## HAZARDOUS SUBSTANCES

# Separation and Identification of Carbon Tetrachloride and Other Halogenated Hydrocarbons from Multicomponent Mixtures

By HARRY SHUMAN (Food and Drug Administration, Room 1204, U.S. Customhouse Bldg., Philadelphia, Pa. 19106)

**Gas chromatography has frequently been used to separate and identify halogenated hydrocarbons in mixtures, and the microcoulometric gas chromatograph, which uses combustion and a detection system sensitive only to chloride, bromide, and iodide, appears well suited to this type of determination. Retention patterns for nonpolar and polar columns differ materially; this difference can be used in identifying individual halogenated compounds. Isothermal operation of columns is suitable for many of these compounds, but some higher boiling compounds would have excessive retention times and partial programming appears advisable. Study will be continued and extended.**

Before the development of gas chromatography, methods for the separation and identification of halogenated hydrocarbon solvents in multi-component mixtures were limited in scope and frequently complex. While pure halogenated hydrocarbons can be identified by physical tests and their infrared spectra, these criteria are of little value with many complex mixtures.

Jacobs (1) and Jacobs and Scheflan (2) have dealt at length with tests and analyses for chlorinated hydrocarbon and have included numerous references. The well known Beilstein flame test serves to indicate the presence of organohalogen compounds. The phenylisocyanide odor test is given by chloroform and carbon tetrachloride. The Fujiwara colorimetric reaction, in one or another modification, has been applied to the detection and determination of chloroform, carbon tetrachloride, trichloroethylene, perchloroethylene, tetrachloroethane, and others.

Traditional methods for the determination of halogenated hydrocarbons in general include thermal decomposition (flame and tube combustion), alkaline hydrolytic procedures, and sodium reduction methods in which the liberated halide is determined by silver nitrate procedures. Schemes have been presented for the separation, identification, and proximate analysis of mixtures involving fractionation, extraction, and the application of chemical and physical tests (1–3).

A number of papers in the literature have described the separation of chlorinated hydrocarbons by gas-liquid chromatography, employing packed columns and both polar and nonpolar liquid phases. Harrison (4) reported retention volumes for 26 chlorinated hydrocarbons with 2 polar and 2 nonpolar liquid phases. Warren and co-workers (5) reported retention times of 12 chlorinated hydrocarbons; they used paraffin wax on Célite 550. Urone, Smith, and Katnik (6) studied the chromatographic behavior of 11 chlorinated hydrocarbons and reported specific retention volumes for 6 liquid phases at 4 temperatures. A 6-foot column of paraffin wax on Chromosorb operating at 97°C achieved excellent separation of the 11 chlorinated hydrocarbons. Gunther, Blinn, and Ott (7) used the Beilstein test to detect organohalogen compounds emerging from a gas chromatograph and proposed a means of using this test as an indicator in conjunction with thermal conductivity detection for quantitative purposes. Archer, Bevenue, and Zweig (8) compared the sensitivity of microcoulometric halogen detection with the thermal conductivity detector on 4 halogenated hydrocarbons with 2 partitioning phases. Burke (9), working with the microcoulometric gas chromatograph and a column with a silicone fluid phase, determined relative retention times for a number of chlorinated

compounds, including some of the common chlorinated solvents used as fumigants.

The microcoulometric gas chromatograph using combustion and a detection system sensitive only to chloride, bromide, and iodide appears well suited to the separation, identification, and determination of halogenated compounds in multicomponent mixtures. Since the detector is blind to nonhalogen compounds, no preparative treatment other than dilution with suitable solvents should be required with many samples. Retention patterns for polar and nonpolar columns differ materially, and this difference can be used to identify individual halogenated compounds.

An investigation of the literature was made for uses (2, 10, 11) and formulations (12) containing chlorinated hydrocarbons. The following categories were covered:

Adhesives and cements
Cleaning compositions (various)
Fire extinguishers
Ink removers
Paint, varnish, and lacquer removers and solvents
Polishes and dressings

Based on the investigation, the first three chlorinated hydrocarbons in the following list appear to be most widely used in the above categories; the remaining compounds are used in approximately descending order:

| | *Name* | *Formula* |
|---|---|---|
| (a) | Carbon tetrachloride | $CCl_4$ |
| (b) | Trichloroethylene | $CCl_2{=}CHCl$ |
| (c) | 1,2-Dichloroethane | $CH_2Cl.CH_2Cl$ |
| (d) | Methylene chloride | $CH_2Cl_2$ |
| (e) | Chloroform | $CHCl_3$ |
| (f) | *o*-Dichlorobenzene | $C_6H_4Cl_2$ |
| (g) | Perchloroethylene | $CCl_2{=}CCl_2$ |
| (h) | Propylene dichloride | $CH_2Cl.CHCl.CH_3$ |
| (i) | 1,2-Dichloroethylene | $CHCl{=}CHCl$ |
| (j) | Tetrachloroethane | $CHCl_2.CHCl_2$ |

Chlorinated hydrocarbon solvent combinations of two and occasionally three (e.g., paint removers) are used together in some formulations.

Urone and coworkers (6) selected 11 chlorinated hydrocarbons "for the frequency of their industrial use as well as their potential toxicity to the human system." While industrial use does not necessarily parallel use in household-type products, 7 of the chlorinated hydrocarbons they selected are included in the above list, and the remaining 4 are as follows:

| | *Name* | *Formula* |
|---|---|---|
| (k) | 1,1-Dichloroethane | $CHCl_2.CH_3$ |
| (l) | 1,1,1-Trichloroethane | $CCl_3.CH_3$ |
| (m) | 1,1,2-Trichloroethane | $CHCl_2.CH_2Cl$ |
| (n) | Monochlorobenzene | $C_6H_5Cl$ |

Less common chlorinated compounds that are contained in a few formulations, notably in paint removers and lacquer thinners, are as follows:

(o) Dichlorobutane
(p) 2-Chloro-1-butene
(q) Amylene dichloride
(r) Dichlorodiethylether (not a chlorinated hydrocarbon but highly toxic (13))

## Experimental

For preliminary study, 5 of the most commonly used chlorinated hydrocarbons, (a) through (e) in the above listing, were selected and their gas chromatographic behavior on 3 columns was observed.

In the Food and Drug Administration microcoulometric gas chromatographs are normally provided with 6 ft, 15–20% silicone fluid columns for use in chlorinated pesticide analyses. Silicone columns, though not found as satisfactory by several authors as paraffin wax columns (5, 6) would be suitable for initial observations in analytical work. In some cases column changeover might be avoided or useful supplemental data obtained.

### *Gas Chromatograph*

The equipment (from Dohrmann Instruments Company) consisted of a modified Micro-Tek Model GC 2500, 2-column, programmed-temperature gas chromatograph; a Brown 1-mv recorder; a Dohrmann Model S-100 sample inlet/combustion unit; a Model C-100 coulometer; and a Model T-200-S silver-silver acetate titration cell.

### *Columns*

All columns were made of coated 30/60 mesh Chromosorb P packed into 6′ by ¼″ o.d. (3/16″ i.d.) aluminum tubing.

(a) *DC 200 silicone fluid* (12,500 cst).— Dissolved in ethyl acetate. Liquid phase 17.5% on acid-washed support. (Column is normally conditioned for chlorinated pesticide analysis at 250°C.)

(b) *Paraffin wax.*—M.p. 48°C. Dissolved in benzene. Liquid phase 25%. Column conditioned 72 hr in laboratory oven at 140°C with $N_2$ flow at 100 ml/min.

(c) *Tricresyl phosphate.*—Dissolved in ethyl acetate. Liquid phase 25%. Column conditioned 24 hr in laboratory oven at 125°C with initial $N_2$ flow at 150 ml/min. (Flow in this column slowed down during heating.)

### *Gas Chromatographic Conditions*

Column temperatures were 60°C for the DC 200 silicone fluid column and 67°C for the paraffin wax and tricresyl phosphate columns. Injection port temperatures were 140°C. The column outlet tube and the block oven temperature of the Dohrmann unit was maintained at 250°C; furnace temperature at 850°C. Nitrogen carrier gas flow was 75 ml/min. The sensitivity range was 32 ohms.

### *Procedure*

ACS grade chloroform and carbon tetrachloride were used. Methylene chloride, 1,2-dichloroethane, and trichloroethylene were obtained from Fisher Scientific Company. All compounds, when chromatographed singly, showed only one peak.

The compounds, weighed into tared volumetric flasks, were diluted with hexane and aliquots were taken so that final dilutions contained 1 $\mu$g/$\mu$l of the compound or 1 $\mu$g of each of the 5 compounds per $\mu$l. Injections of 3 $\mu$l samples were made with a 10 $\mu$l syringe with the column exit vent closed. The solvent could not be vented with low boiling chlorinated compounds, as required with much larger injections for chlorinated pesticides, nor was venting necessary for 3 $\mu$l injections of hexane solution, since there was no evidence of contamination of the titration cell.

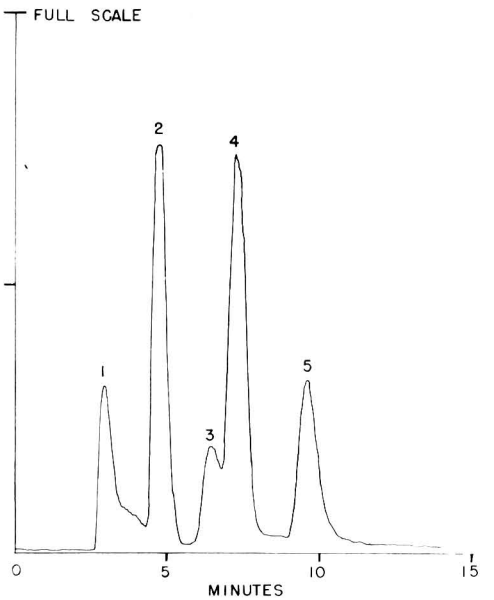


*Fig. 1*—Gas chromatogram of mixture of chlorinated hydrocarbons. Column, 17.5% DC 200 silicone fluid (12,500 cst). Column temp., 60°C. Nitrogen flow, 75 ml/min. Peaks are as follows: *1*, methylene chloride; *2*, chloroform; *3*, 1,2-dichloroethane; *4*, carbon tetrachloride; *5*, trichloroethylene.

This report of the Associate Referee was presented at the Seventy-Seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

## Results and Discussion

Figures 1, 2, and 3 are chromatograms of the 5 chlorinated hydrocarbons obtained with the 3 columns. The nonpolar silicone fluid and paraffin wax columns gave the same elution pattern for the chlorinated hydrocarbons, but the order of elution with the polar tricresyl phosphate column was quite different from the other two. The paraffin column gave the best resolution of the five compounds.

When compounds were chromatographed individually on the silicone fluid column, methylene chloride and 1,2-dichloroethane tailed considerably, and total response for 1,2-dichloroethane was poor. Peaks for the 3 remaining compounds were symmetrical, and responses were of the same order as with the other columns. Whether this is typical or anomalous behavior for this column remains to be determined.

The differences in elution patterns are further emphasized in Table 1, which gives the retention time ratios of the chlorinated hydrocarbons relative to carbon tetrachloride as unity.

**Table 1. Relative retention times (carbon tetrachloride = 1)**

| Compound[a] | DC 200 Silicone Fluid | Paraffin Wax | Tricresyl Phosphate |
|---|---|---|---|
| (1) Methylene chloride | 0.41 | 0.32 | 0.65 |
| (2) Chloroform | 0.65 | 0.60 | 1.39 |
| (3) 1,2-Dichloroethane | 0.88 | 0.81 | 1.83 |
| (4) Carbon tetrachloride | 1.00 | 1.00 | 1.00 |
| (5) Trichloroethylene | 1.32 | 1.28 | 1.74 |

[a] Numbers 1 to 5 correspond to peaks in Figs. 1, 2, and 3.

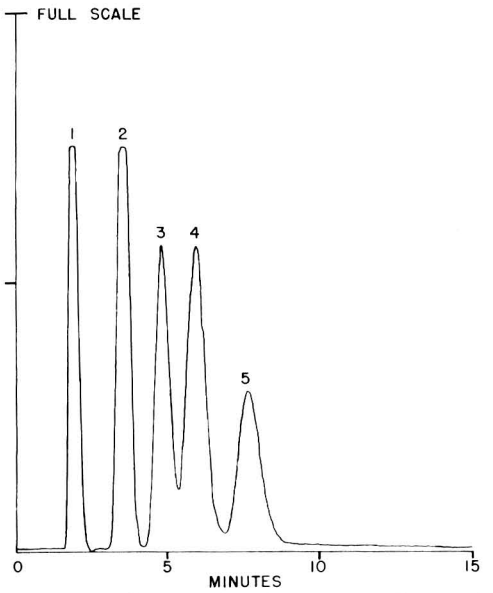


*Fig. 2*—Gas chromatogram of mixture of chlorinated hydrocarbons. Column, 25% paraffin wax. Column temp., 67°C. Nitrogen flow, 75 ml/min. Peaks are as follows: *1*, methylene chloride; *2*, chloroform; *3*, 1,2-dichloroethane; *4*, carbon tetrachloride; *5*, trichloroethylene.

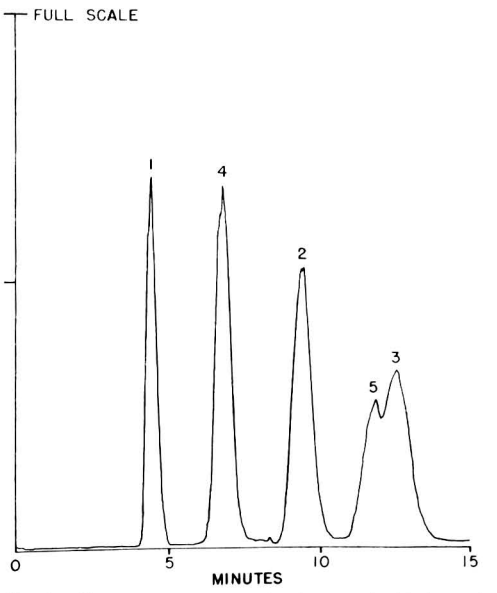


*Fig. 3*—Gas chromatogram of mixture of chlorinated hydrocarbons. Column, 25% tricresyl phosphate. Column temp., 67°C. Nitrogen flow, 75 ml/min. Peaks are as follows: *1*, methylene chloride; *2*, chloroform; *3*, 1,2-dichloroethane; *4*, carbon tetrachloride; *5*, trichloroethylene.

## Recommendations

It is recommended that this work be extended to include most of the other chlorinated hydrocarbon compounds previously listed. Such extension would include several additional halogenated hydrocarbons with boiling points below that of trichloroethylene (87°C), and might require changes in column specifications and also investigation of additional polar phases to obtain the best resolution. On the other hand, the inclusion of higher boiling compounds like perchloroethylene (121°C), the highly toxic (13) 1,1,2,2-tetrachloroethane (146°C), and *o*-dichlorobenzene (180°C) would lead to excessive, if not impossible, retention times with isothermal operating temperatures suitable for the lower boiling compounds. It therefore appears that isothermal operation up to a point, followed by temperature programming, will be required for the range of chlorinated hydrocarbons of concern.

It is also recommended that representative types of multicomponent mixtures containing one, two, or three chlorinated hydrocarbons be prepared and used to test selected procedures.

## References

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(5) Warren, G. W., Priestley, L. J., Jr., Haskin, J. F., and Yorborough, V. A., *Anal. Chem.*, **31**, 1013 (1959).

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These recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A, and were accepted by the Association. See *This Journal*, **47**, 120 (1964).

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(10) Brooks, V. J., and Jacobs, M. B., *Poisons—Properties, Chemical Identification, Symptoms, and Emergency Treatment*, 2nd Ed., D. Van Nostrand Co., Inc., Princeton, N.J., 1958, p. 131.
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(13) Threshold Limit Values for 1962, adopted at the 24th Annual Meeting of the American Conference of Governmental Industrial Hygienists, Washington, D.C.

# Spectrophotometric Characterization of Some Authentic Oil of Bergamot and a Few Related Oils

By FELICE A. ROTONDARO (Food and Drug Administration, 1204 U.S. Customhouse Bldg., Philadelphia, Pa. 19106)

**Steam distillation separates oil of bergamot into a volatile fraction (85–95% of the original pressed oil) which has a qualitatively significant IR absorption but no appreciable UV absorption in the 270–375 mμ range. The relatively small steam nonvolatile fraction, however, has significant absorption in the 270–375 mμ range; maximum at 313 mμ, minimum at 277 mμ, and a ratio of 2.45 ± 0.07 for $A_{313}/A_{277}$. The steam nonvolatile fraction of bergamot consists primarily of the furocoumarin bergaptene. These compounds classed as psoralens have been reported to induce sensitization of the skin under the influence of sunlight or UV irradiation. Initial studies to separate bergamot oil constituents from mixtures have been partially successful. Work on commercial mixtures, and on characterizing the nonvolatile fraction by chemical and physical means, will be continued.**

Oil of bergamot is classed as a strong sensitizer under the Federal Hazardous Substances Labeling Act (sec. 2 k) by Regulation 191.6e (1). The National Formulary (2), in the monograph on p. 31, gives the physical characteristics and limits for the official oil. The chemical assay presumptive of the pure oil requires an ester content of "not less than 36 per cent as Linalyl Acetate," which is determined by a procedure essentially similar to that given in *Official Methods of Analysis* (3) for lemon oil (19.076–19.078). Since esters of linalol and other closely related alcohols form a significant part of many volatile oils, the assay value for ester content has to be evaluated very carefully.

