0	253
10	26.6	18.9	116	162	28.3	24.2	112	164	90.2	8.2	140	238	92.8	9.1	146	248
11	25.9	19.0	120.6	166	25.9	23.6	119.9	169	95.9	7.1	139.3	242	98.5	9.5	155.4	263
12	28.1	22.3	105.4	156	30.1	26.0	93.4	150	98.5	8.2	134.0	241	96.4	8.8	114	219
13	20.4	20.0	114.0	154	21.9	23.3	110.8	156	88.1	9.7	144.5	242	90.5	8.6	142.9	242
Mean	26.4	20.2	120	166	28.4	25.1	115	167	97	8.7	145	250	98	9.6	151	256
Std dev.	1.7	2.8	4.4	7.9	3.3	3.3	5.8	7.2	3.9	2.3	10.6	14.2	5.2	3.9	6.7	18.3
Coeff. of var.	6.5	13.7	3.6	4.6	11.1	12.7	4.8	4.1	4.6	29.0	8.2	6.3	6.2	44.3	4.9	8.0

[a] Car., carotenes; MHP, monohydroxy pigments; DHP, dihydroxy pigments; Sum, sum of reported values. All data are expressed as mg/lb.

[b] Collaborator 5 did not report. Underlined values are not included in mean value, standard deviation, or coefficient of variation, since they were discarded as outliers; *see* text for details.

Table 2. Collaborative test results with hot saponification techniques, using samples containing xanthophyll esters[a]

	Marigold meal — sample V				Com. feed — sample VII			
Coll.[b]	Car.	MHP	DHP	Sum	Car.	MHP	DHP	Sum
1	0	0	5948	5948	9.1	2.2	27.6	39
2	41	59	6603	6703	25	5	17	47
3	66	85	6422	6573	15	8	27	50
4			6300	6300	10.5	2.1	29.5	42
6	0	0	6640	6640	10	2	32	44
7	0	37.7	6630	6668	8.9	3.2	30.4	42
8	120.8		6130	6251	8.2	2.0	31.6	42
9	0	0	5985	5985	9.6	8.9	14.4	33
10	10.4	60.5	6200	6271	8.4	1.9	29.7	40
11	0	87.7	6723	6811	8.9	2.2	32.5	44
12	0	276	5720	5996	10.8	8.2	21.1	40
13	0	144	6160	6304	9.9	8.5	22.4	41
Mean	—	—	6288	6371	9.4	4.1	27.1	42
	Marigold meal — sample VI				Com. feed — sample VIII			
1	0	0	6253	6253	17.2	4.0	60.8	82
2	27	62	6000	6089	50	10	21	81
3	41	106	6362	6509	47	9	33	89
4			5771	5771	15.6	2.7	56.5	75
6	0	0	6180	6180	16	4	57	77
7	0	18.8	6046	6065	16.6	1.2	58.2	76
8	115.0		6030	6145	14.6	3.6	57.5	76
9	0	0	6080	6080	15.5	12.2	33.2	61
10	11.4	56.7	5700	5768	14.3	3.4	54.8	72
11	0	87.7	6177	6265	15.2	3.2	60.2	79
12	0	268	4910	5178	18.8	14.9	44.0	78
13	0	162	5520	5682	17.5	17.6	41.5	77
Mean	—	—	5919	5999	16.2	6.1	54.5	78
Std dev.	—	—	—	39.5	1.6	6.1	7.5	5.1
Coeff. of var.	—	—	—	0.6	12.1	112	18.1	8.5

[a, b] See footnotes, Table 1, for further detail.

Table 3. Variances of the data from 4 pairs of samples

Pair	Extn	Pigments	2-sample mean, %	Precision S_r^2	Std dev. S_d^2	Systematic S_b^2	*F*-ratio
Corn gluten	hot	Carotenes	26.6	1.4	3.0	0.8	0.6
Corn gluten	hot	MHP	20.8	1.6	8.1	3.2	2.0
Corn gluten	hot	DHP	123.	10.9	19.2	4.2	0.4
Corn gluten	hot	Sum	170.	11.8	62.4	25.3	2.1
Corn gluten	cold	Carotenes	29.4	1.3	10.6	4.6	3.6
Corn gluten	cold	MHP	25.8	1.9	10.8	4.4	2.3
Corn gluten	cold	DHP	119.	4.5	33.2	14.4	3.2
Corn gluten	cold	Sum	174.	6.2	51.6	22.7	3.7
Alfalfa meal	hot	Carotenes	84.	11.6	15.0	1.7	0.2
Alfalfa meal	hot	MHP	8.0	0.9	5.4	2.2	2.4
Alfalfa meal	hot	DHP	130.	8.6	112.6	52.0	6.0
Alfalfa meal	hot	Sum	222.	20.9	198.0	88.0	4.2
Alfalfa meal	cold	Carotenes	85.	19.6	27.5	4.0	0.2
Alfalfa meal	cold	MHP	8.8	2.0	15.2	6.6	3.3
Alfalfa meal	cold	DHP	136.	7.9	45.1	18.6	2.4
Alfalfa meal	cold	Sum	228.	34.3	336.	151.	4.4
Marigold meal	hot	Sum	6186.	616.	1560.	472.	0.8
Com. feed	hot	Carotenes	12.8	0.6	2.4	0.9	1.5
Com. feed	hot	MHP	5.4	5.7	37.	15.6	2.7
Com. feed	hot	DHP	41.5	6.8	57.	25.1	3.7
Com. feed	hot	Sum	60.	5.4	26.3	10.4	1.9

inbreds was 5% of the total xanthophylls, or equivalent to about 25% of the monohydroxy pigments. The difference in the MHP 2-sample mean per cent, 25.8 (overnight) vs. 20.8 (hot saponification), apparently reflects this (Table 3). As pointed out by Collaborator 11, this represents an inaccuracy when the official method is applied to corn products. The calculated DHP equivalent for corn gluten samples in this study is 1.1% higher when derived from the hot saponification data than when derived from the overnight data. This known error is probably small in relation to unknown errors in carotenoid methodology. It can be avoided by using the hot saponification step.

Marigold meal presents a special analytical problem because the esterified DHP fraction constitutes over 90% of the carotenoids present (2). A 50 mg sample, an appropriate size for analysis of this fraction, provides only trace amounts of other pigments. The reported data for carotenes and MHP, therefore, are not meaningful. Values from the different laboratories for DHP and sum of pigments were in good agreement.

The sum of pigments extracted by the different analysts from the commercial feed samples was quite consistent, despite the relatively low pigment concentration in these materials. The fractionation data suggest that some collaborators, especially 2 and 3, did not have optimal conditions for saponification, although the majority agreed well in all respects. Control of time and temperature is undoubtedly quite critical.

In the absence of differences in precision and systematic error, and without statistically significant differences in the sums of pigments obtained from the different materials, one concludes that either technique, hot or cold, is usable for samples which do not contain substantial amounts of xanthophyll esters. The hot extraction must be used when esters predominate as in marigold meals or feeds that contain them.

Comments of Collaborators

Collaborator 1, and several others, commented that the method is straightforward and procedural details were followed without difficulty. Collaborator 2 selected all aliquots to give A values of 0.35 to 0.725. He felt that his laboratory had a low bias for MHP. Collaborator 3, and several others, commented that carotene and MHP bands were not visible on the columns of Samples V and VI. He connected a water-cooled condenser to the volumetric flask with Tygon tubing. Collaborator 6 did not receive the dye, and hence did not calibrate his instrument on the day of analysis. He thought that the column of adsorbent should be longer and that a larger volumetric flask should be used for MHP. Collaborator 10 preferred a 50 ml flask for collection of DHP and commented that room temperature extraction for 1 hr appears to be adequate. Collaborator 11 commented (*a*) that hot saponification of alfalfa meal produced large losses of MHP, perhaps due to isomerization and incomplete extraction; (*b*) that hot saponification of corn gluten lowered MPH and tended to raise DHP, probably reflecting the presence of esters and that, if so, the AOAC method is inaccurate for corn products; (*c*) that the collaborative study lacked a basis for determining whether hot saponification may have produced losses in the marigold meal, mixed feed, or practice sample; (*d*) that the alfalfa meal samples were not typical good quality dehydrated meal samples, and that generally alfalfa meals do not give a sharp MHP band on silica gel G; (*e*) that in view of these comments the hot saponification method is unsatisfactory for alfalfa, corn gluten, or mixed feeds. Collaborator 12 used 2 different lots of adsorbent, stating that one gave sharper separation of bands than the other. Collaborator 13 increased the ratio of Super-Cel to silica gel G to allow faster analysis. For a 56° bath, flasks were placed near the perimeter of a pie plate in which ¾″ water was heated with a small hot plate.

Recommendations

It is recommended—

(1) That Section 39.020 of the official first action method for carotenes and xanthophylls in dried plant materials and mixed feeds, 39.018–39.023, be modified to read as follows:

39.020 ***Preparation of Sample***

Grind sample to pass No. 40 sieve. Accurately weigh sample (2 g corn gluten or alfalfa meal; 50 mg marigold meal; 4 g mixed feed) into 100 ml vol. flask. Pipet 30 ml extractant into flask, stopper, and swirl 1 min. For low moisture samples, e.g., marigold meal, dehydrated alfalfa, or corn gluten (not air-dried samples), also pipet 1 ml H_2O/2 g sample into flask, stopper, and swirl 1 min. For high moisture (air-dried) samples, omit addn of H_2O.

(a) *Hot saponification.*—(For rapid extn and for samples contg xanthophyll esters.) Pipet 2 ml (4 ml

for 4 g sample of mixed feed) 40% methanolic KOH into flask, swirl 1 min, and place flask in 56° H_2O bath 20 min. Attach air condenser or cool neck of flask to prevent loss of solv. Cool sample, and let stand in dark 1 hr. Pipet 30 ml hexane into flask, swirl 1 min, dil. to vol. with 10% Na_2SO_4, and shake vigorously 1 min. Let stand in dark 1 hr before chromatgy. Upper phase is 50 ml.

(b) *Cold (overnight) saponification.*—(For samples not contg xanthophyll esters.) Let mixt. stand in dark ca 16 hr. Pipet 2 ml 40% methanolic KOH into flask and swirl 1 min; let stand in dark 1 hr; then proceed with hexane addn as in (a).

(2) That attention be given to newer methods for carotenes in plant materials with a view toward improvement of 39.014–39.017.

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Materials were kindly supplied as follows: marigold meal, Florafil S.A., Liverpool 143–201, Mexico 6, D. F., Mexico; alfalfa meal, Western Alfalfa Co., 1222 Colfax St., Schuyler, Neb.; corn gluten meal and commercial feed, Indiana State Chemist, Purdue University, W. Lafayette, Ind.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D and were adopted by the Association; *see* (1973) *JAOAC* 56, 403.

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Calculation of Turbidimetric Microbiological Vitamin Assay Results, Using an APL/360 Computer Program

GREGORY V. BROLUND, ETHELBERT W. HASKINS, and GEORGE A. HUDSON
Division of Nutrition, Food and Drug Administration, Washington, D.C. 20204

An APL-360 computer program is described for the calculation of results from microbiological vitamin assays. The results are compared to manual calculations specified in official AOAC methods. This comparison shows the validity of the computer program for these calculations.

Computerized calculation of microbiological assay results offers an objectivity and degree of accuracy that may be lacking when manual curve plotting and visual interpolation are performed.

Application of computers to perform this task has been described by Tsuji and coworkers (1) by calculation from a linearized standard response curve. This method may not be generally applicable to all possible curves. Berg and Behagel (2) have also reported the use of a computer to obtain results of microbiological assays. Punch cards were used to enter data into an IBM 360/50 computer. Details of their program were not published.

In writing the following program we have incorporated all criteria of acceptability specified in 39.084 of *Official Methods of Analysis* (3). The program is written in Applied Programming Language (APL). A flow chart and a complete listing of the program are shown in Figs. 1 and 2 respectively.

Five standard dose levels are required. The computer program is designed to accept a varied number of replicates within each dose level. The average per cent transmittance of each standard level is then calculated and treated as a point on a point-to-point curve.

The sample is set up at 4 dose levels in duplicate. The program requires 8 transmittance readings for each sample. If, for any reason, a reading is missing, a zero must be substituted for the missing reading in the input. Per cent transmittance of each sample tube is matched to the appropriate segment of the standard curve. Equivalent standard concentration per tube (Del (I)) is calculated by the formula:

$$\text{Del } (I) = (T\ (I) - S_a)/(S_b - S_a),$$

where $T\ (I)$ is the per cent transmittance of sample tube (I), and S_a and S_b are the 2 points on the standard curve that describe the segment on which $T\ (I)$ is located.

Del (I) for each tube is then checked to determine if it is equivalent to less than 0.5 or more than 4.5 ml standard response. Values outside these limits are discarded, as prescribed in *Official Methods of Analysis*.

The values between 0.5 and 4.5 ml standard response are converted to vitamin content/ml assay solution and averaged. According to *Official Methods of Analysis*, values that vary by more than 10% from this average are discarded. A new average is then taken from the final remaining values and the potency of the sample is calculated.

The calculation of 3 turbidimetric cyanocobalamin assays performed as prescribed in *Official Methods of Analysis* is used to demonstrate the application of this program. Turbidity as per cent transmittance at 550 nm was read for each tube, using a Bausch & Lomb Spectronic 20 spectrophotometer. Readings for standard tubes, standard identity, and potency and, in turn, sample identification numbers, sample tube readings, and dilution factors were manually transmitted to an IBM 360 computer. Transmission was via an IBM 2741 Communications Terminal (Armonk, N.Y.) equipped with a Bell System Data Phone.