For the purpose of the Federal Hazardous Substances Labeling Act, neither the range of the physical constants nor the assay for the ester content is distinctive enough to unequivocally characterize a sample. Oils of coriander, lavender, neroli, sage, etc. contain substantial amounts of linalol and/or closely related alcohols as well as their esters. In addition, many synthetic oils of bergamot on the market closely simulate the natural product.

The monographs on oil of bergamot by Guenther (4), Parry (5), Allen (6), and Villavecchia (7) should be consulted for a clear understanding of the variety and amounts of normal constituents and possible adulterants. The general chemistry of volatile oils and the various methods for determining their physical and chemical characteristics are summarized in Volume I of Guenther, Volume II of Parry, and Chapters 19 and 28 of *Official Methods of Analysis*. Recent articles of interest include those of Kirchner and Miller (8), Fritzsche Brothers (9), Mesnard and Bertucat (10), and Brown and Shyluk (11).

The light sensitization effect of bergamot and other volatile oils has been reported by Lane and Strauss (12), Urbach and Kral

(13), Hopf (14), Cajoule (15), Katz (16), and Becker (17). Guenther, *et al.* have published comprehensive reviews (18).

Early in the past decade authentic oils of lemon were examined spectrophotometrically by Cultera, Buffa, and Trifiro (19) and by Sale (20). Definite absorption characteristics at 270–375 mμ shown by expressed oils are lacking in distilled oils. A number of samples of bergamot oil gave absorption characteristics similar to oil of lemon but more than five-fold greater in density. As with lemon, the volatile constituents do not absorb appreciably at 270–375 mμ. Hence, the absorption characteristics of the nonvolatile fraction of the oil are significant in the examination of essential oils, especially those derived from citrus fruits.

The most convenient method of separating the volatile from the nonvolatile constituents is by steam distillation, as reported by Winkler (21) for lemon oil. The main nonvolatile constituent of bergamot is the coumarin derivative, bergaptene. Caporale and Cingolani (22) reported the ultraviolet spectra of certain furocoumarins, including bergaptene. Theile, Dean, and Suffis (23) used a combination of spectrophotometric and chromatographic assays for bergamot.

## Materials and Method

The results reported here are based on the examination of some samples of authentic Italian oil of bergamot generously supplied by a number of essential oil firms. Some samples were purchased.

An essentially all-glass steam distillation apparatus was assembled from stock standard taper glassware. The steam generator consisted of a 2 L Erlenmeyer flask fitted with an internal resistance wire coil. The 110–120 volt current was supplied and controlled through a Powerstat. A 200 ml round-bottom distillation flask was heated by a Glas-Col with the current controlled by a Powerstat. A separatory trap as described in *Official Methods of Analysis*, p. 259, was used to collect and measure the oil distillate.

The ultraviolet curves were obtained on a Cary Model 11 spectrophotometer and the infrared curves on a Beckman IR-5.

### *Procedure*

Place 2 ml of the oil in a 200–250 ml round-bottom distilling flask with about 25 ml of water and 0.1–0.5 ml 1*N* HCl, and connect the flask. Preheat the sample solution to boiling before letting a slow stream of steam pass through. Distill until the volatile fraction is completely separated (usually 15–20 minutes).

Let the distillate in the trap cool, read the volume of the oil, and then drain the oil into a small tube containing some anhydrous sodium sulfate. Stopper the tube, shake well, and centrifuge or let the sulfate settle. Dilute 0.5–0.1 ml of the clear dry oil with alcohol so that 0.10 ml oil is contained in 100 ml alcohol. Obtain an absorbance curve of the oil in the 270–375 mμ range, using alcohol for a blank.

Transfer the cooled residual aqueous solution from the distillation flask to a 200 ml volumetric flask. Rinse the distillation flask with several small portions of alcohol, warming if necessary to dissolve any residue, add the rinsings to the volumetric flask, and cool.

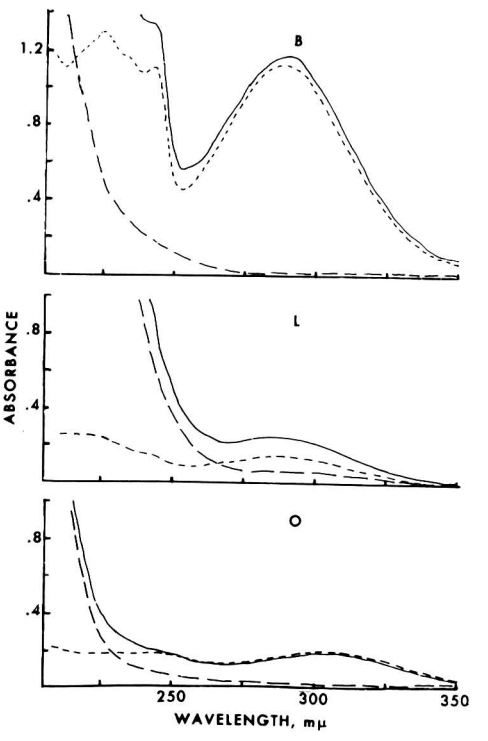


*Fig. 1*—Ultraviolet absorption for oils of (B) bergamot, (L) lemon, and (O) orange. Oil as is: solid line. Steam volatile fraction: dashed line. Steam nonvolatile fraction: dotted line. Concentration for all curves, 0.10 ml/100 ml 95% alcohol in 1 cm matched silica cells run against 95% alcohol blank.

**Table 1. Ultraviolet absorbance of bergamot oil**

| Sample[a] | $A_{313}$ | $A_{277}$ | $\Delta A_{313}-A_{277}$ | $\Delta A$ Baseline[d] |
|---|---|---|---|---|
| 1 | 1.12 | 0.57 | 0.55 | 0.78 |
| 2 | 1.20 | 0.56 | 0.64 | 0.82 |
| 3 | 1.08 | 0.52 | 0.56 | 0.73 |
| 4 | 1.12 | 0.56 | 0.56 | 0.73 |
| 5 | 1.04 | 0.52 | 0.52 | 0.68 |
| 6[b] | 0.94 | 0.49 | 0.45 | 0.50 |
| 7[b] | 0.87 | 0.50 | 0.37 | 0.53 |
| 8[c] | 0.52 | 0.33 | 0.19 | 0.29 |
| 9[c] | 0.19 | 0.15 | 0.04 | 0.09 |
| 10[c] | 0.13 | 0.24 | 0.09 | — |
| Av. 1–5 | $1.112^{+0.098}_{-0.072}$ | $0.546^{+0.024}_{-0.026}$ | $0.566^{+0.074}_{-0.046}$ | $0.750^{+0.070}_{-0.070}$ |
| 1–7 | $1.053^{+0.147}_{-0.173}$ | $0.544^{+0.026}_{-0.054}$ | $0.546^{+0.094}_{-0.176}$ | $0.686^{+0.134}_{-0.186}$ |

[a] All samples 0.10 ml/100 ml 95% alcohol in 1 cm matched fused silica cells. 95% alcohol used for blank. Spectrophotometer used, Cary 11. [b] Samples of undeterminate age. [c] Samples labeled "Imitation Bergamot". [d] Sometimes referred to as the "CD Line Value".

**Table 2. Ultraviolet absorbance of the steam nonvolatile fraction of bergamot oil**

| Sample[a] | $A_{313}$ | $A_{277}$ | $\Delta A_{313}-A_{277}$ | $A_{313}-A_{277}$ |
|---|---|---|---|---|
| 1 | 1.13 | 0.46 | 0.67 | 2.46 |
| 2 | 1.01 | 0.40 | 0.61 | 2.52 |
| 3 | 1.02 | 0.42 | 0.60 | 2.43 |
| 4 | 1.10 | 0.46 | 0.64 | 2.40 |
| 5 | 1.03 | 0.42 | 0.61 | 2.45 |
| 6[b] | 0.94 | 0.49 | 0.45 | 1.91 |
| 7[b] | 0.81 | 0.37 | 0.44 | 2.19 |
| 8[c] | 0.43 | 0.21 | 0.22 | 2.05 |
| 9[c] | 0.18 | 0.11 | 0.09 | 1.64 |
| 10[c] | 0.18 | 0.20 | 0.02 | 0.90 |
| Av. 1–5 | $1.06^{+0.07}_{-0.05}$ | $0.43^{+0.03}_{-0.03}$ | $0.63^{+0.04}_{-0.03}$ | $2.45^{+0.07}_{-0.05}$ |
| 1–7 | $1.02^{+0.11}_{-0.21}$ | $0.41^{+0.08}_{-0.04}$ | $0.57^{+0.10}_{-0.13}$ | $2.34^{+0.18}_{-0.43}$ |

[a] All samples 0.10 ml/100 ml 95% alcohol in 1 cm matched fused silica cells. 95% alcohol used for blank. Spectrophotometer used, Cary 11. [b] Samples of undeterminate age. [c] Samples labeled "Imitation Bergamot".

Fill the flask to the mark with alcohol and mix well. Dilute 5 or 10 ml aliquots to 100 ml with alcohol and obtain an absorbance curve in the 270–375 m$\mu$ range as above.

For the infrared curves fill 0.025 mm NaCl cells with the original oil and dried steam distillate. Use an empty matched cell for a blank.

## Results and Discussion

Figure 1 shows characteristic ultraviolet absorbance curves for oils of bergamot (B), lemon (L), and orange (O). It is clear that the absorbance of the original oils in the 270–375 m$\mu$ region is due primarily to the nonvolatile constituents. The volatile fraction, which constitutes 85–95% of the original oil, shows a significant absorption below 270 m$\mu$.

Sale (20) found the baseline method useful for characterizing and differentiating pressed oil from distilled lemon oils. The differential absorbance procedure graphically shown by Sale as a "C-D" line appears

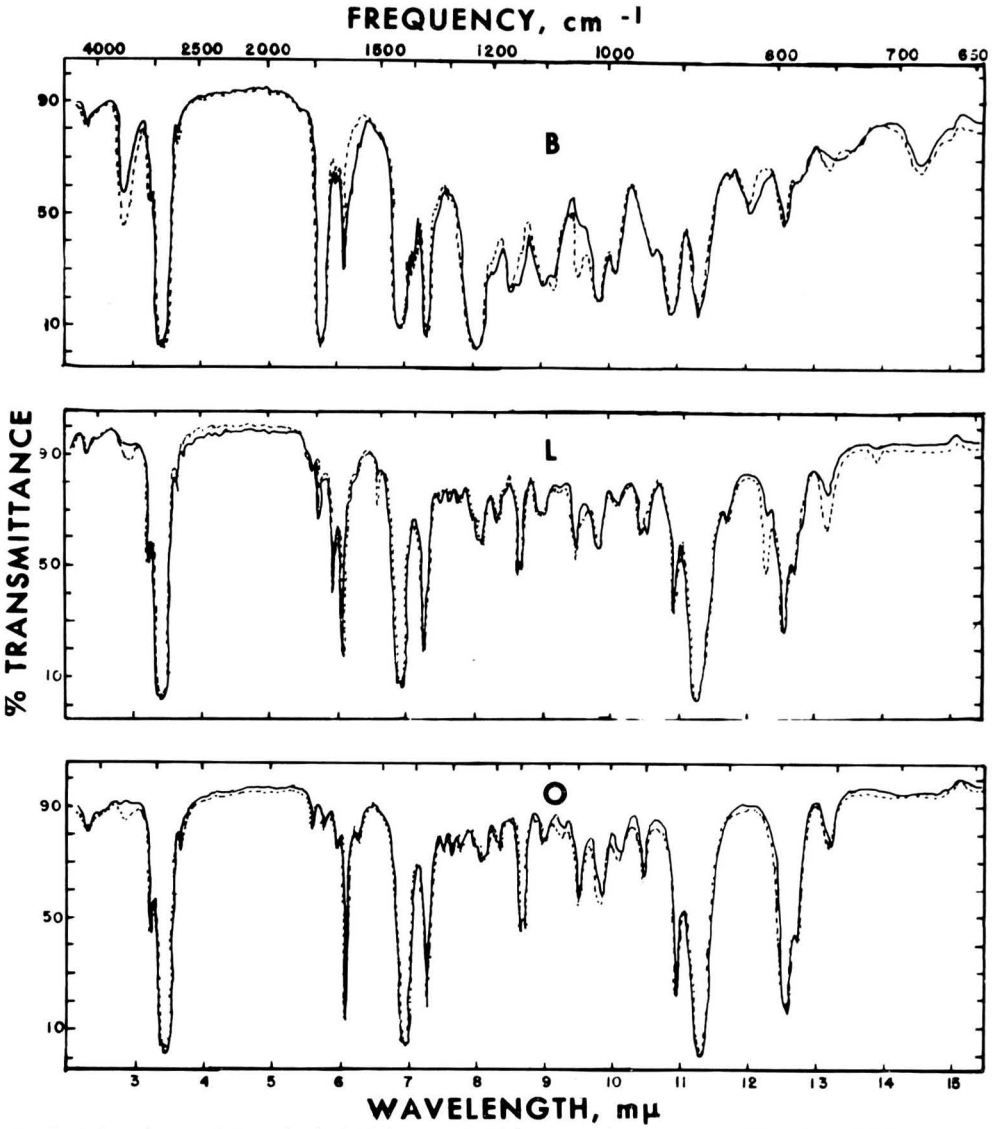


*Fig. 2*—Infrared transmission of oil of (*B*) bergamot, (*L*) lemon, and (O) orange. Oil as is: solid line. Steam volatile fraction: dotted line. Samples measured in 0.025 mm NaCl cells.

to have been adopted and referred to in the essential oil industry as "CD Line Values." However, Table 1 shows that either the total absorbance at the maximum (313 m$\mu$) or the difference between the absorbance at the maximum and that at the minimum (277 m$\mu$) is just as characteristic as the baseline $\Delta A$ ("CD Line Values"). This is true because the volatile fraction, especially with bergamot oil, contributes such a small absorption at the maximum as to be negligible. Sale noted that the maximal values for lemon oil reported by Cultera, Buffa, and Trifiro were in substantial agreement with his findings.

Table 2 gives the values obtained on the steam nonvolatile fraction of bergamot oil. A much greater uniformity in the values is noticeable. Also, the ratio of $A_{313}/A_{277}$ appears to be a valuable purity index. This becomes significant when attempting to evaluate the purity of an oil recovered from old or imitation samples.

Curve O of Fig. 1 shows that oil of orange

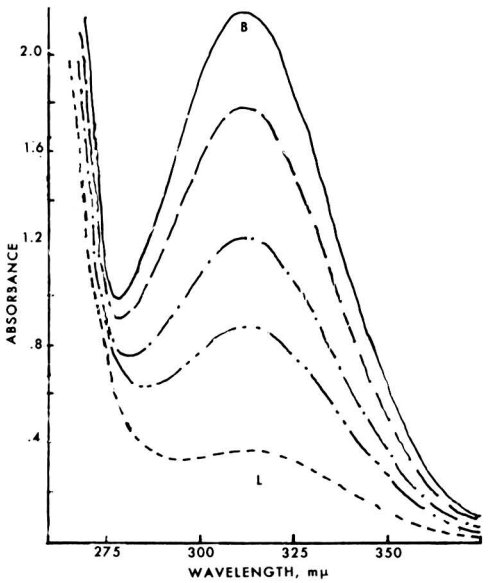


*Fig. 3*—Ultraviolet absorbance of oils of (*B*) bergamot, (*L*) lemon, and v/v mixtures of the two. 75 parts *B* and 25 parts *L*: dashed line. 50 parts *B* and 50 parts *L*: dash-dot line. 25 parts *B* and 75 parts *L*: dash-dot-dot line. All concentrations, 0.10 ml/100 ml 95% alcohol in 1 cm matched silica cells: 95% alcohol used for blank.

has an absorption not unlike that of lemon in intensity except that the maximum is at 325–330 mμ. The minima, however, are at about 295 mμ for lemon and orange, but at 275–277 mμ for bergamot. Thus, oil of lemon is intermediate between orange and bergamot. The curves in Fig. 2 show that the steam volatile fraction is the constituent which gives the original oil its infrared characteristics. Again bergamot (B) is characteristically different from lemon (L) and orange (O). The very small changes produced by distillation are quite noticeable.

Figure 3 shows the ultraviolet absorption of bergamot, lemon, and (75+25), (50+50), and (25+75) (v/v) mixtures of the two, respectively. The curves show that the absorptions of the two oils are additive and in apparent compliance with Beer's Law. Although the peak absorptions for the two oils are quite close (313–315 mμ), the absorbance for bergamot is approximately five times greater than that for lemon. The corresponding minimal absorbances, however, are more distinctive—bergamot at 277 mμ, lemon at 295 mμ. Of greater qualitative significance, moreover, is the rather wide margin in the ratio of $A_{max.}/A_{min.}$; the ratio for bergamot is about twice that of lemon.

Figure 4 shows the infrared absorption of eight of the more common constituents in oils of bergamot, lemon, and orange. These are identifiable in the curves of both the original pressed and the distilled oils.