Figure 3 is a printout of an assay with all 8 tubes acceptable under AOAC criteria. Figure 4 shows an assay with some tubes discarded for failure to meet the $\pm 10\%$ of the average requirement. In Fig. 5, readings were rejected for being equivalent to less than 0.5 ml of the standard. Discarded tubes are represented in the printout as a zero under "ml equiv std conc of tubes used." The number of tubes used to calculate potency is also specified in the printout. An acceptable assay is defined as one in which at least two-thirds of the tubes are within AOAC limitations.

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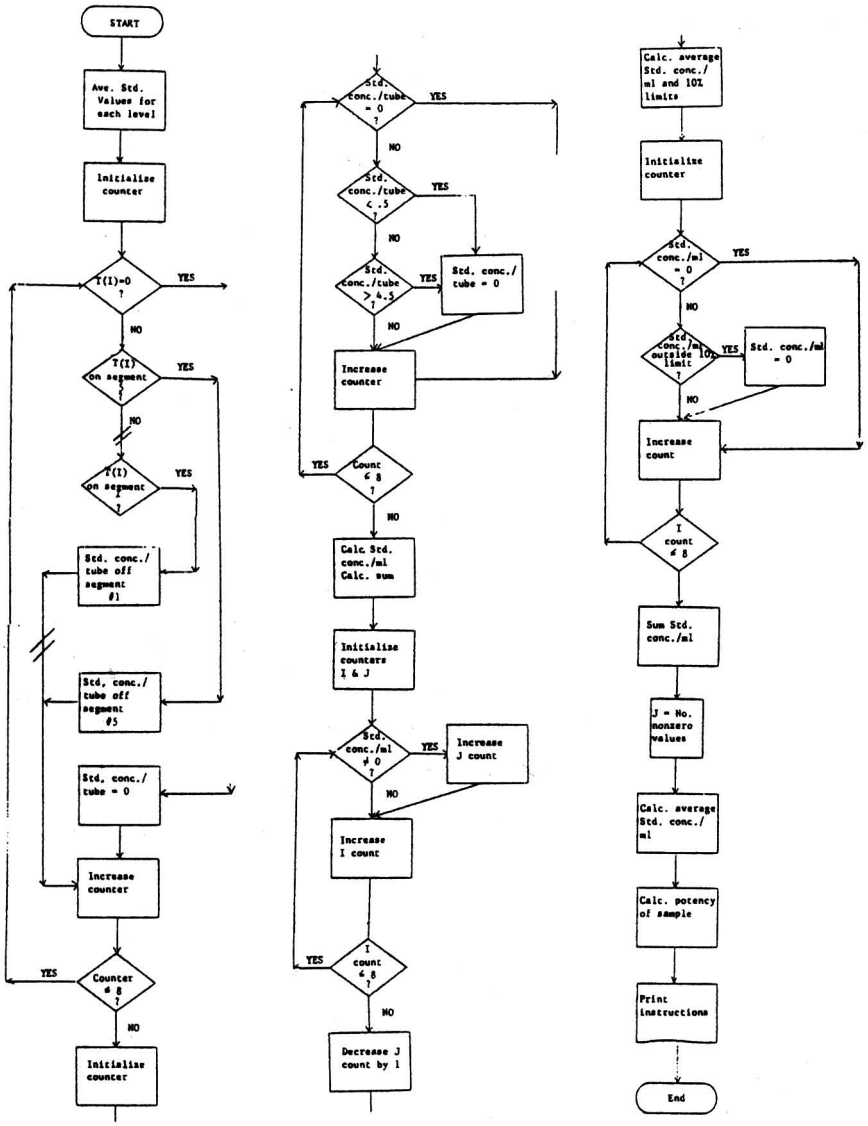

FIG. 1—Flow chart of the APL-360 program.

The printout also includes all input data, the average equivalent standard concentration/ml, the prescribed 10% limits, the standard concentration/ml of each tube used, and the assay potency with the appropriate units.

Table 1 shows the results of each assay calculated manually and by the computer program. The results of the 2 methods of calculation are within 0.17–0.33%.

The described program parallels each step that is performed in manual calculation of microbiological results. Although this program has been described for turbidimetric assays, with modification it would be equally applicable to those per-

```
    ∇ TEST
[1]   S1←(+/SA1)÷(ρSA1)
[2]   S2←(+/SA2)÷(ρSA2)
[3]   S3←(+/SA3)÷(ρSA3)
[4]   S4←(+/SA4)÷(ρSA4)
[5]   S5←(+/SA5)÷(ρSA5)
[6]   DEL←⍳8
[7]   I←1
[8]  GB1:→(T[I]=0)/L20
[9]   →(T[I]<S4)/L5
[10]  →((T[I]<S3)∧(T[I]≥S4))/L4
[11]  →((T[I]<S2)∧(T[I]≥S3))/L3
[12]  →((T[I]<S1)∧(T[I]≥S2))/L2
[13]  →(T[I]≥S1)/L1
[14] L1:DEL[I]←|(T[I]-100)÷(S1-100)
[15]  →L6
[16] L2:DEL[I]←|((T[I]-S1)÷(S2-S1))+1
[17]  →L6
[18] L3:DEL[I]←|((T[I]-S2)÷(S3-S2))+2
[19]  →L6
[20] L4:DEL[I]←|((T[I]-S3)÷(S4-S3))+3
[21]  →L6
[22] L5:DEL[I]←|((T[I]-S4)÷(S5-S4))+4
[23]  →L6
[24] L20:DEL[I]←0
[25] L6:I←I+1
[26]  →(I≤8)/GB1
[27]  I←1
[28] GB2:→(DEL[I]=0)/L8
[29]  →(DEL[I]<0.5)/L7
[30]  →(DEL[I]>4.5)/L7
[31]  →L8
[32] L7:DEL[I]←0
[33] L8:I←I+1
[34]  →(I≤8)/GB2
[35]  DIV← 1 1 2 2 3 3 4 4
[36]  DELA←⍳8
[37]  DELA←DEL÷DIV
[38]  SUM←(+/DELA)
[39]  I←J←1
[40] GB3:→(DELA[I]=0)/L9
[41]  →L10
[42] L9:J←J+1
[43] L10:I←I+1
[44]  →(I≤8)/GB3
[45]  J←J-1
[46]  TENLIM←(SUM÷J)×0.1
[47]  AVE←SUM÷J
[48]  I←1
[49] GB4:→(DELA[I]=0)/L12
[50]  →((DELA[I]<(AVE-TENLIM))∨(DELA[I]>(AVE+TENLIM)))/L11
[51]  →L12
[52] L11:DELA[I]←0
[53] L12:I←I+1
[54]  →(I≤8)/GB4
[55]  SUMA←(+/DELA)
[56]  J←J-1
[57] GB5:→(DELA[I]=0)/L13
[58]  →L14
[59] L13:J←J+1
[60] L14:I←I+1
[61]  →(I≤8)/GB5
[62]  J←J-1
[63]  FAVE←SUMA÷J
[64]  ANS←FAVE×DF×C
[65]  'MICROBIOLOGICAL ASSAY---'
[66]  '      NATIONAL CENTER FOR NUTRIENT ANALISIS';'  ';DATE
[67]  ' '
[68]  TE← 4 2 ρT
[69]  'TEST VALUES'
[70]  TE
[71]  ' '
[72]  'DILUTION FACTOR:';DF
[73]  'THE STANDARD IS ';STD
[74]  ''
[75]  'STANDARD VALUES'
[76]  SA1
[77]  SA2
[78]  SA3
[79]  SA4
[80]  SA5
[81]  ''
[82]  'SAMPLE NUMBER: ';SNO
[83]  J;' TUBES USED IN THIS ASSAY'
[84]  ''
[85]  AVEA←(⌊0.5+AVE×1000)÷1000
[86]  TLL←(⌊0.5+(AVE-TENLIM)×1000)÷1000
[87]  TLH←(⌊0.5+(AVE+TENLIM)×1000)÷1000
[88]  'AVERAGE ';AVEA;'        ';TLL;'↔';TLH
[89]  ''
[90]  'STD CONC/ML OF TUBES USED'
[91]  DELB←(⌊0.5+DELA×1000)÷1000
[92]  DELB
[93]  ''
[94]  ANSA←(⌊0.5+ANS×1000)÷1000
[95]  →(J≥6)/A1
[96]  'ESTIMATE ANSWER ';ANSA;' ';UNITS;'          ';NAME
[97]  →0
[98] A1:'OFFICIAL ANSWER ';ANSA;' ';UNITS;'          ';NAME
    ∇
```

FIG. 2—Complete listing of the APL-360 program.

```
MICROBIOLOGICAL ASSAY---
   NATIONAL CENTER FOR NUTRIENT ANALISES  AUG 16, 1972

        TEST VALUES
     74              73
     47.5            47
     36.5            36
     28.5            29

DILUTION FACTOR: 608
THE STANDARD IS CYANOCOBALAMIN STD CONC .01 NG/ML

STANDARD VALUES
74  70  69.5
47.5  50  47
36.5  36  35.5
29  29  28
24.5  24  24.5

SAMPLE NUMBER: TEST 1
8 TUBES USED IN THIS ASSAY

AVERAGE EQUIV STD CONC 0.987   0.889↔1.086

NL EQUIV STD CONC OF TUBES USED
0.902  0.936  1.027  1.048  0.986  1  1.01  0.989

OFFICIAL ANSWER 6.003 MCG/ML
```

FIG. 3—Sample computer printout showing 8 acceptable sample tubes.

```
MICROBIOLOGICAL ASSAY---
   NATIONAL CENTER FOR NUTRIENT ANALISES  AUG 16, 1972

        TEST VALUES
     74              73
     47.5            55
     36.5            36
     28.5            29

DILUTION FACTOR: 608
THE STANDARD IS CYANOCOBALAMIN STD CONC .01 NG/ML

STANDARD VALUES
74  70  69.5
47.5  50  47
36.5  36  35.5
29  29  28
24.5  24  24.5

SAMPLE NUMBER: TEST 2
7 TUBES USED IN THIS ASSAY

AVERAGE EQUIV STD CONC 0.963   0.866↔1.059

NL EQUIV STD CONC OF TUBES USED
0.902  0.936  1.027  0  0.986  1  1.01  0.989

OFFICIAL ANSWER 5.95 MCG/ML
```

FIG. 4—Sample computer printout showing an assay with one tube rejected for failure to be within 10% of the average.

```
MICROBIOLOGICAL ASSAY---
   NATIONAL CENTER FOR NUTRIENT ANALYSES  AUG 16, 1972

        TEST VALUES
     88                88.5
     77.5              76.5
     66.6              66
     57                58

DILUTION FACTOR: 608
THE STANDARD IS CYANOCOBALAMIN STD CONC .01 MG/ML

STANDARD VALUES
74   70   69.5
47.5  50   47
36.5  36   35.5
29   29   28
24.5  24   24.5

SAMPLE NUMBER: TEST 3
6 TUBES USED IN THIS ASSAY

AVERAGE EQUIV STD CONC 0.4    0.36⟷0.44

ML EQUIV STD CONC OF TUBES USED
0  0  0.39  0.408  0.4  0.408  0.404  0.393

OFFICIAL ANSWER 2.435 MCG/ML
```

FIG. 5—Sample computer printout showing an assay with 2 tubes discarded for being equivalent to less than 0.5 ml of the standard.

formed titrimetrically. The system is valid and rapid and, in additional, elicits a printed record incorporating all assay data. It should be considered as an acceptable alternative means of calculation.

Table 1. Comparison of results of vitamin assays calculated manually and by the APL-360 program

Assay	Calcd potency		
	Manual	Computer program	% Diff.[a]
Test 1	6.02	6.00	0.33
Test 2	5.96	5.95	0.17
Test 3	2.49	2.44	0.20

[a] Manual determination used as reference.

It is also applicable to all microbiological vitamin assays; the only changes that are necessary between assays are the identity and the concentration of the standard.

References

(1) Tsuji, K., Elfring, G. L., Crain, H. H., & Cole, R. J. (1967) *Appl. Microbiol.* 15, 363–367

(2) Berg, T. M., & Behagel, H. A. (1972) *Appl. Microbiol.* 23, 531–542

(3) *Official Methods of Analysis* (1970) 11th Ed., AOAC, Washington, D.C., secs. 39.083–39.092

This paper was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

DRUGS IN FEEDS

Determination of Sulfaquinoxaline in Feeds: Collaborative Study

CHESTER R. SZALKOWSKI[1]
Merck Sharp & Dohme Research Laboratories, Rahway, N.J. 07065

In the method developed, sulfaquinoxaline is extracted from the feed sample with dimethylformamide and separated from interfering substances by chromatography on alumina. The isolated sulfaquinoxaline is diazotized and coupled in the presence of zirconium. The color complex is then extracted with butanol and measured at 550 nm. Recoveries of sulfaquinoxaline from feeds reported by 13 collaborators ranged from 96 to 101%, with a coefficient of variation of 5%. This assay method is suitable for determining sulfaquinoxaline at all levels and has been adopted as official first action, to replace methods 38.139 and 38.140–38.141.