Some exploratory methods to separate and measure the essential oils in mixtures were tried. The samples included a few liquids, lotions, and creams. The methods used consisted primarily of (*1*) extraction with immiscible solvents, with and without centrifugation; (*2*) use of the Babcock bottle method; (*3*) distillation of the extracted oils followed by salting and re-extraction of the steam volatile and nonvolatile fraction; and (*4*) measurement of ultraviolet and infrared absorption of separated fractions. The results obtained thus far on mixtures do not warrant collaborative work.

It is recommended that study continue.

**Acknowledgments**

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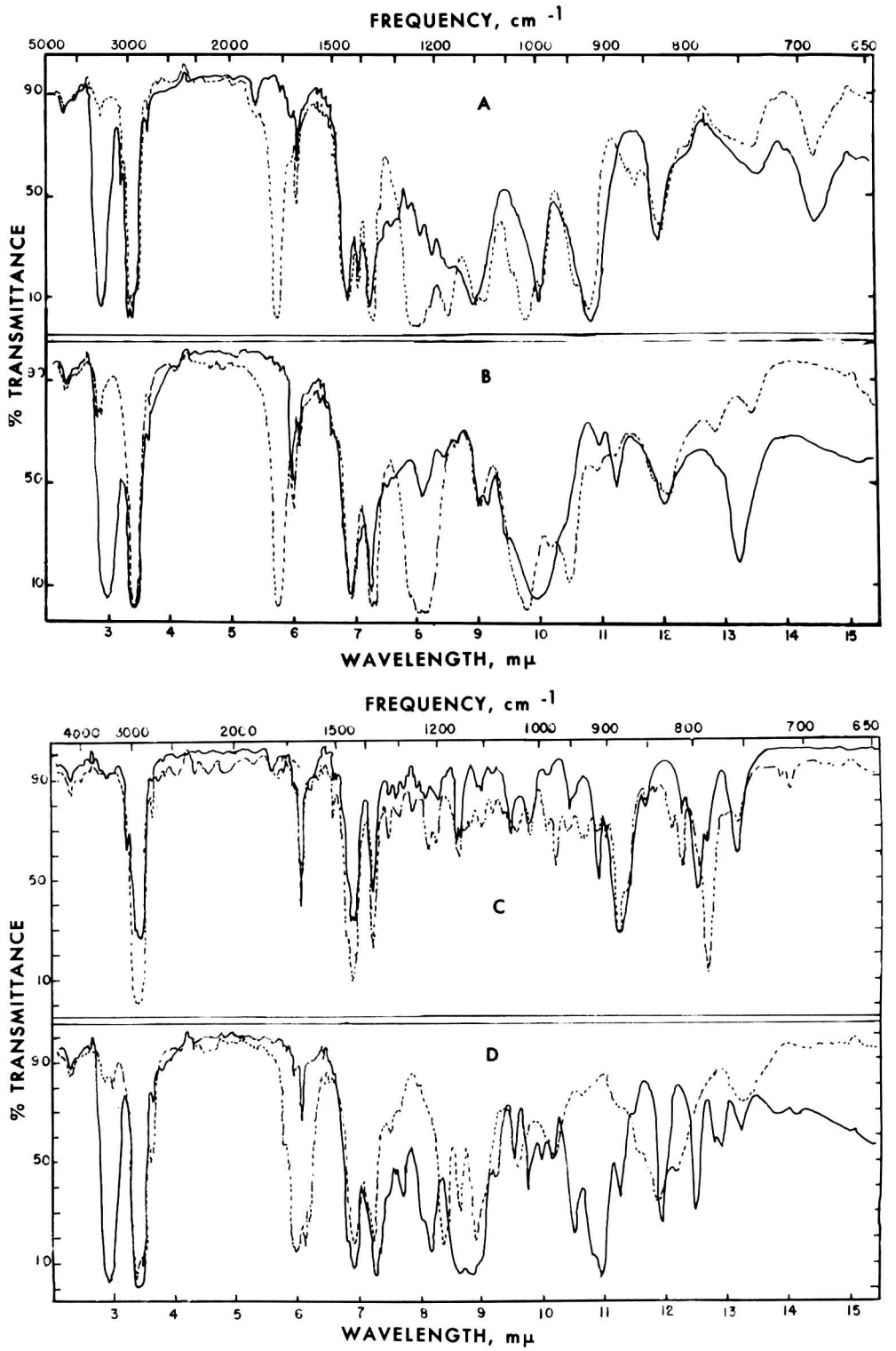


*Fig. 4*—Infrared transmission of (A) linalol, solid line, and linalyl acetate, dotted line; (B) geraniol, solid line, and geranyl acetate, dotted line; (C) d-limonene, solid line, and dipentene, dotted line; (D) terpineol, solid line, and citral, dotted line.

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# Hydrocarbon Propellants in Self-Pressurized Containers

By GEORGE E. KEPPEL (Food and Drug Administration, 1141 Central Parkway, Cincinnati, Ohio 45202)

**A study was undertaken to determine whether the present official flammability tests for self-pressurized containers or aerosols are suitable for those containing hydrocarbon propellants. Fifty aerosols, five containing hydrocarbon propellants, were examined by the official methods for flammability (flame projection and flash point tests), and by other tests. Official tests detected the extremely flammable component in only one of the five highly flammable samples, a paint preparation. Of 24 samples examined for propellant identity, 5 contained hydrocarbon propellants, 10 contained hydrocarbon and fluorocarbon mixtures, and the remaining 9 contained fluorocarbons only. The present official flammability tests are not entirely suitable for self-pressurized containers with flammable propellants, and studies on flammable substances in pressurized containers should be continued.**

Regulations for enforcement of the Federal Hazardous Substances Labeling Act (26 F.R. 11214, Federal Register, November 28, 1961) include methods for testing contents of self-pressurized containers for flammability (Regulations 191.15 and 191.16).

At present, some of these self-pressurized containers, or "aerosols," as they are commonly called, contain low-boiling hydrocarbons as propellants. These pressurizing agents may be propane or isobutane alone or as mixtures. It is quite evident that these substances are very flammable.

The purpose of this study was to determine whether the flammability tests as set forth in the Federal Regulations are suitable for aerosols having hydrocarbon propellants. These tests were developed primarily for pressurized containers using Freons as the propellants. "Freon" is a trade name for a series of nonflammable fluorocarbons developed for aerosol propellants, refrigerants, and other uses. Similar products known as "Genetrons" are also available.

The present flammability tests as given in the regulations for self-pressurized containers specify a test for flame projection, and, depending on the outcome of this test, a flash point determination. In the flame test, contents of the aerosol are sprayed under precisely defined conditions toward a candle flame having a flame height of 2 inches at about 4 inches above the candle base. During the spraying the container is held 6 inches from the flame, and the length of the extended flame is noted. By definition (Regulation 191.1) (2)(1) contents of the self-pressurized containers are "flammable" if a flame projection exceeding 18 inches is obtained at full valve opening or if a flash back (a flame extending back to the dispenser) is obtained at any degree of valve opening. Contents are "extremely flammable" if a flash back is obtained at any degree of valve opening and the flash point is less than 20°F. The method for flash point is specified in Regulation 191.16. The apparatus used is the Tagliabue open cup. The container and apparatus are chilled to −25°F, the container is punctured to exhaust the propellant, the contents are transferred to the cup, and flash point is determined by passing the flame periodically across the cup as the temperature rises.

## Experimental

The above tests were used in examining 50 aerosols obtained locally in retail markets. In addition, several unofficial tests were applied to identify the propellant gas. These tests are not given in the regulations, but were necessary in the present study to determine if the propellant was a hydrocarbon. The tests are listed below in the order in which they are made:

1. Flame projection test.

2. Flame projection test on propellant gas: The container is inverted and the valve system is cleared of fluid by opening the valve several times. The gas stream is then directed at the flame. (This method fails for some types of self-pressurized containers, as some valves are designed to deliver the product from any container position.)

3. Propellant identity: The gas is collected by a modification of Newburger's method (*Manual of Cosmetic Analysis*, Association of Official Agricultural Chemists, 1962, 4.30(b), p. 20), as follows: The container, in an inverted position, is first pressed against the bottom of a beaker to remove fluids. With container still in inverted position, the plastic valve cap is removed and the gas is collected in an apparatus consisting of a 250 ml Erlenmeyer flask, with a stopper containing a glass tube extending to about 1 inch from the bottom of the flask, the top of which has a short length of gum rubber tube whose edges extend slightly above the glass tube to serve as a seal against the bottom of the container. This glass tube contains a second glass tube of such diameter and length that it will open the valve when the container is pressed down firmly. The flask outlet is connected to an infrared gas cell by a short length of polyethylene tubing that contains cotton to remove entrained liquid particles. Before the cell is filled, the system and cell are flushed with the gas. After it is filled, the cell valves are closed, and the gas is identified by its infrared spectrum. If gas concentration is too great, portions are removed by slight suction.

Some container valves, such as those on paints and enamel aerosols, have no external valve tube; the tube is part of the valve cap and is removed with it. In such cases, the above gas collecting system is modified by inserting into the glass tube a glass rod of such diameter and length that it will enter the hole in the container and engage the valve when the container is pressed down.

4. Propellant gas flame test: This test measures the flammability of the gas, and confirms the infrared findings. The container is inverted and the valve system cleared of

fluid as in Test No. 3. The container, still inverted, is immersed in water in a 2 L beaker and is pressed against the bottom of the beaker to release gas, which is collected in a graduate or large test tube previously filled with water. The tube of collected gas is held to a flame to determine if the gas burns. It was expected that the flame projection test on the propellant gas, Test No. 2, would show if the gas was flammable, but such was not the case. With hydrocarbon propellants, no flame formed unless the inverted container was held 1–2 inches from the candle flame. The reason for the absence of flame at 6 inches is not known.

5. Flash point test: method as given in Regulation 191.16. As the propellant gas is lost in this test, it was made the final test, and was determined only on those aerosols that exhibited a flash back in Test No. 1. For this work the term "flash back" was interpreted as any flame that extended back from the candle flame toward the container.

## Results

Of the 50 aerosols tested, 5 contained hydrocarbon propellants. Results are given in Table 1.

The results indicate that the flame projection test will not always detect the presence of an "extremely flammable" hydrocarbon propellant. There was a flash back for only one sample, a color spray used to decorate Christmas trees. Therefore, of the five samples, only one would be tested for flash point if the examination was limited to the flame projection test. However, since the unofficial tests showed presence of hydrocarbon propellants, flash points were determined on all five samples.

The flash point test proved difficult to apply. In the time required to release the propellants, remove the contents, mix, and transfer to the flash point cup, the temperature of the contents had risen rapidly. In spite of this, all flash points were below 20°F or "extremely flammable" by definition, except for one starch spray sample, No. 4. In this case all of the propellant gas had escaped from aqueous suspension. The other starch sample, No. 5, held some of the propellant gas as bubbles which ignited as the test was begun.

In general, the flash point test does not appear reliable for the contents of self-pressurized containers. For example, when a nonflammable propellant such as Freon is present, some of the gas dissolves in the contents if they contain oils, mineral spirits, or alcohol. As the product is warmed in the flash cup, the gas escapes as numerous small

**Table 1. Self-pressurized containers with hydrocarbon propellant**

| Sample No. | Product | Flame Projection Test | | Flashback Flame Extension | Propellant Gas Identity | Propellant Gas Flammability Test | Flash Point |
|---|---|---|---|---|---|---|---|
| | | Contents | Propellant | | | | |
| 1 | Color spray | 29″ | no flame | 5½″ | Mixture of propane and butanes | Burns | 15° |
| 2 | Room deodorant | 20″ | not determined[a] | none | Isobutane | Burns | 15° |
| 3 | Spray starch | no flame | no flame | — | Hydrocarbon mixture | Burns | No flash point |
| 4 | Spray starch | no flame | no flame | — | Hydrocarbon mixture | — | 15° |
| 5 | Spray enamel | 20″ | no flame | none | Hydrocarbon mixture | Burns | 8° |

[a] Unable to clear valve.

bubbles. The surface is coated with the blanket of nonflammable gas so that volatile vapors from the product are trapped. When a flame is passed over the cup, it is repeatedly extinguished or enhanced to a smoky luminous flame with a greenish tinge. (The green tinge is the familiar Beilstein test, and confirms the presence of halogenated organic compounds.)

Flash points were determined on all cans showing flash backs. Those labeled as "flammable" and having Freons as propellants had flash points well above 80°F. The range was 87–128°F.

For an accurate flash point, this dissolved nonflammable gas should be removed, but this cannot be done with the method in its present form. The method serves to detect the flammable propellant if the gas is partially soluble in the aerosol contents or is entrained to some extent. If the contents are a water suspension, such as the spray starches, nearly all of the flammable gas escapes and is not detected.

A total of 24 samples were examined for propellant identity by Test No. 3. These samples were chiefly those that gave flames over 18 inches by the flame projection test, but also included 6 samples with flames less than 18 inches or no flame at all. Of the 24 tested, 5 contained hydrocarbon propellants, 10 contained hydrocarbon and Freon mixtures, and the remaining 9 were Freons. They were identified by comparing the infrared curves with those in Newburger's *Manual of Cosmetic Analysis* and those in "Catalog of Infrared Spectra for Qualitative Analysis of Gases," by Pierson, Fletcher, and Gantz (*Anal. Chem.*, **28**, 1218 (1956)).

The aerosol samples examined consisted of 13 enamels, paints, and coatings; 15 hair sprays; 4 window cleaners; 4 room deodorants; 2 each of rust solvents, lubricants, insecticides, and starches; and 1 each of starting fluid, degreaser, filter coating, defroster, leather stretcher, and disinfectant.

### Recommendation

The studies indicate that the present official flammability tests are not entirely suitable for self-pressurized containers with extremely flammable hydrocarbon propellants. It is recommended that studies be continued.

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. See *This Journal*, **47**, 120 (1964).

## FERTILIZERS AND LIMING MATERIALS

# Spectrophotometric Determination of Aluminum, Iron, Manganese, Phosphorus, and Titanium in Liming Materials

By P. CHICHILO (U.S. Fertilizer Laboratory, Soil and Water Conservation Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.)

**Methods have been developed for the determination of aluminum with ammonium aurintricarboxylate (Aluminon); iron with 2,4,6-tripyridyl-s-triazine (TPTZ); manganese by oxidation to permanganate with $KIO_4$; phosphorus by a heteropoly blue method; and titanium with disodium-1,2-dihydroxybenzene-3,5-disulfonate (Tiron). Determinations are carried out on the filtrate obtained in the AOAC perchloric acid method for silicon. Adequacy of the directions was established by "ruggedness tests," and the procedures were used to analyze carbonate and silicate liming materials. It is recommended that the methods be studied collaboratively.**

Variable, though generally unknown, amounts of aluminum, iron, manganese, phosphorus, and titanium are added to the soil in carbonate and silicate liming materials. As fertilization and liming practices are put on a more scientific basis, increased knowledge of the occurrence of these elements in liming materials becomes desirable.

In the AOAC method for the elemental analysis of liming materials (1, 2) aluminum, iron, phosphorus, and titanium are precipitated in the filtrate from silica separation and weighed as a group; manganese is not determined. Since determination of individual elements would provide additional valuable information, Subcommittee A has recommended that methods for these five elements be studied (3).

The photometric methods presented in this report have been selected and adapted because of their applicability and simplicity. Details of the methods are based on a literature study and preliminary tests. Adequacy of the directions in the methods was established by the use of "ruggedness tests," and the procedures were then applied to types of materials being used for liming. The materials analyzed included the two National Bureau of Standards standard limestone samples, the blast furnace slag, and the cement kiln dust used in a study of silicon determination (4), and an agricultural limestone containing 45% $CaCO_3$ and 35% $MgCO_3$.

## METHODS

### *Principles*

*Aluminum:* Formation of a lake with aurintricarboxylate (Aluminon) (5, 6). *Iron:* Formation of a derivative of 2,4,6-tripyridyl-s-triazine (TPTZ) (7). *Manganese:* Oxidation of manganese salts to permanganates with $KIO_4$ (5). *Phosphorus:* Reduction of molybdophosphoric acid to a heteropoly blue product (8). *Titanium:* Formation of a chelate complex with disodium-1,2-dihydroxybenzene-3,5-disulfonate (Tiron) (9).

Standard curves for iron, manganese, phosphorus, and titanium are straight lines on semilog paper and only two points are needed to establish each curve; however, to insure the accurate locations of the lines, several points are determined for each element.

### *Preparation of Sample and Blank Solutions*

(a) *Sample soln A (filtrate and washings from Si detn).*—Proceed as in the official perchloric acid method for Si (1, 2) with the following modifications:

(1) If material contains org. matter, transfer weighed sample to Pt crucible and place in cold muffle furnace. Raise temp. gradually to 1000° and hold for 15 min. Transfer sample to a 400 ml beaker, moisten cautiously with $H_2O$, and proceed as in the official method.

(2) Omit HCl from the $H_2O$ used in washing silica on the filter paper. Chlorides interfere with photometric detn of Mn. (According to Hillebrand (10), washing with $H_2O$ is as effective as washing with dil. HCl.)