Numerous criticisms concerning the failure of the official AOAC methods, 38.139 and 38.140–38.141 (1), to recover guaranteed amounts of sulfaquinoxaline from feeds necessitated re-evaluation of the official methods. The Associate Referee re-examined all factors which influence the extraction of the drug from the feed, separation from impurities and interfering substances, formation of color, and the photometric measurement. As a result, a modified method for the determination of sulfaquinoxaline at all levels, given below, was developed and collaboratively studied.

METHOD

Sulfaquinoxaline—Official First Action

(Applicable in presence of arsanilic acid)

38.C05 *Principle*

Sulfaquinoxaline is extd from feed with DMF and sepd from interfering substances by column chromatography on Al_2O_3. Isolated sulfaquinoxaline is acidified, diazotized, and coupled in presence of Zr, and colored complex is extd with butanol and measured at 550 nm. Arsanilic acid remains in final aq. soln and can be measured at 540 nm and compared with std treated similarly.

[1] Retired. Present address: P. O. Box 196, Hunlock Creek, Pa. 18621. Address reprint requests to C. R. Szalkowski, Merck Sharp & Dohme Research Laboratories, Rahway, N.J. 07065.

38.C06 *Reagents*

(a) *Aluminum oxide.*—*See* 38.023(b).

(b) *Alkaline salt soln.*—Dissolve 2.0 g NaOH and 100.0 g NaCl in 500 ml H_2O.

(c) *NED soln.*—*See* 38.013(d).

(d) *Zirconium soln.*—Dissolve 5.0 g zirconyl chloride, $ZrOCl_2.8H_2O$ (Fisher Scientific Co.), in 100 ml H_2O.

(e) *Sulfaquinoxaline std solns.*—(*1*) *Stock soln.*—Weigh 40.0 mg Ref. Std Sulfaquinoxaline (available from Merck & Co., Inc.) and dissolve in 50.0 ml DMF. Soln is stable at least 1 month if kept tightly stoppered and protected from light. (*2*) *Intermediate soln.*—80 μg/ml. Dil. 5 ml stock soln to 50 ml with DMF. (*3*) *Working soln.*—8 μg/ml. Dil. 5 ml intermediate soln to 50 ml with DMF. Prep. from freshly prepd intermediate soln just before use.

(f) *Butanol mixture.*—Mix 100 ml *n*-hexane with 400 ml *n*-butanol.

38.C07 *Preparation of Sample*

Weigh 4.00 g ground feed sample into 100 ml vol. flask. Add 50.0 ml DMF, stopper, and agitate by magnetic stirrer or mech. shaker 60 min. Transfer mixt. to 50 ml centrf. tube and centrf. 5 min at 2500 rpm.

38.C08 *Chromatography*

(a) *Preparation of column.*—Constrict end of 50–60 cm length of 9–11 mm id glass tubing by rotating in hot flame until opening is 4–5 mm. Insert small plug of Pyrex glass wool in lower end and compress with glass rod to thickness of 2–3 mm. Transfer 5.0 g Al_2O_3 to dry tube and pack by gentle tapping while applying vac.

(b) *Separation.*—Pipet 10 ml clear ext onto column and let pass thru by gravity. Do not let column run dry; keep 5 mm head of liq. Wash inner walls with two 5.0 ml portions $CHCl_3$. Let final washing drain until no further liq. appears at tip. Discard effluent and washings. Attach column tip to vac. and draw air thru until Al_2O_3 is dry, indicated by tube returning to room temp. Elute column by gravity with 25 ml alk. salt soln, collecting eluate in 25 ml vol. flask. Add 1.0 ml HCl to eluate, dil. to vol. with H_2O, and mix well.

Prep. reagent blank by transferring 10 ml DMF onto fresh column and proceeding as for sample.

Prep. std by transferring 10.0 ml sulfaquinoxaline working std soln onto fresh column and proceeding as for sample.

38.C09 *Determination*

Transfer 10 ml aliquots of each eluate to sep. centrf. tubes. Add 2.0 ml Zr soln and mix. Add 1.0 ml *0.1% $NaNO_2$ soln*, mix, and let stand 2 min. Add 1.0 ml *0.5% NH_4 sulfamate soln*, mix, and let stand 2 min. Add 1.0 ml NED soln, mix, and let stand 10 min. Add 2.0 g NaCl and 10.0 ml butanol mixt., stopper, and shake vigorously until NaCl dissolves. Centrf., carefully transfer portion of clear, colored top solv. layer to 1 cm cell, and read A at 550 nm against butanol mixt. Correct for reagent blank.

% Sulfaquinoxaline in sample = $0.01 \times (A/A') \times W$, where A and A' refer to sample and std, blank corrected, resp., and W = g sample.

Collaborative Study

The 4 feed samples sent to 17 collaborators were prepared from an unmedicated broiler finisher feed. Sample 1S contained 0.0100% sulfaquinoxaline, sample 2S 0.0150% sulfaquinoxaline, sample AS 0.006% arsanilic acid and 0.0100% sulfaquinoxaline, and sample 1AS 0.010% arsanilic acid and 0.0125% sulfaquinoxaline. Each collaborator was supplied with 50 g of each feed sample, 2 g reference standard sulfaquinoxaline, and a copy of the method. The collaborators were requested to perform single assays on each feed on 2 different days and to report the results.

Results and Recommendation

Table 1 gives the recoveries of sulfaquinoxaline from the 4 feed samples reported by the collaborators. Table 2 shows the absorbance obtained on 2 different days for 32 μg sulfaquinoxaline in 10.0 ml butanol. The data in Table 1 were treated statistically according to the techniques suggested by Youden (2). The ranking technique, Table 3, was used to identify collaborators who had consistently high or low results. Table 4 summarizes the results of the statistical study. The results indicate a satisfactory degree of precision for the method. The means and standards of deviation were calculated for all collaborators. The coefficients of variation on the 4 samples ranged from 4.9 to 5.5%.

The Associate Referee feels that more precise and accurate results can be obtained when the analysts are more familiar with the procedure.

When properly performed, the proposed method has 2 advantages over the present official

Table 1. Collaborative results for the determination of sulfaquinoxaline (%) in feeds

		Sulfaquinoxaline found, %			
Coll.	Day	Sample 1S	Sample 2S	Sample AS	Sample 1AS
1	1	0.0100	0.0149	0.0102	0.0124
	2	0.0099	0.0151	0.0100	0.0126
	Av.	0.0100	0.0150	0.0101	0.0125
2	1	0.0096	0.0149	0.0105	0.0128
	2	0.0098	0.0152	0.0105	0.0128
	Av.	0.0097	0.0151	0.0105	0.0128
3	1	0.0098	0.0145	0.0100	0.0121
	2	0.0095	0.0147	0.0100	0.0121
	Av.	0.0096	0.0146	0.0100	0.0121
4	1	0.0096	0.0142	0.0096	0.0117
	2	0.0095	0.0141	0.0094	0.0116
	Av.	0.0096	0.0142	0.0095	0.0116
5	1	0.0096	0.0132	0.0102	0.0115
	2	0.0094	0.0132	0.0092	0.0112
	Av.	0.0095	0.0132	0.0097	0.0114
6	1	0.0088	0.0137	0.0091	0.0111
	2	0.0086	0.0138	0.0094	0.0115
	Av.	0.0087	0.0138	0.0093	0.0113
7	1	0.0110	0.0173	0.0116	0.0140
	2	0.0097	0.0148	0.0101	0.0122
	Av.	0.0104	0.0161	0.0109	0.0131
8	1	0.0100	0.0140	0.0105	0.0125
	2	0.0100	0.0139	0.0104	0.0123
	Av.	0.0100	0.0140	0.0104	0.0124
9	1	0.0087	—	0.0094	0.0113
	2	0.0088	—	0.0096	0.0115
	Av.	0.0088	—	0.0095	0.0114
10	1	0.0090	0.0140	0.0090	0.0120
	2	0.0090	0.0140	0.0100	0.0120
	Av.	0.0090	0.0140	0.0095	0.0120
11	1	0.0096	0.0147	0.0103	0.0127
	2	0.0098	0.0149	0.0104	0.0125
	Av.	0.0097	0.0148	0.0104	0.0126
12	1	0.0090	0.0148	0.0099	0.0121
	2	0.0095	0.0156	0.0107	0.0130
	Av.	0.0092	0.0152	0.0103	0.0126
13	1	0.0099	0.0155	0.0106	0.0138
	2	0.0092	0.0146	0.0098	0.0113
	Av.	0.0096	0.0150	0.0102	0.0126
14	1	0.0093	0.0137	0.0114	0.0137
	2	0.0095	0.0135	0.0098	0.0125
	Av.	0.0094	0.0136	0.0106	0.0131

Table 2. Absorbances of sulfaquinoxaline standard (32.0 μg) in 10.0 ml butanol

Coll.	Cell path, cm	Absorbance Day 1	Absorbance Day 2	Daily variation
1	1	0.491	0.504	0.013
2	1	0.467	0.466	0.001
3	1	0.535	0.510	0.025
4	1	0.539	0.524	0.015
5	1	0.312	0.320	0.008
6	1	0.485	0.485	0.000
7	1	0.388	0.492	0.104
8	2	0.520	0.520	0.000
9	1	0.486	0.480	0.006
10	1	0.505[a]	0.494[a]	0.011
11	1	0.510	0.510	0.000
12	1	0.469	0.405	0.064
13	1	0.425	0.490	0.065
14	½″	0.346	0.389	0.043
Av.				0.035

[a] Standard contained 32.88 μg sulfaquinoxaline/10 ml butanol.

methods, 38.139 and 38.140–38.141: it is more specific and sulfaquinoxaline can be quantitatively determined in presence of arsanilic acid.

It is recommended that this method be adopted as official first action to replace the present official final action methods, 38.139 and 38.140–38.141, which should be repealed.

Acknowledgments

The Associate Referee is deeply grateful to J. B. Garber for his suggestions and criticisms which were most helpful in the development of the method. He also wishes to express his appreciation to the following collaborators:

A. Bestienich and T. M. Kuevers, Canada Department of Agriculture, Calgary, Alberta, Canada

B. E. DiDario, Merck Sharp & Dohme Research Laboratories, Rahway, N.J.

D. F. Erskine and C. A. Luhman, California Department of Agriculture, Sacramento, Calif.

Thomas B. Hall, Arkansas State Plant Board, Little Rock, Ark.

Norma M. Holbrook, Agricultural Experiment Station, Lexington, Ky.

Paul Leicht and J. M. Kauffman, Whitmoyer Laboratories, Inc., Myerstown, Pa.

R. M. Morgan, West Experimental Station, Amherst, Mass.

Table 3. Ranked collaborator results[a]

	Day 1					Day 2					Av.				
	Sample					Sample					Sample				
Coll.	1S	2S	AS	1AS	Score	1S	2S	AS	1AS	Score	1S	2S	AS	1AS	Score
1	2.5	3.5	6.5	7	19.5	2	3	7	3	15	2	4	7	4	17
2	6	3.5	3.5	4	16	3.5	2	3	2	10.5	4	3.5	3.5	2	13
3	4	6	7	8.5	25.5	6	6	7	7	26	4	6	8	6	24
4	6	7	9	10	32	6	8	9	9	32	4	7	11	8	30
5	6	10	6.5	11	33.5	6	13	10	12	41	5.5	11	9	10	35.5
6	8	9	11	13	41	10	11	9	10	40	9	9	11	10	39
7	1	1	1	1	4	4.5	5	5	6	20.5	1	1	2	1.5	5.5
8	2.5	8.5	4	6	21	1	10	4	5	24	2	8.5	3.5	5	19
9	9	—	10	12	31	9	—	8	10	27	8	—	11	10	29
10	7.5	8.5	12	9	37	8	9	7	8	32	7	8.5	11	7	33.5
11	6	5	5	5	21	3.5	4	4	4.5	16	4	5	4	4	17
12	7.5	4	8	8.5	28	6	1	2	1	10	6	2	5	4	17
13	3	2	3.5	2	10.5	7	7	7	11	32	4	3.5	6	4	17.5
14	6	9.5	2	3	20.5	4.5	12	1	4.5	22	5.5	10	1	1.5	18

[a] The approximate 5% 2-tail limits for ranking for 14 collaborators and 4 materials are 8 and 52: 1 = highest value, 14 = lowest value.

Table 4. Results of statistical analysis of the collaborative results for sulfaquinoxaline (SQ)

Sample	SQ Added, %	% Found, day 1	Coeff. of var., day 1	% Found, day 2	Coeff. of var., day 2	Av. found	S_d	S_r	S_b	F (S_d^2/S_r^2)	Coeff. of var., %	SQ Rec., %
1S	0.0100	0.0096	6.2	0.0095	4.4	0.0096	0.000468	0.000322	0.000410	2.11	4.9	96.0
2S	0.0150	0.0146	7.1	0.0144	5.0	0.0145	0.000808	0.000554	0.000697	1.32	5.5	96.6
AS	0.0100	0.0102	7.4	0.0100	5.0	0.0101	0.000498	0.000545	0.000315	0.84	5.0	101.0
1AS	0.0125	0.0124	6.4	0.0121	5.2	0.0123	0.000616	0.000622	0.000431	0.98	5.0	98.2

R. J. Noel, Purdue University, W. Lafayette, Ind.

J. Penrod, Supersweet Feeds, New Ulm, Minn.

S. Dianne Prentice and B. F. Rickey, New York State Agricultural Experiment Station, Geneva, N.Y.

Mary Maud Sharpe, Department of Agriculture, Tallahassee, Fla.

Marilyn Strauss and James McGill, Georgia Department of Agriculture, Atlanta, Ga.

D. M. Viviano, Ralston Purina Co., St. Louis, Mo.

REFERENCES

(1) *Official Methods of Analysis* (1970) 11th Ed., AOAC, Washington, D.C.

(2) Youden, W. J. (1967) *Statistical Techniques for Collaborative Tests*, AOAC, Washington, D.C.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–11, 1972, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee G and was adopted by the Association; see (1973) *JAOAC* 56, 410–411.

ATTENTION

Abstracts of papers or reports to be presented at the 87th Annual Meeting of the AOAC, Oct. 9–12, 1973, MUST be submitted on standard, preprinted forms. The forms are available, on request, from the AOAC Editorial Office.

Since 1971 the Abstract Bulletin has undergone two changes. The abstract submitted by the author is now reproduced directly by a photographic process; it is no longer edited and retyped in the Editorial Office. This new procedure makes it imperative that authors provide an informative, carefully prepared abstract typed on the special form, plus 2 additional copies (carbon copies or photocopies). This form includes general rules for preparing satisfactory abstracts. Abstracts not fulfilling these requirements will be returned to the author for correction, if time permits. Improperly prepared abstracts received too late to allow this will not appear in the Abstract Bulletin. The Abstract Bulletin itself is a new size—4 × 9″, the same size as the AOAC program.

In addition to the abstract, five copies of *all* contributed papers and Associate Referee reports must be submitted to the AOAC office. The manuscripts should be double-spaced throughout. Unless otherwise specified, all manuscripts received will be considered for publication in *JAOAC*, pending satisfactory review. Each Associate Referee should also send one copy of his report to his General Referee.

PLEASE COOPERATE!!!

Write for standard abstract forms (one required for each presentation). Mail your abstract in early.

DEADLINE for receipt of abstracts—August 17, 1973.

Qualitative Identification of Furazolidone, Tylosin, and Zoalene in Feeds

LEWIS A. BAREFIELD
Arkansas State Plant Board, P. O. Box 1069, Little Rock, Ark. 72203

Rapid microscopic methods are described for the identification of furazolidone, tylosin, and zoalene in mixed feeds at levels normally used for feeding. The drugs are identified by the colors produced in solution: furazolidone—intense blue in dimethylformamide and potassium hydroxide, zoalene—bright green in dimethylformamide and potassium hydroxide, and tylosin—pink-orchid in distilled water. Collaborative results showed excellent agreement, and the methods have been adopted as official first action.

The 11th Edition of *Official Methods of Analysis* (1) contains official final action quantitative methods for determining furazolidone, 38.064–38.065, tylosin, 38.223–38.226, and zoalene, 38.158–38.160. The methods for furazolidone and zoalene are spectrophotometric and the method for tylosin is microbiological. The method for zoalene, however, is not applicable in the presence of furazolidone, nitrofurazone, or nihydrazone.

Rapid and reliable qualitative methods are needed as screening procedures for each of these drugs; a method is also needed for the detection of zoalene in the presence of other drugs. The following spot test methods are offered as supplements to the official quantitative methods cited. These spot tests were subjected to collaborative study by members of the Methods Committee of the American Association of Feed Microscopists (AAFM) in 1972 and by a class of twenty collaborators at the biennial Short Course in Feed Microscopy, conducted by the AAFM at Kansas State University in June 1971. The described spot tests (2) depend on the intense blue color produced locally by furazolidone particles (3) and the bright green color produced by zoalene (4) as they dissolve in a solution of dimethylformamide and potassium hydroxide, and the pink-orchid color of tylosin (5) that is produced on standing 5 min in distilled water; each color can be observed under a stereoscopic microscope.

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

METHODS

Furazolidone, Tylosin, and Zoalene—Official First Action

38.C01 *Apparatus*

See 7.099.

38.C02 *Reagents*

(a) *Dimethylformamide (DMF).*—Reagent grade.

(b) *Alcoholic potassium hydroxide soln.*—4%. Dissolve 4 g KOH in 100 ml alcohol. If premixed with DMF (1+9), prep. fresh daily.

(c) *Ethylenediamine.*—Use in hood.

38.C03 *Preparation of Sample*

Gently grind pellet, cube, or crumble forms with mortar and pestle. Sieve thru nest of Nos 10, 20, and 30 sieves with pan. Drugs usually are concd in portion in pan.

38.C04 *Identification*

(a) *DMF test.*—Place 9 drops DMF and 1 drop alc. KOH soln in each of 3 depressions of white spot plate. Sprinkle ca 0.01 g fine feed material into each soln from tip of spatula while observing reaction under microscope. Furazolidone produces intense blue color, easily detected at $\geq$0.0025%. Zoalene gives bright green, easily detected at $\geq$0.0025%. Color of minute particles of zoalene fades rapidly; color of larger particles persists 3–5 min.

(b) *Ethylenediamine test.*—Place dry filter paper at bottom of petri dish and sprinkle ca 0.5 g fine feed evenly over paper. Dispense 2–4 ml ethylenediamine under edge of paper so as to wet entire paper and sample. Examine under stereoscopic microscope at 10× for particles developing bright purple, indicating zoalene, or deep red, indicating furazolidone. (Blood meal, frequently used in livestock feed, also gives deep red color with reagent.)

(c) *Water test.*—Place ca 0.3 g fine feed in petri dish and add 4–5 ml H_2O to moisten to paste-like consistency. After 4–5 min, examine under stereoscopic microscope at 10× with white paper under dish. Pink or orchid particles indicate tylosin. (Shades of many colors may be present in gelatin carrier of tylosin but pink and orchid are least likely to be confused with particles of other feed components.)

Collaborative Studies

Twenty students enrolled in the 1971 Short Course in Feed Microscopy at Kansas State University were given 4 samples, as unknowns, to be tested without consultation. Sample 1 consisted of ground yellow corn with 0.011% tylosin; sample 2, a blank (ground corn only); sample 3, ground corn containing 0.022% zoalene; and sample 4, ground corn containing 0.011% furazolidone. Nineteen collaborators made perfect scores. One collaborator identified zoalene and furazolidone correctly but reported Terramycin in sample 1 (tylosin) and tylosin in sample 2 (the blank). He apparently did not read the instructions.

In a separate collaborative study, conducted by the American Association of Feed Microscopists, 23 collaborators were asked to analyze each of 4 samples marked A, B, C, and D. The test samples were 65% ground yellow corn, 20% layer supplement, and 15% soybean meal (44%) with the following drugs added: A, 0.0055% tylosin; B, blank; C, 0.0055% furazolidone; and D, 0.0125% zoalene.

Test kits were assembled and distributed to 23 collaborators. The kits contained the 4 feed samples, identified as A, B, C, and D; reference samples of tylosin, zoalene, and furazolidone feed grade concentrates; instructions and a report sheet; and about 20 ml of each of 3 test reagents: dimethylformamide (DMF); potassium hydroxide (KOH), 4% in 100 ml 95% ethyl alcohol; and ethylenediamine (EDA).

The collaborators were asked to indicate the presence or absence of zoalene and furazolidone, using the DMF-potassium hydroxide reagent and then using EDA. The presence or absence of tylosin was to be determined also for each of the 4 samples. A total of 20 different determinations were to be reported.

Table 1. Collaborative results for the identification of furazolidone (F), tylosin (T), and zoalene (Z)

					Using EDA[b]	
Coll.	T	B[a]	F	Z	F	Z
Kansas State Short Course (AAFM)						
1	[c]	T	F	Z		
2	T	B	F	Z		
3	T	B	F	Z		
4	T	B	F	Z		
5	T	B	F	Z		
6	T	B	F	Z		
7	T	B	F	Z		
8	T	B	F	Z		
9	T	B	F	Z		
10	T	B	F	Z		
11	T	B	F	Z		
12	T	B	F	Z		
13	T	B	F	Z		
14	T	B	F	Z		
15	T	B	F	Z		
16	T	B	F	Z		
17	T	B	F	Z		
18	T	B	F	Z		
19	T	B	F	Z		
20	T	B	F	Z		
Methods Committee (AAFM)						
21	T	B	F	Z	F	Z
22		T,B	F	Z	F	Z
23	T	B	F	Z	F	Z
24	T	B	F	Z	F	Z
25	T	B	F	Z	F	Z
26	T	B	F	Z	F	Z
27	T	B	F	Z	F	Z
28		T,B	F	Z	F	Z
29	T	B,F		Z	F	Z
30	T		F	Z	T,B,F,Z	Z
31	T	B	F	Z	F	Z
32	T	B	F	Z	F	Z
33	F	B	F	Z	F	Z
34	T	F	F	Z	F	Z
35	T	B	T	Z	F,Z	F,Z
36	T	B	F	Z	F	Z
37	T	B	F	Z	F	Z
38		B		Z	F	Z
39	T	T,B	F	Z	F	Z
40	T	B	F	Z	F	Z

[a] Blank.
[b] Performed by Colls. 21–40 only.
[c] Terramycin reported.

Results and Recommendation

Of the 20 AAFM collaborators, all 20 respondents reported zoalene in the sample (D) to which it had been added both by the DMF-KOH method and by EDA. Nineteen found furazolidone in the correct sample, using EDA, and 17 reported furazolidone, using DMF-KOH. Sixteen respondents reported tylosin in the correct sample. Nineteen of the 20 correctly identified the blank. (*See* Table 1.)

Each of the 40 collaborators made perfect scores on the qualitative analysis for zoalene; 37 identified furazolidone correctly; and 35 correctly identified tylosin. Thirty-eight of the 40 collaborators reported the blank correctly.

The Associate Referee therefore recommends that these methods be adopted as official first action.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee G and was adopted by the Association; *see* (1972) *JAOAC* 56, 410–411.

Acknowledgments

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C. G. Abernathy, California Department of Agriculture, Sacramento, Calif.

O. G. Abrahams and J. H. Dawson, Missouri Department of Agriculture, Jefferson City, Mo.

J. T. Adam, Con Agra, Inc., Omaha, Neb.

R. J. Bax, Ralston Purina Co., St. Louis, Mo.

F. Cottec, Canada Department of Agriculture, Calgary, Alberta, Canada

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A. J. Gehrt, Moorman Mfg. Co., Quincy, Ill.

E. M. Gordon, Canada Department of Agriculture, Ottawa, Ontario, Canada

E. L. Graves, State Chemical Laboratory, State College, Miss.

E. Hammer, Security Mills, Nashville, Tenn.

M. Herrera, Iowa Department of Agriculture, Des Moines, Iowa

W. J. Hill, American Association of Feed Microscopists, Estes Park, Colo.

J. A. Johnson, Jr., Virginia Department of Agriculture, Richmond, Va.

B. Kaufman, Landmark Feeds, Lima, Ohio

J. E. Klenk, Hubbard Milling Co., Mankato, Minn.

A. C. Koontz, Gooch Mfg. Co., Lincoln, Neb.

A. O. Langkait, Farm Bureau Co-operative, Indianapolis, Ind.

R. Light, United Co-operatives of Ontario, Guelph, Ontario, Canada

H. W. McCray, Florida Department of Agriculture, Tallahassee, Fla.

G. D. Miller, Kansas State University, Manhattan, Kan.

P. D. Moore, Central Soya, Decatur, Ind.

J. Musgrave, Harvest Brand, Inc., Pittsburg, Kan.

P. Roebuck, MFC Services, Jackson, Miss.

H. Romo, Anderson Clayton Co., Guadalajara, Mexico

J. G. Russell, Arkansas State Plant Board, Little Rock, Ark.

W. A. Schnack, Moorman Mfg. Co., Quincy, Ill.

M. L. Slater, Canada Department of Agriculture, Ottawa, Ontario, Canada

L. E. Smith, Goldkist, Inc., Lithonia, Ga.

A. M. Spinning, Farm Bureau Cooperative, Indianapolis, Ind.

B. E. Titlow, Ohio Department of Agriculture, Reynoldsburg, Ohio

R. M. Vaden, Southern States Laboratories, Baltimore, Md.

A. Warner, Agway, Inc., Ithaca, N.Y.

T. B. Windsor, Ralston Purina Co., Camp Hill, Pa.

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SPECIAL REPORTS

Report on the 15th and 16th Annual Meetings of the Collaborative International Pesticides Analytical Council

MILTON S. SCHECHTER and ROBERT L. CASWELL*
U.S. Full Member of CIPAC, Agricultural Research Center, U.S. Department of Agriculture, Beltsville, Md. 20705
** U.S. Correspondent of CIPAC, Pesticides Regulation Division, Environmental Protection Agency, Washington, D.C. 20250*

15th Annual CIPAC Meeting

The 15th Annual Meeting of the Collaborative International Pesticides Analytical Council (CIPAC) was held in Washington, D.C., at the U.S. Department of Agriculture, Oct. 4–9, 1971.[1] This was the first time that the United States was host for a CIPAC meeting, which made attendance possible for a number of AOAC Associate Referees who have been participating in collaborative work but who ordinarily have not been able to go to CIPAC meetings in Europe. Also, the meeting was held in October, rather than at the usual time in June, so that CIPAC members could easily attend the 85th Annual Meeting of the AOAC, held Oct. 11–14, the week after the CIPAC meetings.

The CIPAC organization consists of official government chemists representing their respective countries, along with correspondents, observers, and expert witnesses (the latter are invited official and industrial scientists), as well as representatives from WHO (the World Health Organization) and FAO (the Food and Agriculture Organization). The Chairman is R. de B. Ashworth (Great Britain), the Secretary is J. Henriet (Belgium), and the Treasurer is M. J. P Harrington (Great Britain). The major aim of CIPAC is to adopt standardized methods of analysis for technical pesticides and their formulations after international collaborative study. The organization of CIPAC, the manner in which it operates and develops methods, and its close working relationship with the AOAC have been described earlier, (1971) *JAOAC* 54, 846–848.