(3) Dil. filtrate and washings from Si detn to 250 ml instead of 500 ml. More suitable concns of Mn, P, and Ti are thus obtained.

When Al, Fe, Mn, P, and Ti are to be determined individually in the same sample solution used for Si, $R_2O_3$ group, Ca, and Mg, that solution should be prepared as described. This would in no way interfere with the latter determinations. (If the procedures recommended in the report are adopted, the official methods should be altered.)

(b) *Sample soln B.*—Dil. 10 ml sample soln A to 500 ml with $H_2O$.

(c) *Blank soln A.*—Omit sample and proceed as in prepn of sample soln A.

(d) *Blank soln B.*—Dil. Blank soln A in same proportion as sample soln A was dild to obtain sample soln B.

### *Blanks*

Treat aliquots of blank soln (corresponding to the aliquot sizes of sample solns taken for analysis) as in *Determination* for the appropriate elements, and correct values for unknowns accordingly. Transmittance of the blanks should be very near that of the 0 μg element solns obtained in *Preparation of Standard Curve.*

### Aluminum

### *Reagents*

(a) *Standard aluminum soln.*—(*1*) Stock soln (100 μg Al/ml): Place 0.1000 g pure Al metal in 30 ml beaker. Add 6 ml HCl (1+1). Cover with watch glass and warm gently until Al completely dissolves. Dil. to 1 L with $H_2O$. (*2*) Std soln (5 μg Al/ml): Dil. 25 ml stock soln to 500 ml.

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(b) *Aluminon (ammonium aurintricarboxylate) soln.*—Dissolve the following separately in $H_2O$: 0.5 g Aluminon in 100 ml; 10 g acacia (gum arabic) in 200 ml; and 100 g $NH_4OAc$ in 400 ml. Filter the acacia soln. Add 56 ml HCl to the $NH_4OAc$ soln and adjust pH to 4.5 with HCl or $NH_4OH$. Combine the three solns and dil. to 1 L with $H_2O$.

(c) *Antifoam soln.*—Disperse 0.03 g silicone defoamer (Dow-Corning Antifoam A[1]) in 100 ml $H_2O$.

(d) *Thioglycollic acid soln.*—Dil. 1 ml $HS.CH_2COOH$ to 100 ml with $H_2O$.

### *Preparation of Standard Curve*

Transfer aliquots of std soln contg 0, 5, 20, 40, 60, and 80 μg Al to 100 ml vol. flasks and proceed as in *Determination*. Prep. std curve by plotting μg Al against % T on semilog paper.

### *Determination*

Use sample soln A for limestones contg $< 0.2\%$ or silicates contg $< 0.8\%$ Al, and adjust pH of aliquot to 4.5 with $NH_4OH$. For materials contg greater concns of Al, use sample soln B and omit pH adjustment. Transfer an aliquot (20 ml or less contg $< 80$ μg Al) of sample soln A or B to a 100 ml vol. flask. Dil. to 20 ml with $H_2O$. Add 2 ml thioglycollic acid soln, 0.5 ml antifoam soln, and 10 ml Aluminon soln. Place flask in boiling $H_2O$ for 20 min. (a 250 ml beaker contg 125 ml $H_2O$ holds a 100 ml vol. flask conveniently). Remove flask from $H_2O$ and let cool. Dil. to 100 ml with $H_2O$. Read % T at 525 mμ against 0 μg Al soln (prepd for std curve) set at 100% T. Det. Al by referring to std curve, correct for blank, and report % Al.

## Iron

### *Reagents*

(a) *Standard iron soln.*—(*1*) Stock soln (100 μg Fe/ml): Dissolve 0.1000 g pure Fe metal in 5 ml 2*N* HCl and dil. to 1 L with $H_2O$. (*2*) Std soln (5 μg Fe/ml): Dil. 25 ml stock soln to 500 ml.

(b) *TPTZ (2,4,6-tripyridyl-s-triazine) soln.*—Dissolve 0.500 g TPTZ in a few drops of HCl and dil. to 1 L with $H_2O$.

(c) *Hydroxylamine hydrochloride soln.*—Dissolve 50 g $NH_2OH.HCl$ in $H_2O$. Add 10 ml TPTZ soln and 0.5 g $NaClO_4.H_2O$, and dil. to 500 ml with $H_2O$. Transfer to a separatory funnel, add 25 ml nitrobenzene, and shake several min. Let phases sep. and discard nitrobenzene phase contg Fe. Repeat extn 2 or 3 times.

(d) *Sodium acetate-acetic acid buffer soln.*—Dissolve 164 g NaOAc in $H_2O$. Add 115 ml HOAc, 10 ml $NH_2OH.HCl$ soln, 0.05 g TPTZ, and 1 g $NaClO_4.H_2O$, and dil. to 1 L with $H_2O$. Transfer to a separatory funnel, add 25 ml nitrobenzene, and shake several min. Let phases sep. and discard nitrobenzene phase. Repeat the extn 3 or 4 times.

### *Preparation of Standard Curve*

Treat aliquots of std soln contg 0, 5, 50, and 100 μg Fe as in *Determination*. Prep. std curve by plotting μg Fe against % T on semilog paper.

### *Determination*

Use sample soln A for limestones contg $< 0.05\%$ or silicates contg $< 0.2\%$ Fe and sample soln B for materials contg greater concns of Fe. Transfer an aliquot (contg $< 100$ μg Fe) of sample soln A or B to a 100 ml vol. flask. Add 3 ml hydroxylamine soln and 10 ml TPTZ soln. Add $NH_4OH$ dropwise until the Fe derivative remains violet on mixing. Add 10 ml buffer soln and dil. to 100 ml. Read % T at 593 mμ against 0 μg Fe soln (prepd for std curve) set at 100% T. Det. Fe by referring to std curve, correct for blank, and report % Fe.

## Manganese

### *Reagents*

(a) *Standard manganese soln (50 μg Mn/ml).*—Dissolve 0.0500 g pure Mn metal in 20 ml 0.5*N* $H_2SO_4$ and dil. to 1 L with $H_2O$.

(b) *Acid mixture.*—Add 800 ml $HNO_3$ and 200 ml $H_3PO_4$ to $H_2O$ and dil. to 2 L.

### *Preparation of Standard Curve*

Treat aliquots of std soln contg 0, 50, 100, 300, and 500 μg Mn as in *Determination*. Prep. std curve by plotting μg Mn against % T on semilog paper.

### *Determination*

Transfer an aliquot (contg $< 500$ μg Mn) of sample soln A to a 150 ml beaker. Add 25 ml acid mixt. and 0.3 g $KIO_4$. Bring to a boil and keep near boiling temp. for 10 min. after color develops. Let cool, transfer to a 50 ml vol. flask, dil. to vol., and mix. Read % T at 525 mμ against 0 μg Mn soln (prepd for std curve) set at 100% T. Det. Mn by refer-

[1] The inclusion of trade names in this paper does not imply endorsement or preferential treatment of the product by the U.S. Department of Agriculture.

ring to std curve, correct for blank, and report % Mn.

## Phosphorus

### *Reagents*

(a) *Standard phosphorus soln.*—(*1*) Stock soln (100 μg P/ml): Dissolve 0.4393 g $KH_2PO_4$ in $H_2O$ and dil. to 1 L. (*2*) Std soln (5 μg P/ml): Dil. 25 ml stock soln to 500 ml.

(b) *Ammonium molybdate soln.*—Dissolve 20 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 500 ml $H_2O$. Add 285 ml $H_2SO_4$, cool, and dil. to 1 L with $H_2O$.

(c) *Hydrazine sulfate soln.*—Dissolve 2 g $H_2NNH_2H_2SO_4$ in $H_2O$ and dil. to 1 L.

### *Preparation of Standard Curve*

Treat aliquots of std soln contg 0, 5, 50, and 75 μg P as in *Determination*. Prep. std curve by plotting μg P against % T on semilog paper.

### *Determination*

Transfer an aliquot (contg < 75 μg P) of sample soln A to a 100 ml vol. flask. Add 5 ml ammonium molybdate soln and mix. Add 5 ml hydrazine soln, dil. to 70 ml with $H_2O$, and mix. Place flask in boiling $H_2O$ for 9 min. Remove, cool rapidly, and dil. to vol. Read % T at 827 mμ against 0 μg P soln (prepd for std curve) set at 100% T. Det. P by referring to std curve, correct for blank, and report % P.

## Titanium

### *Reagents*

(a) *Standard titanium soln.*—(*1*) Stock soln (100 μg Ti/ml): Place 0.1668 g $TiO_2$ and 2 g $K_2S_2O_7$ in Pt crucible. Heat the covered crucible gently at first and then to dull redness for ca 15 min. Dissolve the melt in 50 ml $H_2SO_4$ (1+1) and dil. to 1 L with $H_2O$. (*2*) Std soln (5 μg Ti/ml): Dil. 25 ml stock soln to 500 ml.

(b) *Sodium acetate-acetic acid buffer soln.*—Mix equal vols of 1*M* HOAc and 1*M* NaOAc. The pH of resulting soln is 4.7.

(c) *Tiron soln.*—$Na_2(OSO_2)_2C_6H_2(OH)_2.H_2O$. Dissolve 4 g Tiron in $H_2O$ and dil. to 100 ml.

### *Preparation of Standard Curve*

Treat aliquots of std soln contg 0, 5, 50, and 75 μg Ti as in *Determination*. Do not add dithionite to stds. Prep. std curve by plotting μg Ti against % T on semilog paper.

### *Determination*

Transfer an aliquot (contg < 75 μg Ti) of sample soln A to a 50 ml beaker. Dil. to ca 25 ml with $H_2O$. Add 5 ml Tiron soln, and then add $NH_4OH$ (ca 10% soln) dropwise until the soln is neutral to Congo red paper. (The Tiron soln must be added before pH is adjusted.) Transfer to a 50 ml vol. flask, add 5 ml buffer soln, dil. to vol. with $H_2O$, and mix thoroly. Add 25 mg dithionite ($Na_2S_2O_4$) and dissolve with a minimum of agitation (to avoid the reappearance of blue). Read % T, within 15 min. after adding dithionite, at 410 mμ against 0 μg Ti soln (prepd for std curve) set at 100% T. Det. Ti by referring to std curve, correct for blank, and report % Ti.

## Ruggedness Tests

The methods were subjected to Youden's ruggedness test (11) with variations in amounts and order of addition of reagents, duration of heating, degree of dilution, rate of cooling, changes in acidity, etc. Aliquots of standard solutions containing 50 μg Al, 50 μg Fe, 200 μg Mn, 45 μg P, or 50 μg Ti were used in each duplicated series of comparisons.

Seven different factors were altered in each method and the particular set of eight combinations of seven factors recommended by Youden was used to study the effects of the alterations. One of the seven factors altered in each method was a meaningless operation such as tapping designated flasks with a pencil.

Except for the titanium method, there were no outstanding differences caused by altering conditions (Table 1). The differences occurring in the method for manganese appear larger than those for aluminum, iron, and phosphorus, but the method for manganese is less sensitive and requires a greater concentration of element. According to Youden (12) an average difference of 8.1 μg Mn would be required for significance. Also, an analysis of variance indicated significant differences only in the case of titanium.

Critical conditions in the titanium determination were the order and extent of adjusting pH and the time elapsed between adding dithionite and measuring transmittance. Further tests were made, and it was determined that when Tiron is added before pH is adjusted, there is no difference between adjusting to pH 3 or 5 and also that there is no difference between measuring transmittance immediately or 18 minutes after

**Table 1. Results of the ruggedness tests of the analytical procedures**

| Conditions Altered | Difference Between Averages (μg of Element)[a] |
|---|---|
| **Aluminum** | |
| Dil. to: 18 vs. 22 ml | 0.3 |
| Add $HClO_4$: 0.05 ml vs. none | 0.1 |
| Add first: Aluminon vs. thioglycollic acid | 0.1 |
| Add antifoam reagent: none vs. 0.5 ml | 0.5 |
| Heat: 18 vs. 22 min. | 0.5 |
| Cool and measure % T: rapidly vs. 2 hr interval | 0.5 |
| Meaningless factor | 0.7 |
| **Iron** | |
| Add first: hydroxylamine vs. TPTZ | 0.2 |
| Add TPTZ: 10.5 vs. 9.5 ml | 0.2 |
| Add hydroxylamine: 2.5 vs. 3.5 ml | 0.7 |
| Add buffer: 10.5 vs. 9.5 ml | 1.1 |
| Add $NH_4OH$: to color development vs. 3 drops beyond | 0.1 |
| Measure % T: after 1 hr vs. immediately | 0.3 |
| Meaningless factor | 0.8 |
| **Manganese** | |
| Add acid mixt.: 23 vs. 27 ml | 0.2 |
| Add $KIO_4$: before vs. after boiling temp. is reached | 2.8 |
| Add $KIO_4$: 0.25 vs. 0.35 g | 2.8 |
| Heat at boiling temp.: 8 vs. 12 min. after color develops | 4.6 |
| Stir during heating: occasionally vs. none | 1.0 |
| Measure % T: after 1 hr vs. immediately | 7.0 |
| Meaningless factor | 0.7 |
| **Phosphorus** | |
| Add molybdate: 5.5 vs. 4.5 ml | 0.4 |
| Add hydrazine: 5.5 vs. 4.5 ml | 0.3 |
| Heat: 10 vs. 8 min. | 0.6 |
| Add first: hydrazine vs. molybdate | 0.2 |
| Dil. to: 75 vs. 65 ml | 0.1 |
| Cool: rapidly vs. gradually | 0.0 |
| Meaningless factor | 0.3 |
| **Titanium** | |
| Dil. to: 23 vs. 27 ml | 0.1 |
| Adjust pH: 5 vs. 3 | 10.3 |
| Adjust pH: before vs. after adding Tiron | 17.3 |
| Add buffer: 5.5 vs. 4.5 ml | 0.3 |
| Add dithionite: 20 vs. 30 mg | 0.3 |
| Measure % T after adding dithionite: 18 min. vs. immediately | 10.1 |
| Meaningless factor | 0.3 |

[a] First alternative minus second.

**Table 2. Analysis of liming materials**

| Material | Element, % Range | Av. |
|---|---|---|
| **Aluminum** | | |
| NBS Limestone 1a | | |
| Present work | 2.19–2.25 | 2.23 |
| NBS values | 2.15–2.26 | 2.20 |
| NBS Dolomite 88 | | |
| Present work | — | 0.05 |
| NBS values | 0.03–0.04 | 0.04 |
| Limestone 145 | 1.36–1.39 | 1.38 |
| Blast furnace slag | 6.43–6.70 | 6.53 |
| Cement kiln dust | 2.68–2.80 | 2.74 |
| **Iron** | | |
| NBS Limestone 1a | | |
| Present work | 1.06–1.08 | 1.07 |
| NBS values | 1.10–1.18 | 1.14 |
| NBS Dolomite 88 | | |
| Present work | 0.06–0.09 | 0.07 |
| NBS values | — | 0.06 |
| Limestone 145 | 0.81–0.92 | 0.87 |
| Blast furnace slag | 0.42–0.53 | 0.46 |
| Cement kiln dust | 2.07–2.12 | 2.10 |
| **Manganese** | | |
| NBS Limestone 1a | | |
| Present work | 0.034–0.036 | 0.035 |
| NBS values | 0.023–0.034 | 0.029 |
| NBS Dolomite 88 | | |
| Present work | 0.002–0.004 | 0.003 |
| NBS values | 0.002–0.007 | 0.005 |
| Limestone 145 | 0.039–0.041 | 0.040 |
| Blast furnace slag | 0.640–0.668 | 0.656 |
| Cement kiln dust | 0.135–0.140 | 0.138 |
| **Phosphorus** | | |
| NBS Limestone 1a | | |
| Present work | 0.059–0.061 | 0.069 |
| NBS values | 0.048–0.079 | 0.066 |
| NBS Dolomite 88 | | |
| Present work | 0.000–0.002 | 0.001 |
| NBS values | 0.001–0.002 | 0.001 |
| Limestone 145 | 0.025–0.029 | 0.027 |
| Blast furnace slag | 0.000–0.004 | 0.002 |
| Cement kiln dust | 0.021–0.027 | 0.023 |
| **Titanium** | | |
| NBS Limestone 1a | | |
| Present work | 0.103–0.105 | 0.104 |
| NBS values | 0.066–0.120 | 0.096 |
| NBS Dolomite 88 | | |
| Present work | — | 0.005 |
| NBS values | 0.002–0.003 | 0.003 |
| Limestone 145 | 0.075–0.079 | 0.007 |
| Blast furnace slag | 0.233–0.239 | 0.235 |
| Cement kiln dust | 0.155–0.160 | 0.158 |

adding dithionite. The necessary precautions have been noted in the procedure for titanium.

**Table 3. Relative standard deviation of the analytical procedures**

| Source of Data | Relative Standard Deviation (%) Al | Fe | Mn | P | Ti |
|---|---|---|---|---|---|
| Ruggedness tests[a] | 1.24 | 1.70 | 2.50 | 1.02 | — |
| Ruggedness tests[b] | 6.38 | 6.10 | 7.65 | 4.93 | 1.26 |
| Analyses of samples[c] | 3.02 | 3.84 | 3.72 | 7.52 | 1.76 |

[a] Std dev. calculated by Youden's formula (11).
[b] Std dev. calculated from the error term of the statistical analysis of data from ruggedness tests.
[c] Std dev. calculated from the error term of the statistical analysis of liming material results.