At the 15th meeting, reports from expert witnesses, based on work by National Subcommittees, Panels, or Groups were presented on the following subjects: Cyclodienes, Malathion, Dithiocarbamates, Copper, Cross-Contamination, the Check-Sample Series of the American Association of Pest Control Officials, Dimethoate, Dinocap, Dicofol, Mercury, Urea Herbicides, Methoxychlor, Standard Waters, Herbicides, Azinphos, Propoxur, Evaluation of Formulations, Wettability, Ethion, Endosulfan, Paraquat and Diquat, Gas-Liquid Chromatography, Trifluralin, Benefin, Compatibility, Identification of Pesticides, Petroleum Oils, Dioxins, Triazines, Fenchlorphos, and Specifications for Pure Pesticides and Reagents. In addition, at the technical sessions, methods for virtually every pesticide of any appreciable use were considered. Specific actions or decisions were taken on more than 70 pesticides, and 15 pesticides were assigned for initiation of new work.

After discussion by officials of both organizations, a draft of a document covering the guidelines to be followed for collaboration between AOAC and CIPAC was drawn up and is currently being considered for adoption.

Since chemists from abroad can now become members of the AOAC, steps were taken to have CIPAC members become AOAC members.

At the 85th Annual Meeting of the AOAC, a Symposium on the Analysis of Pesticides and Their Formulations, (1972) *JAOAC* 55, 907–947, jointly sponsored by the AOAC, the National Agricultural Chemicals Association, and CIPAC, was held on October 12, 1971. A very interesting series of papers was presented, and the Symposium was very well attended.

In addition to the technical sessions of the 15th CIPAC meeting, a number of social affairs were

[1] For previous reports, *see* (1966) *JAOAC* 49, 158–159; (1967) 50, 153–154; (1968) 51, 421–422; (1969) 52, 357–358; (1970) 53, 407–408; and (1971) 54, 412.

This report was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

arranged, including a banquet sponsored by the AOAC, a reception by the National Agricultural Chemicals Association, and a buffet reception at-home given by Mr. and Mrs. Schechter and Mr. and Mrs. Caswell. The members were also taken on tours of the USDA Agricultural Research Center at Beltsville, Md., of the Food and Drug Administration laboratories in Washington, D.C., and of major sights in Washington. Since wives of many of the CIPAC members were present, a program of additional activities was arranged for the ladies.

16th Annual CIPAC Meeting

The 16th CIPAC meeting was also somewhat unusual, in that the first half of the meeting was held in Stockholm, Sweden, June 19–22, 1972, with Siv Renvall as hostess, and the second half in Lyngby, Denmark, June 23–28, with H. H. Povlsen as host. Invited expert witnesses presented reports on Triazines, the Heat-Stability of Diazinon Formulations, Methods for Testing Emulsifiable Concentrates, Chlordane, Dicamba, Thiometon, Dithiocarbamates, Lindane, BHC and DDT, Herbicides, Compatibility, Phytotoxicity, Mancozeb, Diquat, Mercury, Evaluation of Formulations, Cyclodienes, Preparation of Pure Pesticides as Standards, Identification Methods for Dichlofluanid, Fenitrothion, and Propoxur, Methods of Analysis for Phosalone, Vamidothion, Carbetamide, and Oxadiazon, Gas-Liquid Chromatography, Copper, and Dimethoate.

A one-day CIPAC Symposium was held in Stockholm June 21, under the chairmanship of G. Widmark, at which a number of excellent papers relating to the analysis of pesticides were presented.

At the technical sessions in Sweden and Denmark, methods for most of the important pesticides were reviewed, and decisions were taken on necessary modifications, continuation of collaborative work, adoption as provisional or full CIPAC methods, or adoption as joint CIPAC-AOAC or AOAC-CIPAC methods, as appropriate. Thirty-four pesticides were assigned to various countries for initiation of new work. New assignments for the United States were Chloropropylate, Coumaphos, Crufomate, Dioxathion, Methyl Bromide, Bromacil, Dichlofenthion, Lenacil, Trifluralin, Naled, and Fenoprop (jointly with Great Britain). One day was also spent on matters of liaison with the FAO Working Party of Experts on Official Control of Pesticides (Section B, Specifications) and with the Groupement des Associations Nationales de Fabricants de Pesticides (GIFAP).

The *CIPAC Handbook, Vol. 1, Analysis of Technical and Formulated Pesticides* (1970) G. R. Raw (Ed.) is published by W. Heffer and Sons Ltd., Hills Rd., Cambridge, England (price £10). It contains a compilation of CIPAC methods, as well as special chapters on Infrared Analysis, Reagents, Indicators and Solvents, Miscellaneous Techniques, and Pure Pesticides. *CIPAC Handbook, Vol. 1A*, now being prepared, will contain additional methods and information accumulated since the publication of *Vol. 1*. A *CIPAC Monograph on Standard Waters* is also being readied for publication.

In keeping with CIPAC tradition, the hosts in Sweden and Denmark, with their characteristically outstanding hospitality, arranged some delightful social functions to compensate for the hard work carried out by CIPAC and for the long, difficult sessions of its meetings.

It should be kept in mind that analytical methods for pesticides and their formulations, and for impurities in the technical products, are becoming of increasing importance. One need only consider the furor raised by investigations of the dioxin problem. Another important point is that CIPAC and AOAC methods are given preference by the FAO committee working on specifications for pesticides, which are now actively being drafted and adopted by that organization. We are therefore making a plea to those government, industrial, or academic scientists who have an interest in any aspect of analytical work on pesticides to volunteer for consideration as associate referees or collaborators to aid in developing or improving AOAC or CIPAC methods. Any who are so interested should contact the authors of this paper.

Current Efforts of the Food and Drug Administration to Control Mycotoxins in Food

HENRY FISCHBACH and JOSEPH V. RODRICKS
Bureau of Foods, Food and Drug Administration, Washington, D.C. 20204

Twelve years ago when public attention was focusing more and more on the potential harm from wide pesticidal use, a warning was sounded in England about a completely different hazard—aflatoxins in peanut meal (1). Mycotoxins are the offspring of nature, produced by some fungi under certain conditions of temperature, moisture, storage conditions, and the like. The report from England associated the death of about 100 thousand turkey poults with the presence of aflatoxins in an imported peanut meal. Considerable toxicological work on warm-blooded animals was done by the Weybridge Group of the Ministry of Agriculture, Fisheries and Foods in England and by many others. The Tropical Products Institute, Lever Brothers, workers at the Massachusetts Institute of Technology, and others explored the chemical aspects of the problem. Within a couple of years aflatoxin was unveiled as the severest hepatocarcinogen, to some experimental animals, known to man. Obviously practical standards and reliable methodology were essential to determine the scope of the problem and then to safeguard the food and feed channels. In the early 1960's no practical standards were available and the methodology was lengthy and cumbersome. In 1964 the Trace Substances Commission was established by the Food Section of the Applied Chemistry Division in the International Union of Pure and Applied Chemistry.

The first scheduled meeting was held in Paris on July 1, 1965. The Commission was charged with two major areas of concern to the Food Section, namely, Mycotoxins and Smoke Constituents in Foods. In both of these areas, the major obstacle to determining whether a problem existed, and if so, its magnitude, was the lack of reliable methodology of appropriate sensitivity and specificity. Obviously, without such tools meaningful data could not be acquired and responsible monitoring for the safety of food supplies could not be accomplished. Consequently, the major thrust of the Trace Substances Commission was to stimulate the development of such methodology. It was decided that aside from methods development, the aflatoxin group of the Trace Substances Commission would conduct collaborative studies as needed. Today there is a recommended IUPAC method for the determination of aflatoxin in peanuts and peanut products and a method for determination of aflatoxin concentration and purity (2, 3). The Food Section has broadened its interest in mycotoxins and is currently concerned with ochratoxin, sterigmatocystin, aflatoxin M_1, and transmission of aflatoxins to meat tissues.

Mycotoxins may well be among the world's most significant food contaminants. The sources of many of the unexplained diseases of man and animals may lie in their exposure, through food, to these chemicals. But as with so many other food contaminants, the most serious health effects of these chemicals are probably chronic in nature and therefore very difficult to evaluate. Nonetheless when faced with the need to make decisions, we will continue to base our judgments of food contaminants on the principle of maximum safety to the consumer. We also believe that it is reasonable to think of these chemicals as food pollutants not much different from the various industrial chemicals which are sometimes found in foods. Although mycotoxins are of natural origin[1], we know for the most part what techniques are necessary to prevent their appearance in foods. Food pollution resulting from chemicals of natural origin is probably a more serious problem in the technologically developing nations, whereas that resulting from various industrial chemicals is perhaps a more serious problem in the technologically advanced countries. The more technologically advanced nation can afford to demand that food producers maintain a high standard of quality and safety in their products; however, in those nations where the possibility of starvation

Presented by Henry Fischbach, at the IUPAC-sponsored symposium on Control of Mycotoxins, Goteborg, Sweden, August 21–22, 1972.

[1] Many other natural food toxicants are inherent in certain foods, and are treated differently under our law.

is real, questions of food pollution are necessarily of secondary importance. Nevertheless, we hope that eventually efforts to improve the nutritional state of the world's population will be accompanied by an equal concern with the problems of food safety.

In general, mold contamination of foods is considered a violation of a section of the U.S. Food, Drug and Cosmetic Act which defines a food as adulterated "if it consists in whole or in part of any filthy, putrid, or decomposed substance, . . ." (4). However, toxic mold *metabolites* when present in foods, even in the absence of demonstrable mold growth, can be considered as food adulterants under another section of the Act which defines a food as adulterated and therefore in violation of a section of the law "if it bears or contains any poisonous or deleterious substance which may render it injurious to health. . . ." (5). It is this last section of the Act which is now used to bring legal charges against any food containing aflatoxin; this same section of the Act could be used in the future for other mycotoxins as well.

To eliminate mycotoxin-contaminated food from commerce, we need methods to determine mycotoxins and a means of deploying these methods to survey suspected foods on a massive scale. (This latter operation is performed through 17 FDA district laboratories located throughout the United States.) This effort is, at present, devoted primarily to the aflatoxin group, although we have devoted some surveillance effort to ochratoxin and will be conducting such surveys for patulin in the near future. We wish to emphasize, however, that the development of analytical methods and the use of these methods to locate mycotoxin-contaminated commodities in our food supply is only part of our effort. Simply stated, our eventual goal is to identify agronomic and manufacturing techniques which are necessary to minimize the proliferation of toxigenic fungi on foods and to use such information to enlist the cooperation of industry to establish guidelines for the proper handling of mycotoxin-susceptible foodstuffs; thus, prevention is the key word and the various food industries with which we have come in contact because of the aflatoxin problem seem to think along these same lines.

Until this ideal state is reached, we are encouraging affected industries to set up analytical and control operations so that they themselves can eliminate contaminated materials before these substances reach the marketplace and are therefore subject to seizure by the FDA. This approach to the aflatoxin problem has in several important instances been extremely successful in the United States.

All of these approaches must be supported by research: for example, identification of potential mycotoxin problems, development of analytical methods for mycotoxins (both chemical and biological), chemical and toxicological characterization of new mycotoxins, determination of the fate of mycotoxins during various food processing operations, determination of the conditions under which toxin production can occur, determination of the incidence of mycotoxin contamination of foods, and determination of the extent of transmission of mycotoxins to the edible portions of food-producing animals. All of these activities are underway to some extent in FDA, but we rely heavily on research done in universities, the food industry, and other government agencies. Much mycotoxin research of great value to FDA has come from work in other nations; we collaborate with workers in England, Japan, Denmark, France, Turkey, Iran, the Philippines, South Africa, Germany, Poland, India, Canada, Brazil, and many others, the results of which have greatly aided in reducing this threat to human health.

As a result of past and present surveillance work we have concluded that the potential for aflatoxin contamination is established for the following commodities: peanuts, cottonseed, copra, corn, and various tree nuts (pistachios, almonds, walnuts, Brazil nuts, and pecans). Consequently our district laboratories operate continuing programs to ensure that any of these products that contain aflatoxin are removed from commerce. We have also been able to encourage the development of a variety of industry self-regulating systems of the type we have referred to earlier. For each of the industries affected by our regulatory programs we have instituted an educational program in cooperation with the U.S. Department of Agriculture so that the means can ultimately be found to prevent aflatoxin contamination of foods to an extent compatible with safety and the most advanced technological facilities available to the producers. For these types of interim control operations a recent useful development is a rapid minicolumn qualitative method for aflatoxin, developed by the U.S. Department of Agricul-

ture and extended to a wide variety of commodities by the Food and Drug Administration (6). We are beginning to use this technique in our district operations, and a number of industrial control operations have incorporated it as a rapid screening tool.

As is well known, aflatoxin B_1 is metabolized in dairy cattle and its metabolite, M_1, can be excreted in milk (1, 7). Aflatoxin can also be transmitted to the eggs of laying hens and to various tissues of animals which are used as food (7). Since the presence of aflatoxin in milk, eggs, or meat reaching the consumer would be an extremely unsatisfactory situation, this remains as a less certain area in our program. We are especially concerned about such a prospect since the discovery of the potential for aflatoxin contamination of corn, copra, and cottonseed, all widely used in animal feed. Accordingly we have devised experiments to systematically study the rate of transmission of aflatoxin to milk and meat; the U.S. Department of Agriculture has undertaken a similar project for eggs. The level of aflatoxin that appears in milk, eggs, or meat can be controlled by a careful control of the use of contaminated feed, and these studies, which are just getting underway, are aimed at defining such controls. These studies will be accompanied by a survey for aflatoxins in various milk products taken from those areas of the United States where contaminated animal feed is a serious problem. Methods with sensitivities of the order of 0.1 μg/kg will be applied, and the very sensitive confirmatory test recently reported by Stack *et al.* (8) will accompany the assay. As in all our surveillance work we consider confirmation an integral part of the assay. Results of such surveillance activity will afford some reliable data on the seriousness of this problem in the United States.

One preliminary transmission study, the results of which are still tentative, has demonstrated the transmission of aflatoxin to the milk of dairy cattle when aflatoxin is present in feed at a level of about 33 μg/kg (9). This same study also demonstrated that ammoniation of cottonseed to destroy aflatoxin may lead to a product useful for feeding dairy cattle, since a method sensitive to 0.1 μg/kg detected no aflatoxin in the milk from cows fed such a material. To what extent other toxic products might be formed during the ammoniation process is an important but still unanswered question. We are attempting to find an answer to this question through a study in which tissues and milk from animals receiving this feed will be toxicologically evaluated by feeding studies using the rainbow trout.

Our approach to the problem of mycotoxins in foods in the future will most probably follow the same general patterns that have proved so successful for aflatoxin. At present our concerns center on patulin, penicillic acid, sterigmatocystin, and ochratoxin.

Methods for ochratoxins (10) and sterigmatocystin (11) have been developed in our laboratories and both have undergone successful international collaborative studies. The method for ochratoxin has a sensitivity of 10 μg/kg and that for sterigmatocystin a sensitivity of 50 μg/kg; both methods determine these materials in grain and include confirmatory techniques, as we recommend for all such collaborative studies. A survey of barley for ochratoxin contamination was carried out in our laboratories and low levels (10–37 μg/kg) were found in about 13% of the 180 samples assayed; among those found contaminated was a sample of malting barley. Since our primary concern is human food and since earlier work in our laboratories showed that ochratoxin carried through to the beer during the production process, we conducted a survey for ochratoxin in malted barley as used by beer producers and in the beer they produced. Using a method with a demonstrated sensitivity of approximately 10 μg/kg, we established the absence of ochratoxins A and B in samples of beer and malted barley from every one of the 130 breweries in the United States. Future surveys for ochratoxin in dried beans are now in the planning stage, as are surveys for patulin in various apple products. Methods for patulin require improvements in sensitivity, and our current efforts to improve methods are centered on the use of liquid-liquid chromatography. Preliminary results appear very promising: as little as 10 ng patulin can be detected. We are pursuing some structural studies on the metabolites of *Stachybotrys atra* and thus far have uncovered and purified two potent toxins which have been partially characterized (12). Because of the widespread occurrence of *Penicillium viridicatum* we are attempting to unravel the vast array of secondary metabolites produced by this mold.

Another mold-related problem does not seem

to be a mycotoxin problem, namely, the toxic compounds produced in the sweet potato as a consequence of mold invasion (Prof. B. J. Wilson of Vanderbilt University has made some excellent contributions to this area in recent years). Four compounds have been identified, two of which are liver toxins and two of which are lung toxins (13, 14). These compounds, the major one of which is the hepatoxic furanoterpene known as ipomeamarone, are not present in sound sweet potatoes, but are only present when the potato is mold-infected or otherwise damaged. We are currently developing the methodology for ipomeamarone.

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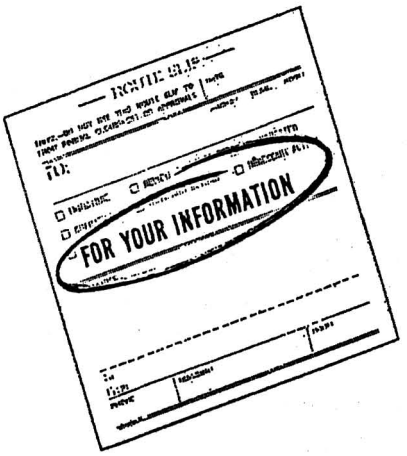


First International Congress on Mercury

The Instituto Technólogico Metalúrgico "Emilio Jimeno" of the University of Barcelona has announced the First International Congress on Mercury, Sept. 2–7, 1973. Sessions as well as symposia and panel discussions will be held on the topics of history, geology and mining, extractive metallurgy, physical metallurgy and metallography, uses (pharmaceutical, metallurgical, and agricultural), toxicity, and miscellaneous. Sessions are planned to stress world resources, the future of mercury, and its wise use.

For further information write to Primer Congreso Internacional del Mercurio, Instituto Technólogico Metalúrgico "Emilio Jimeno" de la Facultad de Ciencias, Universidad de Barcelona, Barcelona, 14, Spain.

International Conference on Land for Waste Management

The Canadian Society of Soil Science is organizing an International Conference on Land for Waste Management to be held in Ottawa, Canada, Oct. 1–3, 1973.

The purpose of the conference is to collect and disseminate information on waste disposal and waste utilization in soils, to evaluate systems of waste management on land, and to identify the problem areas requiring research and development. For achieving this purpose the 3-day conference will be organized around the following themes: (a) climate, vegetation, and soils as factors in waste disposal, including special problems in the North; (b) soil properties and processes in relation to waste recycling and disposal; (c) hydrogeology and geomorphology as factors in waste management; (d) nature of wastes in relation to disposal on land; (e) socio-economic and land use planning for waste disposal, including health and legal aspects; and (f) land waste disposal systems—present and future designs.

The program will feature keynote speakers and will include volunteered papers, discussion, and printed proceedings which pertain to the above themes. The conference will take place in the Ottawa Conference Centre and will include simultaneous translations in English and French.

Address requests for further information and instructions for submitting papers to Mr. M. K. Ward, Executive Secretary, International Conference on Land for Waste Management, National Research Council, Ottawa, Ontario, Canada K1A OR6.

Advances in the Analysis of Lipids and Lipoproteins

The American Oil Chemists' Society (AOCS) is sponsoring a special summer conference entitled "Advances in the Analysis of Lipids and Lipoproteins." The conference will convene at the Ramada Inn, Champaign, Ill., June 17–20, 1973. Dr. E. G. Perkins, Professor of Chemistry, Department of Food Science, University of Illinois, is program chairman.

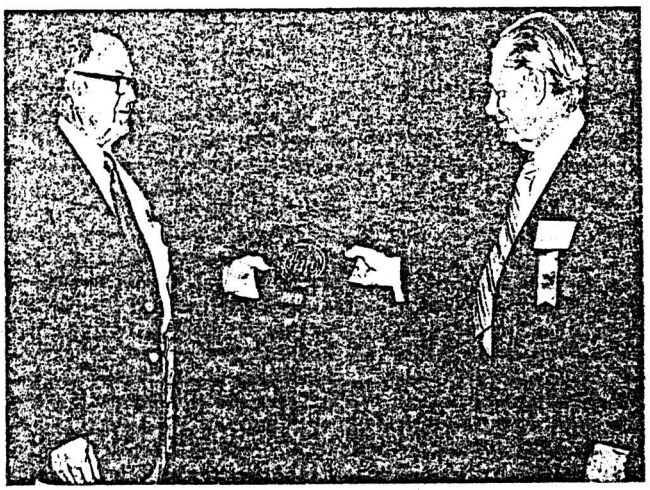
Registration fees for the conference have been set at $110 for AOCS members and $140 for nonmembers. More detailed information is available from the Executive Director, AOCS, 508 South Sixth Street, Champaign, Illinois 61820. Telephone (217) 359-2344.

1972 Meeting

The 86th Annual Meeting of the AOAC was held October 9–12, 1972, at the Marriott Motor Hotel, Twin Bridges, Washington, D.C. More than 230 papers were presented and about 1200 scientists attended, representing Federal, State, and local governments, Canadian organizations, and private industry.

President L. L. Ramsey was given the Presidential Plaque by Donald J. Mitchell, the 1973 President. In his address, "The Role of the Scientist in an Era of Consumerism," President Ramsey detailed the books published, laws enacted, and other significant events that reflect the power of the consumer in the marketplace today. He also emphasized the responsibility of the scientist to continue research to expand man's knowledge, to evaluate risks and benefits to the public health, and to assist in consumer education.

Also at the General Session on Monday morning, Richard Meyer and Stanley Nesheim were presented with certificates in recognition of many years of special contributions to the Association. Dennis Martin, University of Nebraska, Lincoln, was



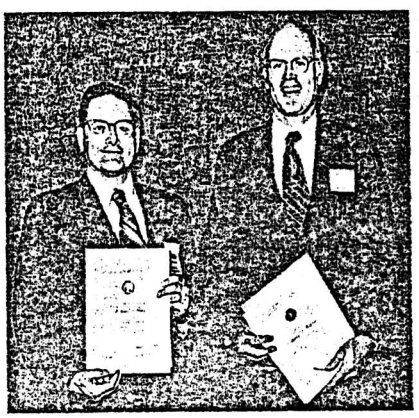
Presentation of Presidential Plaque by Donald J. Mitchell (left) to L. L. Ramsey.

1972 Fellows of the AOAC. From left to right: Paris M. Brickey, Jr., Mike J. Deutsch, Mary Heckman, J. J. Mayernick, M. Percy McKinley, Harold Salwin, and Theron E. Strange.

announced as the 1972 scholarship award winner, the 7 scientists chosen as the 1972 Fellows were presented with their certificates, and Charles C. Clark was honored as Associate Referee of the year.

Clyde L. Ogg delivered his Wiley Award address, "The Human Element in Collaborative Studies," at the General Session. Dr. Ogg stressed the primary importance of the collaborator in the methods validation work of the AOAC, and suggested ways in which we can make better and more efficient use of the experience, suggestions, and evaluations of the collaborators. He also suggested that collaborators and their work responsibilities be considered when collaborative studies are planned and sent out, and that collaborators be promptly informed of the overall results of the study and the performance of their particular laboratories.

Three symposia were held at the 1972 meeting: the Joint AOAC-SCC Symposium on Cosmetic Ana-



Richard Meyer (left) and Stanley Nesheim, with special certificates.

lytical Techniques; the Symposium on Industrial Chemicals as Food Contaminants; and the Forensic Science Symposium.

Thanks to Reviewers

The journal system of publication relies heavily on the services of manuscript reviewers—scientists who serve without pay, with little or no recognition, and often, because of deadlines and pressures of publication, with no formal expression of thanks. A good review of a manuscript is a difficult and time-consuming task, and we wish it were possible to reward our reviewers in some way. The following are those who have reviewed manuscripts for *JAOAC* during the past year. We hope that we have not overlooked anyone, but if we have, please forgive us and accept our sincere thanks for your services.

I. L. Adler, L. L. Alber, T. G. Alexander, R. Allen, S. Ames, B. Armbrecht, B. L. Auerbach, S. Barkan, W. F. Barthel, M. Beroza, A. Bevenue, F. E. Boland, W. R. Bontoyan, A. Bracey, C. L. Bramlett, Jr., W. Brammell, R. F. Brown, R. L. Brunelle, C. A. Brunner, M. Bueno, J. A. Burke, L. I. Butler, A. D. Campbell, A. A. Carlstrom, J. Caro, R. Carr, N. A. Carson, G. G. Carter, R. L. Caswell, E. Christensen, F. S. Chu, D. E. Coffin, E. H. Cohen, R. H. Collier, H. B. S. Conacher, R. A. Conkin, P. Corneliussen, A. F. Cucullu, C. G. Cunningham, H. Cunningham, A. Curley, F. Czech, P. DeCamp, H. DeLuca, E. De Ritter, M. Deutsch, M. Dow, R. H. Dougherty, T. D. Doyle, L. R. Dusold, D. O. Eberle, P. Eisenberg, H. F. Enos, E. A. Epps, Jr., T. J. Farrell, F. R. Fazzari, E. Fernandez-Flores, J. Fiorino, D. Firestone, V. Folen, W. B. Furman, R. J. Gajan, W. M. Gantenbein, A. M. Gardner, J. Gaul, C. W. Gehrke, A. J. Gehrt, L. Gershman, M. E. Getz, M. E. Getzendaner, C. Graichen, J. Grieb, L. Haddaway, L. G. Hambleton, J. Hamilton, H. Hammond, L. Hankin, J. Harrell, M. Heckman, K. S. Heine, R. R. Heinze, M. W. Heitzman, A. H. Hofberg, I. Hoffman, E. R. Holden, W. L. Hoover, T. M. Hopes, W. Horwitz, W. D. Hubbard, R. J. Hummell, H. K. Hundley, O. Hutzinger, M. Ihnat, M. C. Ivey, N. F. Ives, S. Kahan, L. Kamm, L. Kamps, A. B. Karasz, D. Kassera, R. Katz, S. Katz, J. M. Kauffman, F. W. Kavanagh, J. A. Kawatski, P. C. Kearney, J. R. Koons, T. Korbelak, T. C. Kram, R. T. Krause, B. Krinitz, G. Lakata, D. Larry, J. B. Leary, D. J. Lee, J. Levine, A. Lietze, D. J. Lisk, P. Lombardo, C. A. Luhman, A. J. Malanoski, D. M. Marmion, R. M. McCready, K. A. McCully, B. McMahon, V. C. Midkiff, M. M. Millard, H. J. Miller, J. P. Minyard, L. R. Mitchell, D. Moredock, F. J. Mulder, B. M. Mulhern, R. K. Munns, J. Myrdal, P. P. Nair, R. G. Nash, D. C. Nearpass, S. Nesheim, R. J. Noel, S. M. Norman, M. S. Oakley, I. Okuno, O. E. Olson, J. H. Onley, B. Oser, D. E. Ott, J. Pardue, N. R. Pasarela, G. Paulus, H. L. Pease, O. Pelletier, C. Perrin, W. Plank, P. L. Poelma, A. E. Pohland, W. A. Pons, R. H. Price, F. W. Quackenbush, H. L. Reynolds, G. H. Richardson, R. Rippere, J. A. G. Roach, J. V. Rodricks, W. M. Rogers, L. D. Rollins, A. Romano, D. Rosebrook, D. J. Roseth, B. A. Ross, R. C. Rund, J. J. Ryan, J. G. Saha, G. Sakell, H. Salwin, E. Sarnoff, R. M. Saunders, R. L. Schoeneman, A. E. Schulze, G. Schwartzman, P. M. Scott, M. M. Sharpe, A. J. Sheppard, O. L. Shotwell, M. Singh, E. Smith, D. M. Smith, H. Smith, W. H. Stahl, D. L. Stalling, W. F. Staruszkiewicz, Jr.; L. Stoloff, R. W. Storherr, C. R. Szalkowski, S. Tannenbaum, C. W. Thorpe, G. H. Tjan, J. C. Underwood, T. Urbanyi, J. F. Uthe, P. Van Soest, A. E. Waltking, A. E. Wasserman, R. R. Watts, R. S. Wayne, R. G. Webb, J. Weber, C. Weeks, J. R. Wessel, D. West, R. W. Weik, G. Wilamowski, P. Wilkes, C. H. Wilson, B. J. Williams, J. S. Winbush, A. L. Woodson, H. W. Woodson, J. Wragg, W. W. Wright, G. Yip, R. W. Young, and S. J. V. Young.