### Analysis of Liming Materials

The five liming materials were analyzed in triplicate. The three limestones were ignited at 1000° before treatment with $HClO_4$, and the sample solution cleared more rapidly than without ignition. No difficulties were apparent in applying the methods. Results are shown in Table 2.

Phosphorus in the dolomite and blast furnace slag and manganese in the dolomite were near the limits of measurement. Under the normal use of liming materials, lesser amounts would not be of significance. All five elements are sufficiently abundant in most liming materials to fall within the measurable ranges of the methods.

### Precision and Accuracy

The standard deviation, expressed as relative standard deviation (coefficient of variation), was used to judge the precision of the methods (Table 3). Estimates of precision varied according to the procedure used for calculating standard deviation. Youden's procedure (11) (in which the differences of Table 1 are squared and the square root of 2/7 of the sum of squares is taken as the standard deviation) indicated better precision than did estimates based on analyses of variance of the ruggedness data and the analytical results on the 5 liming materials. For the latter two estimates, standard deviation was calculated as the square root of the variance of the error term of the analyses of variance. Precision for titanium appeared better than did precision for the other

elements, but was not estimated by Youden's procedure because of outstanding differences in the ruggedness tests.

In the analysis of variance of the analytical results for the liming materials, replicates were not significant, but, as known in advance, samples were significantly different.

A comparison of ranges and averages obtained on the two National Bureau of Standards samples with the values certified by the Bureau (Table 2) indicated a reasonable degree of accuracy.

### Recommendation

The methods outlined are worthy of further consideration, and it is recommended that they be studied collaboratively.


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This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. See *This Journal*, **47**, 119 (1964).

# Free Water in Fertilizers by Karl Fischer Titration

By JOSEPH H. CARO (U.S. Fertilizer Laboratory, Soil and Water Conservation Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md 20705)

**A method is proposed for free water in fertilizer, which involves extraction with *p*-dioxane and titration of the extract with Karl Fischer reagent. Preliminary tests established the most suitable experimental conditions and showed *p*-dioxane to be the most feasible extractant for fertilizers. In comparison with the official vacuum desiccation method, Karl Fischer extraction-titration gave results for water averaging 0.22% lower on 11 test materials. Because of the inherent positive error of weight loss methods, the lower values are considered to be reasonable.**

The official method for determination of free water in fertilizers—vacuum desiccation for 16–18 hours (1)—has enough inherent drawbacks (2) to warrant a search for an alternative method. Since it depends on weight loss, it is not specific for water. Volatile components of the fertilizer other than water will contribute to weight loss and lead to a positive error. Even materials having a low vapor pressure may be evolved in significant amount during the lengthy desiccation time. Moreover, the amount of water removed depends on the efficiency of the desiccant employed (3) and on the rate at which water diffuses from the test sample. Finally, the long time required is an obvious shortcoming.

The method reported here involves extraction of the fertilizer with *p*-dioxane and titration of the extract with Karl Fischer reagent. It is simple, relatively rapid, and specific for water.

## Experimental

*Extraction.*—Extraction of free water is necessary because direct titration of the sample with Karl Fischer reagent yields a measure of total (free + hydrate) water. A search for extractants adaptable to Karl Fischer titrations (4) led to four possibilities —ethylene glycol, methanol, pyridine, and *p*-dioxane. The eventual choice of *p*-dioxane was dictated by a systematic test conducted on a typical fertilizer (Table 1). The free water content of this material was determined after extraction with each extractant at three different temperatures and with three modes of agitation. Results clearly showed that temperature or type of agitation had little effect on the amount of water removed by *p*-dioxane. Extraction with the alcohols produced a temperature-dependent hydrolysis, while with pyridine some sensibility to both variables was evident.

**Table 1. Apparent free water content of a triple superphosphate[a] under various conditions of extraction[b]**

| Temperature, °C | Type of Agitation: Quiescent, % $H_2O$ | Wrist-Action,[c] % $H_2O$ | Magnetic Stirrer, % $H_2O$ |
|---|---|---|---|
| Extractant: Ethylene Glycol | | | |
| 0 | 1.54 | 4.08 | 3.87 |
| Room | 2.61 | 5.13 | 5.73 |
| 60 | 5.42 | 8.29 | 8.05 |
| Extractant: Methanol | | | |
| 0 | 1.99 | 2.15 | 2.24 |
| Room | 2.29 | 2.40 | 4.34 |
| 60 | 6.01 | 8.81 | 8.16 |
| Extractant: Pyridine | | | |
| 0 | 1.13 | 1.22 | 1.41 |
| Room | 1.44 | 1.71 | 1.47 |
| 60 | 2.24 | 1.89 | 2.05 |
| Extractant: *p*-Dioxane | | | |
| 0 | 1.73 | 1.66 | 1.76 |
| Room | 1.69 | 1.63 | 1.74 |
| 60 | 1.69 | 1.62 | 1.70 |

[a] Free water content by official method = 1.88%.
[b] 2 g sample, 20 ml extractant, 30 min. extraction.
[c] Burrell wrist-action shaker.

The time required for quantitative extraction of water by *p*-dioxane was established in another preliminary test (Table 2). The water is apparently entirely extracted in something under 15 minutes, but a 30-minute extraction time is specified in the method as a safety factor.

**Table 2. Apparent free water content of diatomaceous earth extracted for varying periods of time with *p*-dioxane[a]**

| Extraction Time, min. | % $H_2O$ Karl Fischer |
|---|---|
| 15 | 1.40 |
| 45 | 1.39 |
| 60 | 1.47 |
| 120 | 1.55 |

[a] 2 g sample, 20 ml *p*-dioxane.

The maximum amount of water that can be quantitatively extracted in 30 minutes by 20 ml of *p*-dioxane is indicated in Table 3 to be about 60 mg. This quantity is therefore specified as the maximum in the method.

*Titration.*—While a number of titration procedures are available for use with Karl Fischer reagent, best results were obtained in this work with the electrometric direct dead-stop technique (4, p. 94). The titration vessel was a sawed-off 150 ml beaker, 65 mm deep, sealed with a No. 11 rubber stopper. The glass-enclosed platinum electrodes, the reagent delivery buret (50 ml), and a vent tube were inserted through the stopper and sealed with RTV silicone rubber sealant. The electrical circuit, a simple system consisting of a dry-cell, variable resistor, and galvanometer, was connected to the electrodes and assembled according to the scheme of Carter and Williamson (5).

The end point in Karl Fischer electrometric direct titration methods depends on depolarization of the cathode by the free iodine that persists in the solution after all the water in the test sample has reacted. While water is present, the cathode remains polarized and no current flows. Near the end point, the depolarization causes a flow

**Table 3. Apparent free water content of varying amounts of a 10–10–10 mixed fertilizer extracted with *p*-dioxane[a]**

| Sample Weight, g | $H_2O$ Extracted, mg | % $H_2O$, Karl Fischer |
|---|---|---|
| 2.02 | 207 | 10.26 |
| 1.04 | 109 | 10.51 |
| 0.51 | 57 | 11.22 |
| 0.25 | 28 | 11.13 |

[a] 20 ml *p*-dioxane, 30 min. extraction time.

of current, which is observed on the galvanometer. However, the system is unstable and, if no further reagent is added, the galvanometer needle soon returns to the point of zero deflection. The exact end point thus becomes a matter of arbitrary choice within the general area of the first current surge. In the work reported here, the end point was designated as that point at which the addition of one drop of reagent maintained the galvanometer needle beyond the midpoint of the scale for at least 30 seconds. This end point is tantamount to that of commercial "magic eye" titrimeters. A sharp, reproducible end point was consistently obtained when a small volume of aniline was added to the solution being titrated.

## METHOD

### *Reagents*

(a) *p-Dioxane.*—Reagent grade.

(b) *Karl Fischer reagent.*—Stabilized single solution, Fisher Scientific Co. Cat. No. So-K-3, or equivalent.

(c) *Sodium tartrate dihydrate.* — Reagent grade.

(d) *Methanol.*— Reagent grade, containing no more than 0.1% $H_2O$.

(e) *Aniline.*—Reagent grade.

### *Apparatus*

(a) *Centrifuge.*—High-speed angle type.

(b) *Titration apparatus.*— Electrometric, as described above and fitted with a magnetic stirring device. Commercial titration apparatus is probably adaptable to this method but has not been used by the author.

### *Determination*

Weigh 0.5–2.0 g fertilizer (−20 mesh containing no more than 60 mg $H_2O$) into a 50 ml Erlenmeyer flask, add 20 ml of *p*-dioxane, stopper, swirl once, and let mixture stand 30 min. Swirl once again and centrifuge.

Remove a 5 ml aliquot of the clear centrifugate and transfer into the reaction vessel of the titration apparatus. Add 10 ml methanol and 1 ml aniline, and affix the vessel to the titration apparatus. Titrate, with stirring, to the end point given above with standardized Karl Fischer reagent. (Standardize periodically by separately titrating with the reagent about 0.2 g sodium tartrate dihydrate in 15 ml methanol.) Conduct a reagent blank titration with pure *p*-dioxane in place of the centrifugate. Calculate % $H_2O$ in the sample from the difference in titer.

### Results

The method as described was applied to a variety of fertilizers and compared to

**Table 4. Free water in fertilizers by Karl Fischer titration and by vacuum desiccation method 2.015**

| Lot No. | Material | Free $H_2O$, %, by K. F. Titr. | Free $H_2O$, %, by Vac. Des. | Difference, % |
|---|---|---|---|---|
| F–4 | Triple superphosphate | 1.72 | 1.81 | −0.09 |
| F–5 | Triple superphosphate | 3.20 | 3.47 | −0.27 |
| F–10 | Normal superphosphate | 1.57 | 1.69 | −0.12 |
| 3363a | Normal superphosphate | 1.00 | 1.03 | −0.03 |
| 3466 | Diammonium phosphate | 0.42 | 0.64 | −0.22 |
| N–575 | 10–10–10 (no urea) | 4.23 | 4.64 | −0.41 |
| N–576 | 10–10–10 (75% urea) | 11.18 | 11.49 | −0.31 |
| — | Phosphate rock | 0.90 | 1.27 | −0.37 |
| 3543 | Monoammonium phosphate | 0.43 | 0.62 | −0.19 |
| N–621 | Fresh triple superphosphate | 15.50 | 15.82 | −0.32 |
| N–622 | Normal superphosphate cured 11 days | 2.99 | 3.04 | −0.05 |

results obtained on the same materials with use of the official vacuum desiccation method (Table 4). Although these are single determinations, the preliminary work showed that the precision of the Karl Fischer method was excellent, with replicate results generally agreeing within 0.05% $H_2O$.

Of the two methods, Karl Fischer titration gives the lower free water content in every case. Differences average about 0.22% $H_2O$. Because of the inherent positive error of weight loss methods, these lower results were expected, with accompanying increase in accuracy. On the basis of the data in Table 4, the Karl Fischer method described here should be tested by other analysts, with a view to its eventual adoption as an official method.


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# PESTICIDE RESIDUES

## Determination of Arsenic in Foods

By I. HOFFMAN and A. D. GORDON (Analytical Chemistry Research Service, Canada Department of Agriculture, Ottawa, Ontario, Canada)

Several aspects of the arsine-molybdenum blue and the silver diethyldithiocarbamate methods adopted as official, first action in 1962 were investigated following inquiries directed to the Associate Referee.

### *Effect of Antimony*

Stibine gives a red reaction product with silver diethyldithiocarbamate reagent; this product has a peak absorption at 510 mμ as compared with 522 mμ for the arsine complex. It was found that the presence of KI and $SnCl_2$ in the generating flask depressed the evolution of stibine, but there was the possibility that large quantities of antimony might cause a positive interference in the normal method of generating arsine.

In the silver diethyldithiocarbamate method the addition of 100 μg of $Sb_2O_3$ to 20 μg of $As_2O_3$ caused an apparent increase of 0.5 μg in the arsenic recovery. This interference increased as the Sb:As ratio was increased.

Since $SnCl_2$ reduced the evolution of stibine, the possibility of total prevention by increasing the amounts was investigated. It was found that an increase in added $SnCl_2$ from the usual 8 drops to about 35 drops prevented any interference from 1000 μg of $Sb_2O_3$ on 20 μg of $As_2O_3$.

In the arsine-molybdenum blue method there was no interference even when 500 μg of $Sb_2O_3$ was added to 50 μg of $As_2O_3$. No doubt this is due to the fact that stibine does not form a molybdenum blue complex as does arsine under these experimental conditions. Even when 500 μg of $Sb_2O_3$ was generated in the absence of KI and $SnCl_2$, a colored complex was not formed.

It can be concluded that antimony does not interfere with the determination of arsenic by the arsine-molybdenum blue method and that interferences can be prevented in the silver diethyldithiocarbamate method by adding more $SnCl_2$ to the generating mixture.

### *Recovery of Arsenic from Refractory Substances*

(a) *Fish tissues.*—Recoveries of arsenic from salmon, finnan-haddock, and kipper tissues were found to be erratic and low following nitric and sulfuric acid digestion. This was overcome by the use of perchloric acid according to the following procedure:

"Digest 5 g tissue with nitric and sulfuric

acids in the normal manner until darkening does not occur. Cool, add 0.5 ml 70% perchloric acid solution, and heat until fuming occurs and the digest is clear. Cool, and add two additional 0.5 ml portions of perchloric acid as above. Finish the digestion with water and ammonium oxalate as usual."

Recoveries of arsenic added to salmon tissues which were digested as above and run by the silver diethyldithiocarbamate method are as follows:

| Arsenic Added, μg | Arsenic Recovered, μg |
|---|---|
| 0.50 | 0.43 |
| 1.00 | 0.99 |
| 2.00 | 1.98 |
| 2.50 | 2.55 |

(b) *Tobacco.*—Digestion of flue-cured tobacco with nitric and sulfuric acids resulted in low recoveries (approximately 60%) of added arsenic. Full recoveries were obtained, however, with the above perchloric acid procedure.

The interference of pyridine in the generation of arsine was confirmed when it was found that adding 0.5 ml of pyridine to both a standard arsenic solution and a perchloric acid digest lowered the recoveries of arsenic by approximately 20%.

It is recommended that study of methods for arsenic in foods be continued.

This report of the Associate Referee, I. Hoffman, was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee E, and was accepted by the Association. See *This Journal*, **47**, 135 (1964).

# Summary of Results of the Collaborative Tests on the Rhodamine B Colorimetric Method for Antimony[1]

By J. C. BARTLET (Food and Drug Directorate, Canada Department of National Health and Welfare, Tunney's Pasture, Ottawa, Ontario) and J. L. MONKMAN[2] (Occupational Health Division, 45 Spencer St., Ottawa 4, Ontario)

**In acid solution, pentavalent antimony forms a colored complex with Rhodamine B that can be extracted with an organic solvent such as benzene or toluene. The method was tested by ten collaborators on three samples. The average recovery was 105% with a coefficient of variation of about 10%. The method has been recommended for adoption as official, first action.**

For several years, the American Conference of Governmental Industrial Hygienists has been studying a method for the determination of antimony in air filters and biological materials under the refereeship of J. L. Monkman. The method is based on the formation of a colored complex between antimony and Rhodamine B in acid solution followed by extraction with benzene, toluene, or isopropyl alcohol (1–8), and has been published in *This Journal* (9) and by the A.C.G.I.H. (10). The complete statistical evaluation of the test was presented at the meeting of the A.C.G.I.H. in May 1963. It is the purpose of this paper to present a summary of the results.

## Collaborative Study

Three sample solutions containing 3.0, 5.0, and 8.0 μg antimony per ml were sent to ten collaborating laboratories. Sample 2, in addition to the antimony, contained 750 μg Fe per ml for investigation of possible iron interference. The collaborators were instructed to follow the method of Maren (2), and to use some additional notes by the Referee. Each sample was to be analyzed in triplicate. Of the ten collaborators, eight

[1] The tests were sponsored by the American Conference of Governmental Industrial Hygienists.

[2] A.C.G.I.H. Referee on Antimony.

This report of the Associate Referee, J. C. Bartlet, was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

**Table 1. Analysis of variance of collaborative test of Rhodamine B method for antimony**

| Source of Variation | Degrees of Freedom | Mean Squares | Std Dev. | Coeff. of Var. 100 × S.D./Mean |
|---|---|---|---|---|
| 1. 3.0 μg (mean recovery 3.13 μg) | | | | |
| Between collaborators | 7 | 0.635[a] | | |
| Within replicates | 16 | 0.0129 | 0.11 | 3.5 |
| Total | 23 | 0.204 | 0.45 | 14.4 |
| 2. 5.0 μg (mean recovery 5.24 μg) | | | | |
| Between collaborators | 7 | 0.116 | | |
| Within replicates | 16 | 0.101 | 0.32 | 6.1 |
| Total | 23 | 0.105 | 0.33 | 6.3 |
| 3. 8.0 μg (mean recovery 8.53 μg) | | | | |
| Between collaborators | 7 | 1.71[a] | | |
| Within replicates | 16 | 0.156 | 0.40 | 4.7 |
| Total | 23 | 0.63 | 0.80 | 9.7 |

[a] Significant at the 1% level.

completed the assignment with results suitable for statistical analysis. Several analysts returned four or more results for each sample, but only the first three were used for statistical treatment.

The analysis of variance for each sample is given in Table 1. It was found that there was a difference (significant at the 1% confidence level) between collaborators for the first and third samples but not for the second. The average coefficient of variation within replicates for each analyst was 5% and the over-all coefficient of variation was 10.5% when the differences between analysts are included. For most analysts there was a positive bias, with an average recovery of 105.2%.

In testing for the precision of the methods, Bartlett's test was used to check the homogeneity of variance within the results of each analyst. The chi-square value was within the 95% confidence limits for the 3.0 μg sample only. Aberrant results of two of the eight analysts inflated the chi-square values slightly beyond the limits for the 5.0 and 8.0 μg samples. The reproducibility reported by the other six analysts was within the expected variation.

One of the analysts returned four results for each sample with a coefficient of variation (within his own results) of less than 1%. His comments on the method stressed the importance of a low temperature during color development and a few other minor points of technique. These comments have since been incorporated into the method as published (9).

The reasons for the slight positive bias have not been investigated. However, since each analyst used his own antimony powder for calibration, the purity of the various samples of antimony compared to that of the Referee's sample would affect the results.

### Recommendation

The method has been adopted by the American Conference of Governmental Industrial Hygienists and is recommended for adoption as official, first action by the Association of Official Agricultural Chemists.

### Collaborating Laboratories

The authors wish to thank the following laboratories which assisted in this project:

1. Air and Industrial Hygiene Laboratory, Department of Public Health, Berkeley 4, Calif., U.S.A.

This recommendation of the Associate Referee, who is also General Referee on Metals and Other Elements, was approved by Subcommittee E and adopted by the Association. See *This Journal*, **47**, 136 (1964).

2. The Consolidated Mining & Smelting Co., Analytical Laboratories, Trail, B.C., Canada

3. Detroit Dept. of Health, 8801 John C. Lodge Expressway, Detroit 2, Mich., U.S.A.

4. Department of National Health & Welfare, Food & Drug Directorate, Ottawa 4, Canada

5. Georgia Department of Public Health, Industrial Hygiene Service, 47 Trinity Ave., Atlanta, Ga., U.S.A.

6. Indiana State Board of Health, 1330 West Michigan St., Indianapolis, Ind., U.S.A.

7. Michigan Department of Health, Industrial Hygiene Division, Lansing, Mich., U.S.A.

8. State Board of Health, Division of Disease Control, Helena, Mont., U.S.A.

9. State of New York Department of Labor, Division of Industrial Hygiene, Lab. Section, 80 Centre St., New York 13, N.Y.

10. Pennsylvania Department of Health, Occupational Health Laboratory, P.O. Box 90, Harrisburg, Pa., U.S.A.

11. Utah Department of Health, 45 Fort Douglas Blvd., Salt Lake City 13, Utah, U.S.A.


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# The Metabolism and Persistence of Systox, Diazinon, and Phosdrin on Field-Sprayed Lettuce

By D. E. COFFIN and W. P. McKINLEY (Food and Drug Laboratories, Tunney's Pasture, Ottawa, Canada)

**Three organophosphate pesticides (Systox, Diazinon, and Phosdrin) were applied individually to field plots of lettuce. Samples were removed at various times after spraying and examined for residues and metabolites of the pesticides.**

**The two isomers of Systox rapidly disappeared from the lettuce, but two toxic metabolites were formed and persisted up to 2 weeks after spraying. No metabolites were discovered on lettuce which had been sprayed with Phosdrin or Diazinon. Residues of Phosdrin had decreased to less than 0.1 ppm 3 days after spraying. Residues of Diazinon were less than 0.1 ppm at 10 days and were present at detectable levels 14 days after spraying.**

The formation of metabolites of organophosphate pesticides presents several problems in the analysis of residues of these pesticides. There is very little information concerning the metabolites of many of these compounds and there is even less information regarding their behavior in the procedures used for analysis of pesticide residues.

Metcalf, *et al.* (1, 2) and Fukuto, *et al.* (3–5) showed that the sulfoxide and sulfone of the thiono isomer and at least one other compound were formed in plants treated

with the thiono isomer of Systox. They also showed that the sulfoxide and sulfone of the thiol isomer were formed from the thiol isomer. Metcalf, *et al.* (1) also presented evidence to indicate that the thiono isomer isomerizes to the thiol isomer in bean plants. Getz (6) found that the sulfoxide and sulfone of the thiol isomer were present in kale that had been sprayed with Systox. No toxic plant metabolites have been demonstrated for Phosdrin or Diazinon.

This paper presents data obtained from lettuce that had been sprayed with Systox (a mixture of O,O-diethyl O-2-ethylthioethyl phosphorothioate and O,O-diethyl S-2-ethylthioethyl phosphorothioate), Diazinon (O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate), or Phosdrin (O,O-dimethyl O-1-carbomethoxy-1-propen-2-yl phosphate). These data are restricted to metabolites of these compounds and to the persistence of the compounds and their metabolites. No attempts have been made to identify the hydrolytic degradation products of the pesticides or metabolites.

Systox, Phosdrin, and Diazinon were applied as sprays at the rate of 400 g of active ingredient per acre to separate field plots of lettuce. Representative samples were removed from the treated plots and control plots at 4 hours and 1, 2, 3, 7, 10, and 14 days after spraying. These samples were stored at −20°C until the analyses were completed.

## Materials and Methods

### *Chromatographic Systems*

(a) *System 1.*—Whatman No. 1 paper. Immobile phase: 15% (v/v) propylene glycol in absolute ethanol. Mobile phases: (*1*) benzene; (*2*) cyclohexane.

(b) *System 2.*—Whatman No. 1 paper. Immobile phase: 15% (v/v) propylene glycol in absolute ethanol. Mobile phase: cyclohexane + benzene (6 + 4) (v/v).

(c) *System 3.*—Whatman No. 1 paper. Immobile phase: 4% (v/v) light mineral oil in peroxide-free ether. Mobile phase: acetonitrile + water (3 + 7) (v/v).

### *Reagents*

(Redistill all solvents before use.)

(a) *Polyethylene-coated alumina.*—(Kensington Scientific Corp., Berkeley, Calif.)

(b) *Magnesol.*—Industrial regular (Canada Colors and Chemicals Ltd., Toronto, Ontario).

(c) *Beef liver homogenate* (7).—Homogenize 1 g beef liver with 9 ml water. Freeze to store. Immediately before use dilute with 30 ml water.

(d) *Chromogenic reagent* (7).—Prepare fresh just before use. (*1*) 25 mg 1-naphthylacetate dissolved in 10 ml absolute ethanol. (*2*) 50 mg Azoene Fast Blue RR salt dissolved in 20 ml water and 20 ml BDH buffer (pH 7.0) added. Add solution (*1*) to solution (*2*).

(e) *Iodoplatinate reagent* (8).—Dissolve 1 g platinic chloride in 10 ml water and add to a solution of 10 g KI in 250 ml water. Prepare working solution by mixing 1 volume of this stock solution with 6 volumes of water.

(f) *Isobutyl alcohol-benzene.*—Mix equal volumes of isobutyl alcohol and thiophene-free benzene.

(g) *Molybdate reagent.*—Dissolve 25 g ammonium molybdate in 200 ml 10*N* $H_2SO_4$ and dilute to 500 ml with water.

(h) *Ethanolic* $H_2SO_4$.—Dissolve 20 ml concentrated $H_2SO_4$ in 480 ml absolute ethanol.

(i) *Stannous chloride.*—Dissolve 10 g $SnCl_2 \cdot 2H_2O$ in 25 ml concentrated HCl and store in a glass-stoppered brown bottle. This solution is stable for several months. Dilute 1 ml of this stock solution to 200 ml with 1.0*N* $H_2SO_4$ solution immediately before use.

(j) *p-Nitrophenol indicator.*—Dissolve 25 mg *p*-nitrophenol in 25 ml water.

### *Apparatus*

(a) *Flash evaporator.*—(Buchler Instruments, N.Y.)

(b) *Chromatographic columns.*—Glass tube 2.5 cm o.d., 35 cm long, with a 150 ml reservoir at the top and a medium sintered glass filter plate at the bottom. The plate leads into a drip tube surrounded by a 24/40 inner member of a glass joint. This outer tube is equipped with a side arm for attachment to vacuum.

(c) *Oxygen flask ignitor.*—(Arthur H. Thomas Co., Philadelphia, Pa.)

### *Standards*

(a) *Organophosphates.*—0.01*M* solutions of each of the organophosphates in chloroform. The phosphate analogue of Diazinon was prepared by bromination of Diazinon.

(b) *Phosphorus.*—Prepare from oven-dried

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$KH_2PO_4$ and dilute to give 1 μg phosphorus/ml.

***Extraction and Cleanup***

Extract and clean up 50 g samples of lettuce by the method of Coffin and McKinley (9) modified as follows for the recovery of the sulfoxide of the thiol isomer of Systox: After elution of the magnesol column with chloroform, elute with 50 ml acetone and discard the eluate. Then elute with 100 ml methanol and evaporate the methanol eluate to dryness on the flash evaporator at 35°C. Dissolve the residue in chloroform and transfer to a 10 ml volumetric flask with chloroform.

***Separation and Identification***

Concentrate suitable aliquots of the cleaned up extracts under a stream of nitrogen and transfer to paper chromatograms, using chloroform to complete the transfer. Develop the chromatograms in the appropriate systems, dry, and employ one of the following procedures for identification of the organophosphates present:

(a) The enzymatic detection technique using beef liver homogenate, 1-naphthylacetate, and Azoene Fast Blue RR salt as described by McKinley and Johal (7).

(b) The iodoplatinate detection technique as described by MacRae and McKinley (8).

Cut areas corresponding to the organophosphates out of the chromatograms and estimate the amount of organophosphate present by determination of total phosphorus.

***Phosphorus Determination***

Place 10 ml 7.5*N* nitric acid solution in a 1000 ml Schöniger flask. Wrap the sample in black paper and place in the platinum holder. Flush the flask with oxygen, insert the platinum holder, tighten a screw-clamp on the top of the flask, and ignite the sample, using an infrared lamp. After ignition, shake the flask and contents thoroughly, cool, release the screw-clamp, and transfer the contents to a 50 ml beaker, using small portions of water to rinse the flask and the platinum holder. Evaporate to about 0.5 ml on a hot plate, cool, add 3 drops *p*-nitrophenol indicator, and then add 7.5*N* ammonium hydroxide solution dropwise until a yellow substance appears. Add 1*N* nitric acid dropwise until the yellow substance disappears. Transfer the solution to a 10 ml glass-stoppered graduate cylinder and make the volume to 5 ml. Add 5 ml isobutyl alcohol-benzene solution and 1.7 ml molybdate reagent. Stopper the graduate and shake vigorously for 15 seconds. Let the phases separate. Pipet a 3 ml sample of the upper isobutyl alcohol-benzene layer into a 5 ml volumetric flask and wash the pipet with 1–1.5 ml of ethanolic $H_2SO_4$ solution, adding the washings to the 5 ml volumetric flask. Add 0.1 ml stannous chloride solution, dilute to volume with ethanolic $H_2SO_4$ solution, and mix well. Read the absorbance of the color produced at 625 mμ. Determine the phosphorus content of the sample by comparison with phosphorus standards containing up to 2.5 μg phosphorus and processed in the same manner as the samples.

**Results and Discussion**

When the enzymatic detection procedure was employed, three aliquots of each extract were applied to the paper chromatogram. After development, one of these was used to detect the pesticides and metabolites, and the areas corresponding to the compounds detected were cut out of the other two and used for phosphorus determinations. This was necessary since both the liver homogenate and the chromogenic agent contained phosphorus. It was not necessary with the iodoplatinate detection technique, where phosphorus could be determined directly from the sprayed chromatogram.

The modification of the cleanup procedure was necessary to recover the sulfoxide of the thiol isomer of Systox. The sulfoxide was retained on the magnesol column after elution with chloroform and was not removed by acetone elution, which removed most of the pigments from the magnesol. After acetone elution of the pigments, the sulfoxide was quantitatively recovered by elution with methyl alcohol. The three pesticides and the sulfone of the thiol isomer of Systox were recovered by the chloroform elution of the magnesol column.

Known amounts of the three pesticides and the sulfone and sulfoxide of the thiol isomer of Systox were added to samples of lettuce from the untreated plot just before extraction. After cleanup, these compounds were chromatographed in the appropriate systems and the quantities determined by the total phosphorus technique. The recoveries of these standard compounds are shown in Table 1. This table shows that the procedures are satisfactory for the quantitative

recovery of these compounds from lettuce; 5 of the compounds are recovered in the chloroform eluate, and one in the methanol eluate from the magnesol column. This partial separation during cleanup may prove to be very useful, since the sulfoxide and sulfone of the thiol isomer of Systox are not separated by many of the more generally used paper chromatographic procedures.

The two isomers of Systox and the two oxidation products were separated paper chromatographically by system 1. The chromatograms were first developed with benzene as the mobile phase. After drying, the papers were rotated 180° and then developed in the cyclohexane mobile phase. Both isomers of Systox moved to the solvent front in the benzene mobile phase, while the thiol sulfoxide and thiol sulfone had $R_f$ values of 0.69 and 0.88, respectively. The sulfoxide and sulfone were not mobile in cyclohexane, but the thiol and thiono isomers of Systox had $R_f$ values of 0.78 and 0.95.

The combination of these two mobile phases, developed in opposite directions, provided separation of the four compounds. Since the cleanup procedure isolated the sulfoxide from the other compounds, the residue from the methanol eluate was chromatographed in one direction only, with benzene as the mobile phase. The sulfoxide proved to be relatively insensitive to the enzymatic detection technique and particularly sensitive to iodoplatinate detection.

The enzymatic detection after bromination was used for chromatograms obtained from the chloroform eluates and iodoplatinate detection for the methanol eluates from the magnesol column. None of the other possible oxidation products of Systox, shown in Fig. 1, were available as standards. Figure 1 also illustrates the possible isomerization of the thiono isomer to the thiol isomer.

These compounds all undergo hydrolysis at varying rates, but the most stable of them seems to be the sulfoxide of the thiol isomer (3). Figures 2 and 3 show the chromatographic separation of these compounds and the chromatograms obtained from samples at various times after application of Systox to lettuce. No compounds other than the two isomers of Systox and the sulfoxide and sulfone of the thiol isomer were detected. The quantities of these compounds recovered from lettuce are shown graphically in Fig. 4. The 2 isomers of Systox disappeared within 24 hours of spraying, but the sulfoxide and sulfone appeared within 4 hours. These metabolites reached a maximum within 24 hours, persisted at measurable levels for at least 10 and 7 days, and could not be detected at 14 and 10 days. The total phosphorus content of the cleaned up extracts agreed within experimental error with the total phosphorus content of the separate components on the chromatograms. It would appear that the four identified compounds were the only phosphorus-containing materials present in the cleaned up extract.

**Table 1. Recovery of organophosphate compounds added to lettuce and determined after paper chromatography**

| Compound | Amount Added, μg | Recovered[a] μg | % |
|---|---|---|---|
| Systox-thiol isomer | 34.5 | 30.8 | 89 |
| Systox-thiono isomer | 50.3 | 45.8 | 91 |
| Systox-thiol sulfoxide | 48.0 | 47.5 | 99 |
| Systox-thiol sulfone | 45.6 | 44.5 | 98 |
| Phosdrin | 20.7 | 19.2 | 93 |
| Diazinon | 39.8 | 35.0 | 88 |

[a] Each value is the average of 4 determinations.

Diazinon is a thiophosphate compound which may possibly form the corresponding phosphate by oxidation. There is no possibility that any sulfoxides or sulfones may form by oxidation of this pesticide. It has been shown (Table 1) that Diazinon is quantitatively recovered from lettuce by the procedures employed. The corresponding phosphate, however, was not eluted from magnesol by chloroform. It was quantitatively recovered by acetone elution, but this elution also removed pigments and other materials that interfered with the subsequent paper chromatographic separation. The phosphate was removed by elution with 3% acetonitrile in chloroform, which also removed some pigments, but paper chromatographic separation of the constituents of this eluate was possible.