Special recognition, for reviewing 5 papers or more in a single year, is due M. Beroza, R. L. Brunelle, J. A. Burke, G. G. Carter, R. L. Caswell, E. A. Epps, W. L. Hoover, D. Larry, J. Levine, A. J. Malanoski, R. K. Munns, L. Stoloff, R. W. Storherr, and G. Yip.

Award Fund

A contribution to the Harvey W. Wiley Award Fund has been received from Dr. and Mrs. William Horwitz in memory of Henry A. Lepper. Dr. Horwitz is Executive Director of the AOAC. The AOAC also made a contribution in Mr. Lepper's memory. The fund is used to support the AOAC scholarship award for needy college students and to reward scientists for outstanding contributions to analytical chemistry. The Association matches each contribution to this worthwhile fund. Contributions may be sent to: Luther G. Ensminger, Executive Secretary, AOAC, Box 540, Benjamin Franklin Station, Washington, D.C. 20044.

NEW PUBLICATIONS

World Health Organization Technical Report Series. Available through the American Public Health Association (APHA), Inc., 1015 Eighteenth St., N.W., Washington, D.C. 20036.

No 462, *Evaluation of Food Additives:* 14th Report of the Joint FAO/WHO Expert Committee on Food Additives, 1971. 36 pp. Price $1.00.

Various aspects of problems related to the establishment of specifications are discussed, particularly with respect to solvents used in food preparation and to mercury. Recommendations for further action are made.

No. 487, *WHO Expert Committee on Specifications for Pharmaceutical Preparations:* 24th Report, 1972. 88 pp. Price $1.50.

Proposals for changes and additions to the *International Pharmacopoeia* are discussed, particularly improved test procedures and "good practices in manufacture." Also, 31 new monographs for drug substances are included along with several reagent specifications and test procedures.

No. 495, *Opiates and Their Alternates for Pain and Cough Relief,* 1972. 19 pp. Price $0.75.

The effectiveness of natural and semisynthetic alkaloids is reviewed, in view of the tremendous abuse problem, by a WHO scientific group. Specific ailments are considered individually and recommendations are made. In addition, it was strongly urged that further research be done on alternatives and on opiate addiction.

No. 498, *International Drug Monitoring: The Role of National Centres,* 1972. 48 pp. Price $1.00.

With the increasing problem of adverse reactions to drugs, it is imperative that information be obtained. The objectives and operations of national centers are discussed. Some information is given on the centers of 12 nations. Centers in Sweden and the United Kingdom are discussed in detail and the programs of the Food and Drug Administration are also described.

No. 500, *Oral Enteric Bacterial Vaccines,* 1972. 34 pp. Price $1.00.

While oral vaccination had been generally considered to be an ineffective means of immunization, the success of the live poliomyelitis vaccine has caused attention to be directed to this more convenient means of administering medication. The reader is brought up-to-date by means of a rather extensive review, particularly with respect to developments in enteric fevers, shigellosis, cholera, and *E. coli* enteritis. Recommendations are made for further research and specific topics are suggested.

No. 502, *Pesticide Residues in Food,* 1972. 46 pp. Price $1.00.

Recent developments in the establishment of acceptable daily intakes (ADI) of pesticides are discussed extensively. In addition, current recommended ADI's are conveniently tabulated. Recommendations of the joint FAO/WHO study group are also presented.

No. 503, *Nutritional Anaemias,* 1972. 29 pp. Price $1.00.

Deficiencies of iron, folate, and vitamin B_{12} are discussed, particularly with regard to the occurrence and absorption of hemopoietic nutrients. Recommended daily intakes are presented.

No. 505, *Evaluation of Certain Food Additives and the Contaminants Mercury, Lead, and Cadmium:* 16th Report of the Joint FAO/WHO Expert Committee on Food Additives, 1972. 32 pp. Price $1.00.

Sources of contamination by these metals plus some food additives are discussed, along with means of control and regulation.

The following publications are also available through APHA.

Nutrition: A Review of the WHO Programme, 1965–1971, 1972. 36 pp. Price $0.50.

WHO programs are reviewed with particular emphasis on protein-calorie malnutrition, xerophthalmia, nutritional anemias, and endemic goiter. Proposals for further action are also discussed.

Biological Substances: International Standards, Reference Preparations, and Reference Reagents, 1972. 56 pp. Price $1.00.

The pamphlet consists of tables listing substances available, units of measure, form in which dispensed, year of establishment, references, and addresses of sources. An alphabetical index is included.

Toxicological Evaluation of Some Enzymes, Modified Starches and Certain Other Substances, 1972. 109 pp. Price $1.50.

Monographs are presented for 24 food additives, each containing biological data and references.

Vector Control in International Health, 1972. 144 pp. Price $8.00.

This soft-cover book addresses itself to the problem of the spread of diseases by international transportation of insects and rodents. Control in ports and airports and on ships and aircraft is extensively discussed. The appendices contain illustrations on insect anatomy, tables on pesticides, and information on the safe use of pesticides.

Vocabulary of Surface Active Agents, 2nd Ed., Secretariat, Comite International des Derives Tensio-Actifs, 64, Avenue Marceau, 75008-Paris. 135 pp. Price 20 FF ($4.01), plus shipping.

Definitions of 213 terms are given in English, French, and German. Typical terms are "ampholytic surface active agent" and "winding oil". An index is provided.

Official Publication: Association of American Pesticide Control Officials Incorporated, 1972–1973. 109 pp. Price $4.00. (Order from Robert H. Guntert, State Board of Agriculture, Topeka, Kan. 66612).

The annual publication contains the proceedings of the 26th meeting of the Association (addresses, committee reports), reference material (model bills, regulations), and directory of officials.

International Nonproprietary Names for Pharmaceutical Substances: Cumulative List No. 3. 1971. 189 pp. Price $6.00.

The proposed international nonproprietary names and chemical names or molecular formulas of 2733 compounds are listed in tabular form. Supplemental information, in the back of the pamphlet, lists the procedures for selection of recommended names and also contains a molecular formula index.

CORRECTIONS

JAOAC 55, 984–985 (1972), "Revised Kjeldal Total Nitrogen Method for Feeds and Premixes," by Robert Odland (current address: Scott-Pro Inc., P.O. Box 587, Scott City, Kan. 67871), p. 984, right column, reagent (f), line 2	Change "0.0263N" to "0.028571N."
Ibid., p. 985, left column, lines 5 and 9	Change as above.
JAOAC 55, 1168–1170 (1972), "Effect of Maleic Acid on the Ultraviolet Absorption of Some Antihistamine Maleate Salts," by James L. Hamilton, Jr., Helen S. Naviasky, and William M. Ment, p. 1168, left column, abstract, line 13	Delete the word "sample."
JAOAC 55, 1377–1387 (1972), "Subject Index to Volume 55," p. 1385, entry for Pesticide formulations, chlorinated hydrocarbon contaminants in, TLC	Change "581" to "851."

BOOK REVIEWS

Photometric Organic Analysis: Basic Principles with Applications. Pt. 1. E. Sawicki. Wiley-Interscience, New York, 1970. 679 pp. Price $32.50.

This book is essentially a comprehensive literature search, keying the reader to some 2,100 references. The author's viewpoint is that of a "life scientist," and he often draws examples of applications from tissue or cellular chemistry and from trace analysis techniques. Sawicki avoids the use of quantum mechanics in laying down the principles. Instead, he relies on the extensive use of resonance hybrid structures and positive or negative resonance nodes to explain spectra, shifts in absorbance maxima due to substitution or other reactions, how to move bands toward the red and away from background interference, and how to intensify them for maximum spectral sensitivity in derivative formation.

An excellent discussion is given, showing how ultraviolet-visible spectra may be used to infer whether enol or keto forms are present in cases of complex tautomerism. Sawicki's treatment of electronic interactions between nonconjugated groups is especially interesting, making use of the spatial geometry of the molecule (group overlap or lack thereof). A good treatment on the effects of temperature on spectral shifts and on solvent-solute interaction is also presented.

The volume's greatest value lies in its explanations of how colored reaction products may be formed to analyze various classes of compounds. In an attempt to assess the book's practical usefulness, the reviewer used it to look up information on 2 colorimetric reagents in daily use in our laboratory—the reactions of alkaline picrate reagent with digitoxin, and 2-thiobarbituric acid reagent with oxidized digoxin. An extensive amount of information was located on the whole series of nitrated benzene reagents, including a literature reference to the reaction of digitoxin with 2,2',4,4'-tetranitrodiphenyl. Even more pleasing, the structure of the final color product between 2-thiobarbituric acid and malonaldehyde (a product in the oxidation of digoxin) was given with reference to the literature.

In addition to this book's obvious practical value in designing new analytical methods or improving old ones, its reader can count on the satisfaction of knowing the chemistries of many colorimetric reactions previously regarded as being "not too well understood."

WILLIAM B. FURMAN
National Center for Drug Analysis
Food and Drug Administration
St. Louis, Mo.

Chemie der Pflanzenschutz- und Schädlingsbekämpfunds-mittel. R. Wegler (Ed.). Springer-Verlag, New York, 1970. Vol. 1: 671 pp., price $49.50. Vol. 2: 550 pp., price $40.70.

These 2 ambitious volumes deal with a very timely subject—modern chemicals used to control life on this earth. These chemicals are described in an orderly, documented, and detailed manner. This wealth of information on the chemistry of pesticides fills a real need in the scientific community and particularly for the government scientists regulating these chemicals. Even though the editor and many of the contributors are from Europe, a considerable amount of American literature is covered.

The book title indicates that chemistry is the main subject but included with the chemistry is a wealth of information on mode of action, synergists, resistance, plant diseases, toxicity, and proper use of pesticides. The purposes of these volumes are to give the student a textbook, give the industrial chemist a general view, and offer the specialist help and advice. These it accomplishes very well. However, I feel that the various chapters are so well documented by citations to the open literature that the book comes closer to being a reference work up through early 1969. Those interested in structure-activity relationships (toxicologists, entomologists, plant physiologists, chemists, etc.) will find the compilations on relative toxicities in each chapter of use because they appear in one set of books. For the synthetic chemists and applications scientists, the book offers a subject covered generally only in patents, i.e., manufacture and use of pesticide. Patents are often neglected in both text and reference books. Thus, extensive patent coverage is a definite advantage not found elsewhere.

The German language offers a definite disadvantage to those not familiar with it but the many chemical reactions, figures, tables, etc. alone are almost all self-explanatory. The technical words are often identical or nearly so with the English equivalent. The insights gained will more than make up for the somewhat slower progress of some readers due to this language barrier.

The last chapter by Helmut Frehse is exceptionally well written but one will need to go to the original literature for details on methodology. The laws he discusses have since evolved and so have methods. For example, new pesticide legislation has only recently been enacted in the United States (1972).

Under techniques for the determination of residues, Table 5, p. 461, Vol. 2, mass spectrometry is listed as not sensitive. This is not true today. When a mass spectrometer is combined with a gas chroma-

tograph (for separation) and a minicomputer (with its many varied programs), pesticides can be measured with greater speed, specificity, and sensitivity than by using almost any other technique.

Pesticide petition reviewers and pesticide analytical chemists in general will find the books useful because they can look at a set of compounds already explored and transfer this knowledge to an unknown or older pesticide. They may find questions answered in the published data for the old compounds while this same question may be unanswered in the literature presented for review for a structurally similar compound. The high quality of the 12 main chapters or headings is maintained throughout the 2 volumes. The reader will have to go elsewhere for information on the pesticides in the environment (*see* Chapter 12). However, he will return to these 2 volumes for the basic chemistry of most of the pesticides available before 1969.