## OXIDATION OF SYSTOX

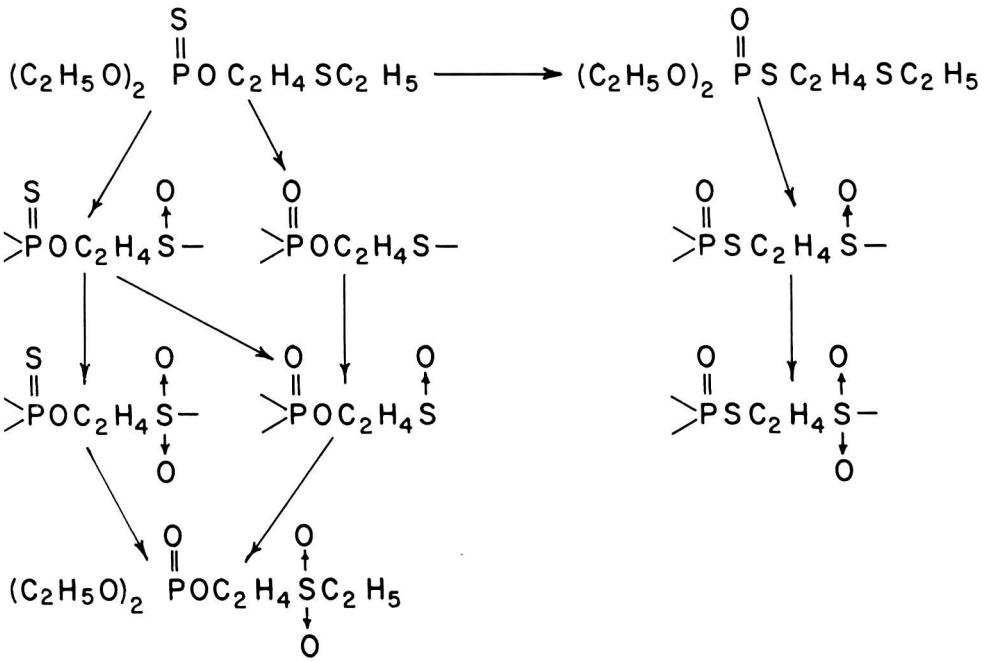


*Fig. 1*—The possible oxidation products of Systox.

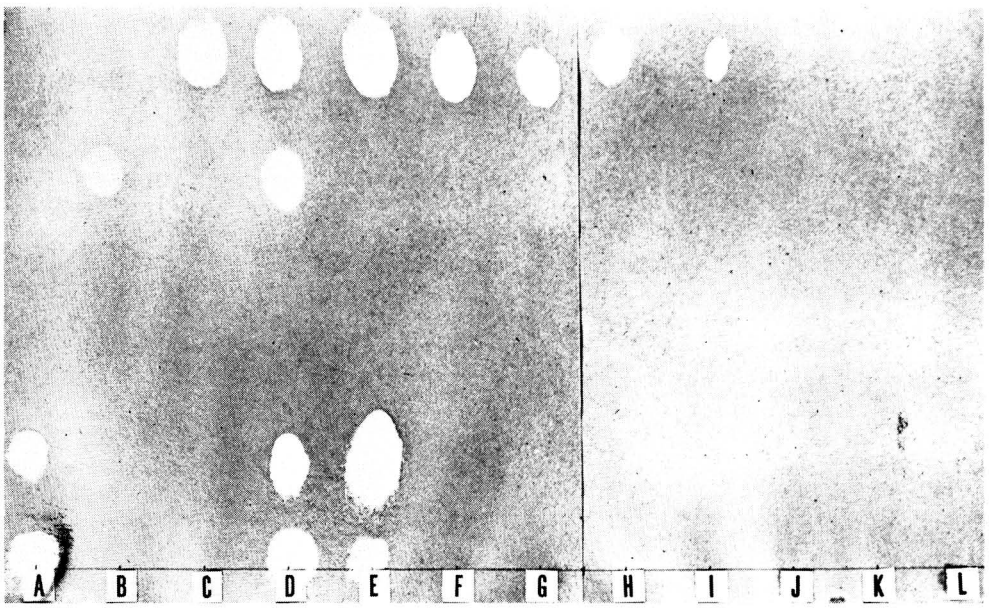


*Fig. 2*—Paper chromatograms developed in system 1 showing the separation of the isomers and some metabolites of Systox (A-D) and the Systox and its thiol sulfone obtained from 10 g samples of lettuce at various times after spraying with Systox (E-L). A, 5 μg Systox (thiono and thiol isomers in ascending order). B, 5 μg Systox-thiol sulfoxide. C, 5 μg Systox-thiol sulfone. D, mixture (A+B+C). E, 4 hours. F, 1 day. G, 2 days. H, 3 days. I, 7 days. J, 10 days. K, 14 days. L, control.

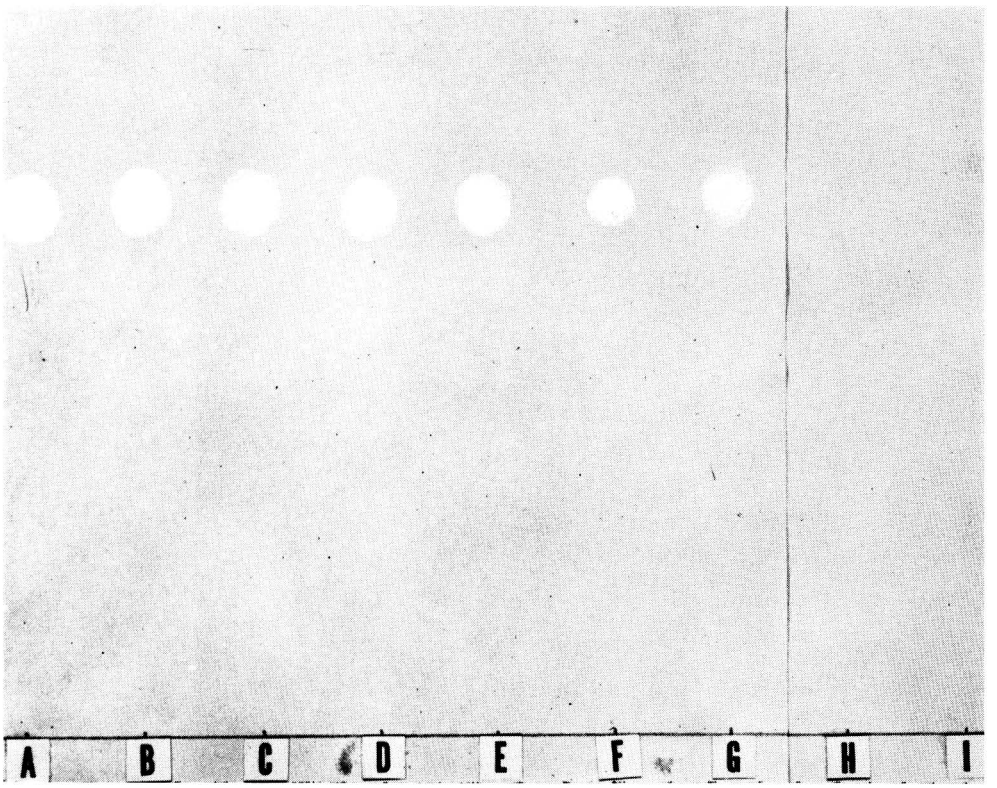


*Fig. 3*—Paper chromatograms showing the Systox-thiol sulfoxide obtained from 10 g samples of lettuce at various times after spraying with Systox. A, 5 μg Systox-thiol sulfoxide. B, 4 hours. C, 1 day. D, 2 days. E, 3 days. F, 7 days. G, 10 days. H, 14 days. I, control.

Chromatographic system 3 was employed for the separation and the enzymatic detection after bromination of the chromatograms, for identification of Diazinon and its oxygen analogue. Figure 5 shows the chromatographic separations obtained and the chromatograms of the residues in the chloroform eluates obtained from lettuce samples. Diazinon and possibly three other esterase-inhibiting compounds appear as a result of Diazinon application. Of these, only Diazinon appeared in measurable quantities and phosphorus could not be detected in any of the other three. Further elution from magnesol with 3% acetonitrile in chloroform failed to remove detectable quantities of any esterase-inhibiting compounds. This elution with acetonitrile in chloroform had given

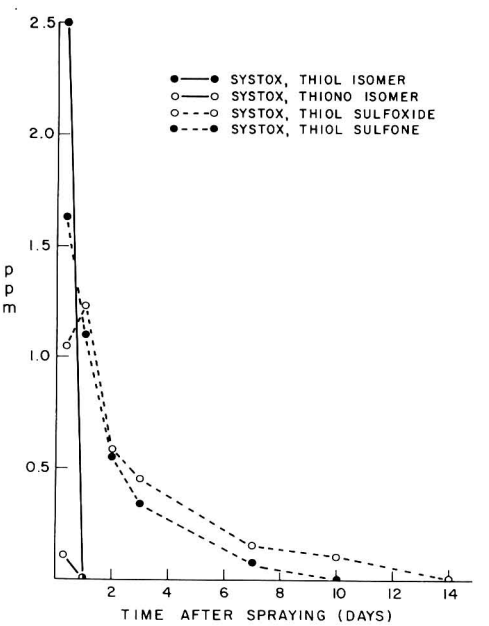


*Fig. 4*—The quantities of the isomers and metabolites of Systox recovered from samples of lettuce after spraying with Systox.

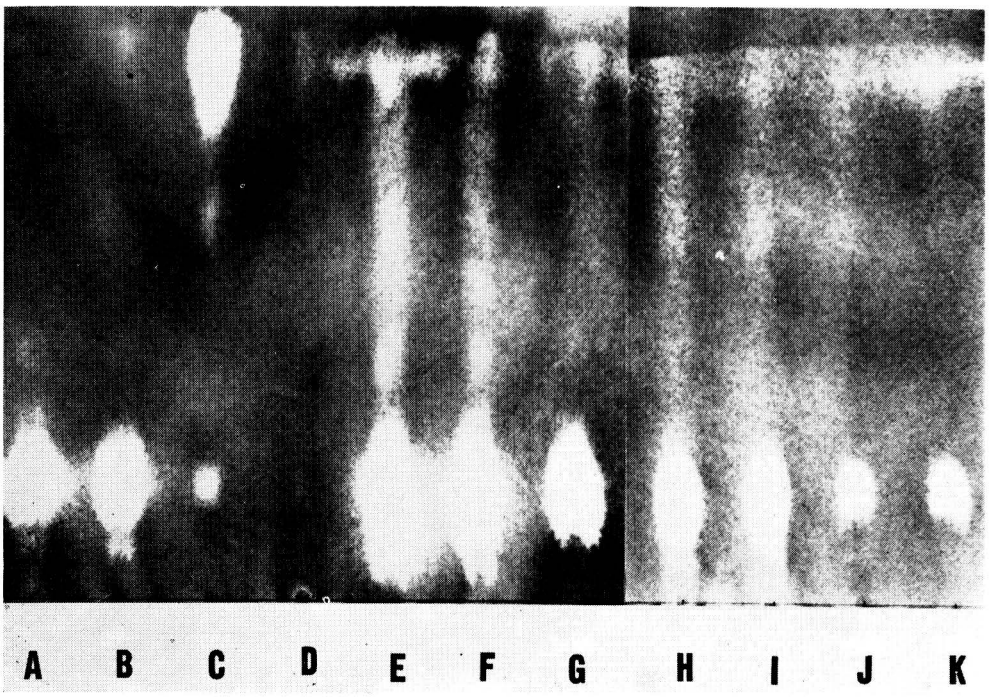


*Fig. 5*—Paper chromatograms developed in system 3 showing the Diazinon obtained from 2.5 g samples of lettuce at various times after spraying with Diazinon. A, 5 μg Diazinon. B, 20 μg Diazinon spray concentrate. C, 5 μg oxygen analogue of Diazinon. D, control. E, 4 hours. F, 1 day. G, 2 days. H, 3 days. I, 7 days. J, 10 days. K, 14 days.

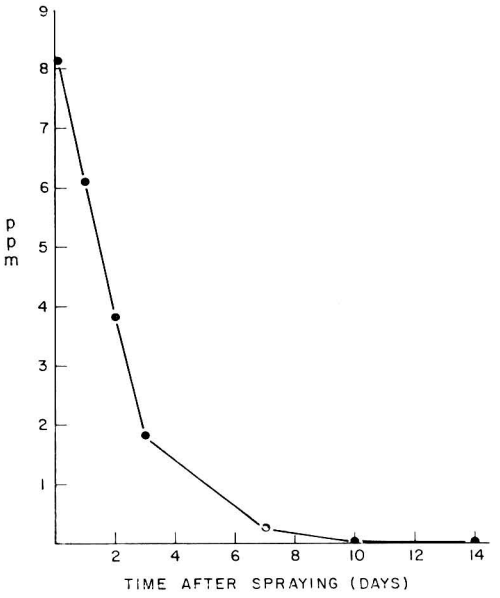


*Fig. 6*—The quantities of Diazinon recovered from samples of lettuce after spraying with Diazinon.

95% recovery of the oxygen analogue of Diazinon added to lettuce samples. Since the rate of hydrolysis of the phosphate is approximately 10 times that of Diazinon (10), there is no reason to expect measurable quantities of the phosphate to appear in plant material. Figure 6 shows graphically the quantities of Diazinon recovered from lettuce at various times after application of Diazinon. Measurable residues of Diazinon persisted for 7 days and detectable amounts were present for at least 14 days. Again the total phosphorus content of the cleaned up extract was in close agreement with the phosphorus content of the Diazinon on the chromatogram, indicating that Diazinon was the only phosphorus-containing compound present in measurable quantities after clean-up.

Phosdrin is a phosphate from which no oxidation products would be expected and the residues of which have been reported to


*Fig. 7*—Paper chromatograms developed in system 2 showing the Phosdrin obtained from 2.5 g samples of lettuce at various times after spraying with Phosdrin. A, 5 $\mu$g Phosdrin. B, 4 hours. C, 1 day. D, 2 days. E, 3 days.

be rapidly dissipated. Chromatographic system 2 was used for the separation of Phosdrin, and the enzymatic detection technique was utilized. Figure 7 illustrates the separation obtained and the residues in lettuce that had been sprayed with Phosdrin. Phosdrin and one other esterase-inhibiting compound were detected. The quantities of residues obtained after spraying with Phosdrin are shown graphically in Fig. 8. This figure illustrates the rapid disappearance of Phosdrin residues with a decrease from 12 ppm at 4 hours to less than 0.1 ppm at 3 days after application. No phosphorus could be detected in the other esterase-inhibiting compound which appeared in the sample taken 4 hours after spraying. The Phosdrin on the chromatogram accounted for all of the phosphorus present after cleanup.

It should be pointed out that it is possible that other metabolites were formed from these pesticides and were not recovered by the procedures employed in this study. Although these pesticides might be expected to behave in a similar manner in most plants, there is no assurance that the same metabolites would be formed in all other crops. The rate of dissipation of residues would vary considerably, depending upon the crop and possibly upon climatic conditions.

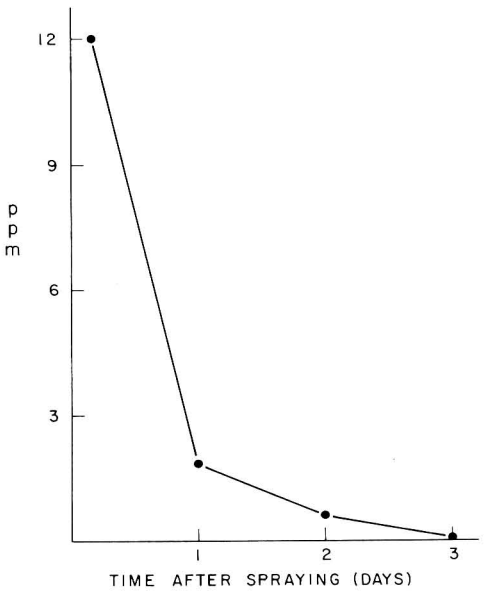


*Fig. 8*—The quantities of Phosdrin recovered from samples of lettuce after spraying with Phosdrin.


## Summary

Results have been presented to show the persistence of three organophosphate pesticides and some of their metabolites on lettuce under field conditions. Phosdrin was rapidly dissipated with residues decreasing from 12 ppm at 4 hours to less than 0.1 ppm at 3 days after application of Phosdrin. Diazinon residues decreased from 8.1 ppm to 0.3 ppm from 4 hours to 7 days after spraying, and detectable quantities of Diazinon were present at 10 and 14 days. No metabolites of Diazinon were detected, and the oxygen analogue was not present in detectable quantities. Systox residues were characterized by the rapid disappearance of both isomers of the pesticide and by the rapid formation of the sulfoxide and sulfone of the thiol isomer, which persisted in measurable quantities up to 10 days but were not detectable 14 days after application of Systox.



## Acknowledgments

The authors are indebted to Mrs. S. I. Read for her constructive criticism of the manuscript, to Mr. G. Savary for technical assistance, and to Dr. A. P. Chan, Research Branch, Canada Department of Agriculture, Ottawa, for providing the plots of lettuce used in this study.


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# The Oxidative Conversion of Thimet to Its Oxygen Analog Sulfone

By ROGER C. BLINN (Metabolism Laboratory, Agricultural Division, American Cyanamid Co., Princeton, N.J.)

**A qualitative and quantitative evaluation was made of the ability of twelve oxidants to convert Thimet Systemic Insecticide to its oxygen analog sulfone. Thin-layer chromatographic and infrared spectrophotometric techniques were utilized for this purpose. The superiority of *m*-chloroperbenzoic acid as the oxidant of choice is demonstrated.**

When O,O-diethyl S-ethylthiomethyl phosphorodithioate (I) (Thimet®) exists as a spray residue on and in plants, the metabolic products shown in Fig. 1 occur (1–3). Two published methods, a cholinesterase inhibition procedure (4–6) and a colorimetric determination of the hydrolytic product of II sulfone (7, 8), are nonspecific. Recently a method was presented for identifying residues resulting from treatment with I, using thin-layer chromatography in conjunction with infrared spectrophotometry, or alternatively, colorimetry (9), which is also dependent on conversion of I and its metabolites to II-sulfone.