WALTER R. BENSON

Division of Drug Chemistry
Food and Drug Administration
Washington, D.C.

Computer Assistance in the Analysis of High-Resolution NMR Spectra. P. Diehl, H. Kellerhals, and E. Lustig. Springer-Verlag, New York, 1972. 96 pp. Price $15.30

The present work is Volume 6 of a 6-volume series entitled *NMR—Basic Principles and Progress/ Grundlagen und Fortschritte.* Volume 5 of this series, *Analysis of NMR Spectra, see* (1972) *JAOAC* 55, 902, presented the chemist with a method for the rapid analysis of an NMR spectrum without requiring the use of a computer. Volume 6 goes one step further and examines the role of a computer in the analysis of high-resolution NMR spectra.

The volume is divided into 2 sections. The first, consisting of 9 chapters, deals with the general presentation of the subject; the second, consisting of 5 appendices, serves as a supplement to the first section. The quantum mechanics which the reader will need to follow the concepts throughout the text are well covered in the first chapter. The heart of the volume consists of a description of the basic computer methods in use as of late 1970 and a discussion of various means used to improve these methods. When the methods are compared, the authors conclude that the most convenient method, for practicing spectroscopists, is that of Castellano and Bothner-By. Other topics in this section include the analysis of spectra unaided by a computer, the calculation of parameter errors, rate studies, and double resonance techniques. The second section covers vectors and matrices, diagonalization of symmetrical matrices, least-squares fitting, parameter errors from least squares fits, and density matrices.

The authors do not intend this volume to serve as a textbook but more for a readership already acquainted with the fundamentals of high-resolution NMR spectroscopy. Since the analysis of NMR spectra is becoming more and more dependent on the computer, this volume should be a welcome addition to the serious spectroscopist. As the authors point out in the text, "Analysis of spectra is rarely possible without recourse to a computer. Exceptions are spectra arising from systems consisting of a small number of spins or possessing certain simplifying features such as symmetry of weak couplings." This is a rapidly changing field; this volume will provide the reader with the basic principles needed to take advantage of the computer and its ever-changing technology.

ERIC B. SHEININ

Division of Drug Chemistry
Food and Drug Administration
Washington, D.C.

Current Concepts in the Pharmaceutical Sciences: Biopharmaceutics. James Swarbrick (Ed.). Lea & Febiger, Philadelphia, 1970. xii + 304 pp. Price $16.50.

This first volume in a contemplated series of reviews in pharmaceutical sciences is a largely successful effort to survey progress in the study of the dynamics of drug-receptor site interactions. The 6 chapters, each contributed by a recognized authority, exhibit a uniformity in treatment and lack of overlap which are unusual in such undertakings.

The initial chapter on pharmacokinetics introduces the formulation and manipulation of the mathematical models necessary for any quantitative description of drug kinetics. This is followed by chapters dealing with aspects of drug intake (gastrointestinal absorption), interaction at receptor sites (the molecular orbital approach is emphasized), and drug disposition and response (as controlled by metabolism and excretion). An otherwise useful chapter on the methods of analysis of drugs and their metabolites is marred by the failure to include the increasingly dominant technique of combined GLC-mass spectrometry. A final chapter discusses in vitro models of drug dissolution. References are adequate; a useful overall index has been included.

None of the chapters attempt exhaustive review of their areas; the emphasis is instead on critical discussion of the fundamental concepts, experimental methods, and most significant results in this relatively new scientific discipline. The result is an attractive volume which will have broad appeal to

mathematicians, chemists, biologists, and medical scientists—a true reflection of the multidisciplinary nature of biopharmaceutics.

THOMAS D. DOYLE

Division of Drug Chemistry
Food and Drug Administration
Washington, D.C.

Thermomicroscopy in the Analysis of Pharmaceuticals. Maria Kuhnert-Bradstätter. Pergamon Press, New York, 1971. x + 409 pp. Price $28.50.

Thermomicroscopy involves the use of a microscope to observe the behavior of a compound or mixture of compounds as it is heated or cooled on a microscope slide. The material on the slide may melt, sublime, decompose, or exhibit other phenomena at particular temperatures or temperature ranges. For many years, the research efforts of the late Ludwig Kofler were directed toward demonstrating how individual compounds could be characterized and identified by means of the changes they undergo under the influence of temperature. Maria Kuhnert-Brandstätter was a student and, later, a collaborator of Professor Kofler, and in her book, she endeavors to emphasize how thermomicroscopy may be used to determine the identity and purity of pharmaceuticals.

The main feature of the book is its "Identification Table for the Hot Stage," which contains almost a thousand pharmaceuticals and related compounds listed by melting points. The melting points of the eutectics obtained when these compounds are fused with standard reference substances and the temperatures at which the refractive indices of the melted compounds match those of standard glass powders are also provided. In a column labeled "Special Remarks," miscellaneous data, useful for the identification of particular substances, are tabulated. These include information about the formation of sublimates, the phase transformations, the growth of crystals from the melt, the decomposition of the substance before its melting point, the loss of water, and the evolution of gases. The colors, crystalline forms, or odors of some substances are described when they are diagnostic, and a small number of supplemental chemical tests are given when they are considered necessary. For a number of the newer pharmaceuticals, the ultraviolet maxima and minima are included. The format of the table closely follows that used by the Koflers in their *Thermo-Mikro-Methoden* (Wagner, Innsbruck, 1954) and some of the data appearing in the present book has been taken from that source.

Besides the above table, the book has a number of short introductory chapters which briefly describe the available apparatus, the preparation of samples, the observations which can be made as the samples are heated or cooled, and a number of special techniques. In other chapters, useful background information concerning crystal morphology, phase diagrams, and polymorphism is presented. The Kofler hot bench and its use are described and a table is provided which gives melting points and eutectic temperatures with standard substances as they are obtained on the hot bench. There is, finally, a table which lists alphabetically all the compounds appearing in the hot stage and hot bench tables along with their melting points as determined by either or both methods.

This book is recommended as a convenient source of melting point data for pharmaceuticals and related compounds. It should be useful to all who are concerned with the identification and control of drugs.

JOSEPH E. KOLES

Bureau of Narcotics and Dangerous Drugs
U.S. Department of Justice
Washington, D.C.

Chelates in Analytical Chemistry, H. A. Flaschka and A. J. Barnard, Jr. (Eds.). Marcel Dekker, Inc., New York. Vol. 3: 231 pp. Price $19.75. Vol. 4: 316 pp. Price $23.50.

Volume 3

In extending the series on the application of chelation to analytical chemistry, the editors have provided a useful addition with this volume. The individual chapters have all been contributed by members of the 'Czech school,' thus providing some material and viewpoints not previously readily available in the English scientific literature. A. Galik has examined the criteria requisite for successful titrimetic use of a water-immiscible organic chelate-containing solvent for visual, photometric, and radiometric analysis of single or multiple metal ions in solution. Examples of typical procedures for inorganic and biologic specimens are provided. A chapter by J. Dolezal, K. Stulik, and J. Zyka deals with the basis of amperometric titrations and briefly describes the nature of the requisite equipment and general methodology. This is followed by a useful review of specific applications to enumerated metal ions with individuals sections devoted to 17 of the more frequently used reagents. A final chapter by M. Kopanica and J. Zyka provides a more broadly based discussion of the study of chelate reactions using potentiometric, polarographic, amperometric, conductometric, and high frequency impedimetric procedures. A significant section provides examples of the application of these techniques to the study of the mechanism and kinetics of chelation reactions.

An index provides ready access to significant matters in the text which is unfortunately marred (in the reviewer's copy) by some poorly printed pages.

This slim volume will be useful to analytical chemists in general and of special interest to those working in the field.

Volume 4

This volume is made up of 2 chapters. The first by S. Shibata is a masterful summary of the present status of the application of pyridylazo compounds in analytical chelation chemistry. A thorough treatment is presented of this important family of compounds with many specific detailed examples of their use. Much valuable information has been brought together in a clearly presented form. The preparation of the reagents has also been included. Literature citations deal with the period prior to 1967 for the most part. The second chapter by J. W. Robinson, P. F. Lott, and A. J. Barnard, Jr. is on the application of chelates to flame analytical techniques. The focus is on the use of various chelates to eliminate the interferences of undesired metals.

The fourth volume of the series capably extends the utility established by the previous books.

MARTIN RUBIN

Georgetown University Hospital
Washington, D.C.

Techniques of Chemistry. Vol. II, Organic Solvents, 3rd Ed. John A. Riddick and William B. Bunger. Wiley–Interscience, New York, 1971. xiii + 1041 pp. Price $24.95.

In this third edition, the authors have added 100 organic compounds, increasing the total number of solvents to 354, and the number of pages has increased to over 1000. These solvents are classified according to their chemical structures into the following major classes: hydrocarbons, hydroxy compounds, ethers, carbonyls, acids, acid anhydrides, esters, halogenated hydrocarbons, nitrogen compounds, sulfur compounds, and compounds with more than one characteristic atom or group.

Each solvent compound is assigned a code number for easy identification and reference. The same number appears in bold face type with each compound in every tabulation of the solvents and use of the name throughout the book. Over 5,000 references to the original literature source of the information in the tables and text is contained in a bibliography section comprising 231 pages.

An alphabetically arranged index, of 30 pages, cross-references the organic solvent compound with the page number containing the tabulation of its physical properties, and with the page number containing information on the methods of preparation, purification, criteria of purity, and safety in the chapter on purification methods.

The tables of physical properties for these organic compounds comprise 445 pages and contain a listing of 44 physical properties, starting with molecular weight, boiling point, etc. and ending with spectral data (ultraviolet, infrared, Raman, mass, and nuclear magnetic resonance). The physical properties reported in the tables and the criteria for their selection are discussed in a chapter on physical properties under the following headings: boiling point, vapor pressure, density, refractive index, viscosity, surface tension, heat of vaporization, critical constants, heat capacity, optical activity, freezing point constant, boiling point constant, acidity and basicity, electrical properties (conductivity, dielectric constant, and dipole moment), flash point, and spectroscopy.

In the chapter on physical properties the authors give the definition of the physical property and the basic equations used in the determination of the value for the property from the experimental data. The discussion of the physical property and the criteria for its selection and inclusion are of particular importance to analytical chemists for evaluating the purity, characterization, stability, etc. of these organic solvent compounds.

Highlights of the book are the concise summaries with literature references, the broad coverage of the literature, and the selection for best values for the tables from contributions of major laboratories working in the areas of preparation, purification, purity evaluation, and physical property measurements of those organic compounds. The third edition of *Organic Solvents* is recommended by the reviewer as an excellent reference source for analytical chemists in the selection and use of these organic compounds as solvents in thin layer chromatography, preparative solvent extraction, etc.

AUGUSTUS R. GLASGOW, JR.

Office of Pesticides Programs
Environmental Protection Agency
Washington, D.C.

Methods of Air Sampling and Analysis: Intersociety Committee. Published by American Public Health Association, 1015 18th St., N.W., Washington, D.C. 20036, 1972. 480 pp. Price $12.50 + 0.50 handling.

This volume contains 57 "tentative" methods proposed by the Intersociety Committee as useful for air pollution control. Methods will become "standard" upon completion of collaborative test programs underway at present. The AOAC is represented on the Committee whose objective is to pro-

duce standardized methods of sampling and analysis for routine investigations of air pollution.

The methods are organized in 7 major divisions (with some examples of substances of interest to regulatory chemists in the agricultural and public health fields in parentheses): (1) carbon compounds (hydrocarbons, benzo(a)pyrene and related polynuclears, formaldehyde, phenolics); (2) halogens (chloride, fluoride); (3) metals (antimony, arsenic, beryllium, iron, lead, manganese, molybdenum, selenium); (4) oxidant and inorganic nitrogen compounds (nitrate); (5) particulate matter; (6) radioactivity (gross alpha and beta content, iodine-131, lead-210, strontium-89 and -90, and tritium); (7) sulfur compounds (hydrogen sulfide, sulfur dioxide, mercaptans).

Of general interest to most analytical chemists is the 129 page Part I which contains general precautions and techniques. Some particularly interesting discussions include an extensive section of adsorption effects on container walls and connecting tubes which can be read with profit by all analysts; observational errors; care and use of volumetric glassware (presumably for completeness); preparation and standardization of common standard solutions; a section emphasizing the use of a recovery series for evaluating the applicability of a method to a particular sample which is a relatively unique but important item in a methods handbook; a very interesting section on handling interferences; an extensive section on photometric methods, including atomic absorption, and short sections on gas chromatographic and radioactivity analysis, and precision and accuracy; and an excellent section on safety, particularly organized around unit operations in analytical chemistry and instruments.

Since the selection of methods was in the hands of knowledgeable committees, they may well serve as starting points for standardizing methods for other types of analyses. The relatively low price of this manual is welcome. Most analytical laboratories will find it worth acquiring for the introduction sections and the selection of methods alone.

WILLIAM HORWITZ

Office of Sciences
Food and Drug Administration
Washington, D.C.

PROCEEDINGS
INTERNATIONAL SYMPOSIUM ON THE IDENTIFICATION AND MEASUREMENT OF ENVIRONMENTAL POLLUTANTS
OTTAWA, CANADA
JUNE 14, 15, 16, AND 17, 1971

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