Two procedures have been used for the oxidative conversion of I and its metabolic products (Fig. 1) to II-sulfone. Previous investigators have used peracetic acid (4, 6), originally proposed for enhancing the cholinesterase inhibition properties of Trithion carbophenothion (10), and perbenzoic acid (7, 8) for I, and permanganate for metasisosystox (11, 12) and Systox (13). We obtained relatively poor recoveries by these methods. Further, the use of perbenzoic acid proved inconvenient because of the lengthy synthesis required and uncertainty about stability of the acid in storage. Therefore, a number of alternative oxidizing conditions have been studied in an effort to improve this critical step in oxidative procedures for Thimet.

The thin-layer chromatographic system described previously (9) has been used in conjunction with infrared spectrophotometry to evaluate the various oxidizing conditions chosen for this study, both qualitatively and quantitatively where required.

## METHOD

### *Reagents*

(All reagents were reagent grade unless otherwise specified.)

(a) *Silica Gel-G and Silica Gel-HF* (1:1).—(Brinkmann Instruments, Inc.) Slurry 1 part with 2 parts of pH 6 buffer solution to prepare thin-layer plates.

(b) *Buffer solution, pH 6.*—Mix 5.7 ml 0.1*N* NaOH with 50 ml 0.1*M* potassium dihydrogen phosphate solution, and dilute to 100 ml with distilled water.

(c) *Solvents.* — Chloroform, acetone, and *n*-hexane; all redistilled before use.

(d) *Developing solution.*—Add 3.5 ml methyl alcohol to chloroform and dilute to 200 ml. Use fresh solution for each chromatogram.

(e) *Chromogenic agent.*—Dissolve 5 ml 5% palladium chloride solution and 1 ml concentrated HCl in 95% ethyl alcohol, then dilute to 100 ml.

### *Oxidants*

(Prepare all oxidants fresh just before use.)

(a) *Peracetic acid.*—Mix 1 ml 30% hydrogen peroxide with 5 ml glacial acetic acid.

(b) *Perpropionic acid.*—Mix 5 ml propionic acid with 1 ml hydrogen peroxide.

(c) *Performic acid.*—Mix 5 ml formic acid and 1 ml hydrogen peroxide.

(d) *Monoperphthalic acid.*—Stir, at room temperature, 15 g phthalic anhydride in 100 ml ethyl ether with 25 ml 30% hydrogen peroxide. Dry separated ether solution over anhydrous calcium sulfate and store under refrigeration until use (14).

(e) *m-Chloroperbenzoic acid.* — (Available from Food Machinery and Chemical Corp. (15).) Dissolve 1 g in 10 ml chloroform just prior to use.

(f) *Potassium permanganate solution.*—0.1*M*.

(g) *Chromic anhydride.*—0.1*M* in glacial acetic acid.

(h) *Sodium dichromate solution.*—0.1*M* in 0.1*M* acetic acid.

(i) *Bromine water.*—Saturated.

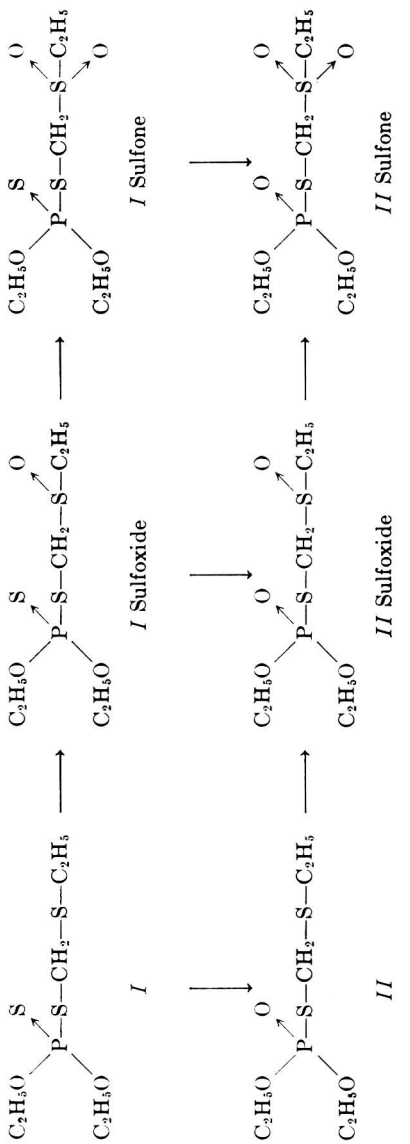


Fig. 1

(j) *Sodium meta-periodate.*—0.1*M* in 0.1*M* acetic acid.

(k) *Sodium meta-periodate.*—Saturated solution in glacial acetic acid.

(l) *Ceric sulfate.*—0.1*M* in 6*N* sulfuric acid.

### Determination

*Oxidation.*—Immerse an 18 × 150 mm test tube containing 100 μg I, either as a residue in the bottom of the tube for oxidation in the aqueous phase or as a solution in 5 ml chloroform for reactions in nonpolar solvents, in a water bath of the appropriate temperature. Add oxidant solution, mix thoroughly, and let the reaction proceed for 15 minutes. Then transfer the tube contents to a 500 ml separatory funnel with Teflon stopcock, using 25 ml chloroform. Stop reaction by shaking thoroughly with 25 ml saturated sodium sulfite. If organic peracids were used, add 25 ml either saturated sodium bicarbonate solution or 3*N* ammonium hydroxide solution and shake the mixture thoroughly. Pass the chloroform solution through anhydrous granular sodium sulfate into a Kuderna-Danish evaporative concentrator; re-extract the aqueous phase twice with fresh 25 ml portions of chloroform. Concentrate the resulting chloroform solution to a few ml in a Kuderna-Danish evaporative concentrator on a steam bath, and evaporate the last traces of solvent from the concentrator tube under vacuum on a Rinco rotating evaporator.

*Thin-layer chromatography.*—To a mixture of 30 g Silica Gel-G and 30 g Silica Gel-HF in a 500 ml glass-stoppered Erlenmeyer flask, add 120 ml pH 6 buffer solution. Immediately shake the mixture vigorously for 1 minute, pour the slurry into a DeSaga-Brinkmann applicator, and apply to the acetone-rinsed glass plates in the usual manner. After air drying, prewash the plates twice with freshly distilled acetone by allowing migration up the plate. By means of a micropipet, transfer the residue as a spot to the origin of a prepared plate with three 0.1 ml portions of methylene chloride. Develop the plate in a fresh solution of 1.75% methanol in chloroform, keeping the atmosphere saturated with solvent vapor by a solvent-saturated blotting-paper curtain. After drying, again develop the plate in chloroform to improve separation of II-sulfone from I-sulfoxide and to remove interferences due to solvents and reagents. For qualitative evaluation, spray with palladium chloride solution so that the sulfur-containing compounds can be seen. (Since its chromogenic action is quite slow,

**Table 1. Composition of oxidation products resulting from reaction of Thimet (I) with various oxidants**

| Oxidant | Temperature, °C | Products Found[a] I S | I SO | I $SO_2$ | II S | II SO | II $SO_2$ | Remarks[b] |
|---|---|---|---|---|---|---|---|---|
| 4 ml 0.2*M* $KMnO_4$ in 20% $MgSO_4$ | 0 | 1 | | Tr | | | Tr | |
| | 25 | 1 | | Tr | | | Tr | |
| | 100 | Tr | | Tr | | | 1 | Poor recovery |
| 4 ml 0.2*M* $KMnO_4$ in 30% acetic acid | 0 | 2 | Tr | 1 | | | Tr | |
| | 25 | | | 1 | | | Tr | |
| | 50 | | | | | | 1 | Poor recovery |
| | 100 | | | | | | 1 | Poor recovery |
| 4 ml 0.2*M* $KMnO_4$ in 0.1*M* $H_3PO_4$ | 0 | | | 1 | | | Tr | |
| | 25 | | | 1 | | | Tr | |
| | 50 | | | Tr | | | 1 | Poor recovery |
| | 100 | | | | | | 1 | Poor recovery |
| 4 ml 0.2*M* $KMnO_4$ in 0.5*M* $H_3PO_4$ | 0 | 2 | | 1 | | | Tr | |
| | 25 | 2 | | 1 | | | Tr | |
| | 50 | Tr | | | | | 1 | Poor recovery |
| | 100 | | | | | | 1 | Poor recovery |
| 0.1*M* $KMnO_4$ in acetone | 0 | 1 | 2 | Tr | | Tr | | |
| | 25 | Tr | | 1 | | | 2 | |
| | 50 | Tr | | 1 | | | 2 | |
| 0.1*M* $CrO_3$ in glacial acetic acid | 0 | Tr | 1 | Tr | | Tr | | |
| | 50 | Tr | 1 | Tr | | Tr | | Emulsion problems |
| | 100 | Tr | 1 | Tr | | Tr | | Poor recovery; Emulsion problems |
| 0.1*M* $Na_2Cr_2O_7$ in 0.1*N* acetic acid | 0 | 1 | | | | | Tr | |
| | 25 | 1 | | | | | Tr | |
| | 50 | 1 | | | | | Tr | |
| | 100 | Tr | | | | | Tr | Poor recovery |
| 0.1*M* $Ce(SO_4)_2$ in 6*N* $H_2SO_4$ | 0 | 1 | 2 | | 3 | Tr | | |
| | 25 | 1 | 1 | | 2 | 2 | | |
| | 50 | 2 | 1 | 3 | | 4 | 5 | |
| | 100 | Tr | Tr | Tr | | | | Poor recovery |
| Bromine water, saturated | 0 | Tr | | | Tr | | | Very poor recovery |
| | 25 | Tr | | | | | | Very poor recovery |
| | 50 | Tr | | | | | | Very poor recovery |
| | 100 | Tr | | | | | | Very poor recovery |
| Bromine water, dilute, in 0.1*M* $H_3PO_4$ | 0 | 1 | | | Tr | 2 | 3 | 1 minute reaction time |
| | 0 | 1 | | | Tr | 2 | 3 | 5 minutes reaction time |
| | 0 | 1 | | | | 1 | 1 | 10 minutes reaction time |
| 0.1*M* Sodium *meta*-periodate in 0.1*N* acetic acid | 0 | 1 | Tr | | 2 | 2 | | |
| | 25 | 1 | 2 | | 2 | Tr | | |
| | 50 | 3 | 2 | Tr | 1 | Tr | | |
| | 60 | 2 | 1 | Tr | | 3 | | Repeat run |
| | 100 | 3 | 2 | 5 | 1 | 4 | Tr | |
| Sodium *meta*-periodate saturated solution in glacial acetic acid | 30 | 1 | | 2 | Tr | | Tr | |
| | 50 | 1 | | 2 | Tr | | | |
| 2 ml of ether solution of monoperphthalic acid (see *Experimental* for concentration) | 0 | Tr | 1 | Tr | | Tr | | |
| | 25 | Tr | 1 | 1 | | 1 | 1 | |
| | 50 | Tr | | | | | 1 | |
| | 70 | Tr | | | | | 1 | |
| Peracetic acid (see *Experimental* for concentration) | 68–70 | 2 | 4 | 5 | | 3 | 1 | |
| | 75–80 | 2 | | | | 3 | Tr | |
| 0.2 g *m*-chloroperbenzoic acid in chloroform | 0 | | | | | | 1 | |
| | 25 | | | | | | 1 | |
| | 50 | | | | | | 1 | |

[a] See Fig. 1 for chemical structure of I and II. S means sulfide, SO means sulfoxide, $SO_2$ means sulfone. 1, 2, 3—refer to relative amounts of occurrence; Tr means trace amounts resulted.

[b] Estimate of recoveries is by visual observation of chromogenic areas on chromatograms.

often components of the chromatographed mixture can not be seen until the next day.) For quantitative evaluation by infrared spectrophotometry, spray chromatographed standards on the edges of the chromatographic plate with chromogenic agent to locate areas of interest.

*Infrared spectrophotometry.*—Scrape the palladium chloride-stained portion of silica gel of interest from the thin-layer plate with a spatula into a medium-porosity fritted glass filter, extract with five 1 ml portions of hot acetone, and filter by vacuum directly into a 25 ml pear-shaped flask. Evaporate the solution to dryness on a Rinco rotating evaporator; add 1 ml hexane, and re-evaporate the solution to dryness. Add a boiling chip to minimize bumping during this operation. Dissolve the residue in the flask in 0.3 ml carbon disulfide, transfer to a 5 mm NaCl cavity cell, and determine the infrared characteristics from 1400 to 700 $cm^{-1}$ with solvent compensation (Perkin-Elmer Model 421). The sharp sulfone absorption band at 1325 $cm^{-1}$ for II-sulfone had an absorbance of 28.8 $\mu g$ per 0.1 absorbance unit.

## Results and Discussion

Table 1 presents the composition of the oxidation products resulting from the reaction of I with the various oxidants. Peracetic acid and perpropionic acid gave essentially identical qualitative and quantitative results, but performic acid oxidation resulted in complete loss of products responding to palladium chloride. Table 2 presents the recovery data compiled from the three most promising oxidants tested. The recoveries are for II-sulfone only; other oxidation compounds that might have been formed have been chromatographically separated but are not included. Table 3 shows the recovery values for II-sulfone obtained with *m*-chloroperbenzoic acid oxidations of I, II, and II-sulfone.

**Table 2. Efficiency of conversion of I to II-sulfone**

| Oxidant | No. Runs | Average % Recovery of II-Sulfone |
|---|---|---|
| Peracetic acid | 3 | 54 ± 1 |
| Monoperphthalic acid | 9 | 42 ± 10[a] |
| *m*-Chloroperbenzoic acid | 3 | 72 ± 3 |

[a] Determined over a period of several weeks. Evidence of instability of the oxidant ether solution under refrigeration.

**Table 3. Recoveries of II-sulfone from oxidation of I and metabolites with m-chloroperbenzoic acid**

| Compound | No. Runs | Average % Recovery of II-Sulfone |
|---|---|---|
| I | 3 | 72 ± 3 |
| II | 3 | 92 ± 3 |
| II-Sulfone | 3 | 85 ± 3 |

In the course of this study, it was noted that the efficiency of elution of I and its oxidation products from silica gel decreased with time. Using dilute acids to prepare the thin-layer plates corrected this elution problem, suggesting the hydrolytic decomposition of the organophosphorus esters. Use of silica gel plates buffered at pH 6 as recommended resulted in the stability of the organophosphorus esters and the optimum mechanical condition of the thin-layer plate.

As Table 1 shows, a variety of oxidation products of I can be obtained, either selectively if a certain compound is desired, or as an over-all mixture. It must be emphasized, however, that while obvious experimental conditions were carefully controlled, most of the oxidation reactions were relatively unreliable and yielded varied qualitative results upon repetition. This was especially true of reactions in the aqueous phase.

Examination of Table 2 shows that *m*-chloroperbenzoic acid is superior to peracetic and monoperphthalic acids in oxidizing I to II-sulfone. Only II-sulfone is found as a product of *m*-chloroperbenzoic acid oxidation, regardless of the reaction conditions (time, temperature, or concentration), whereas with the other oxidants, if such conditions were not carefully controlled, considerable quantities of the intermediate oxidation products of I invariably formed. When a mixture of all of the oxidation products of I was oxidized with *m*-chloroperbenzoic acid, only II-sulfone was produced.

Some losses are encountered as a result of II-sulfone instability to the reaction conditions (Table 3).

The qualitative and quantitative superiority of *m*-chloroperbenzoic acid over other commonly used oxidants, coupled with its stable (15), crystalline nature, make it the

oxidant of choice in the conversion of I and its metabolites to II-sulfone, and suggest that that it may prove useful in oxidative conversion of other sulfur-containing organophosphorus pesticides.

**Acknowledgment**

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# Measurement of Residues of Cygon Insecticide and Its Oxygen Analog by Total Phosphorus Determination after Isolation by Thin-Layer Chromatography

By W. A. STELLER and A. N. CURRY (Metabolism Laboratories, Agricultural Research Center, American Cyanamid Co., Princeton, N.J.)

**A total phosphorus method incorporating thin-layer chromatographic isolation is described for the determination of Cygon Insecticide (generic name, dimethoate) and its oxygen analog. Residues of the insecticides are extracted from macerated plant tissue, and the extract, after solvent partitioning and concentration, is spotted on a thin-layer chromatographic plate. After development of the plate, the areas corresponding to the two compounds are scraped and eluted. The phosphorus content of the respective eluates is determined by an improved molybdenum blue method, and concentrations of dimethoate and its oxygen analog in the original samples are calculated.**

Cygon® [O,O-dimethyl S-(N-methylcarbamoylmethyl) phosphorodithioate], dimethoate, is an organic phosphate insecticide for use under a variety of conditions. Several methods (1-4) are available for the determination of dimethoate residues in various crops. However, none of these is capable of distinguishing dimethoate insecticide from its oxygen analog [O,O-dimethyl S-(N-methylcarbamoylmethyl) phosphorothioate], which Dauterman, *et al.* (5) have shown to be the major toxic metabolite in plants. Evaluation of potential hazards as a result of dimethoate application to food crops required the development of methods capable of determining residues of the oxygen analog in plant

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tissues in the presence of considerably larger residues of the parent compound.

The work of Blinn (6), Walker and Beroza (7), Bäumler and Rippstein (8), and others suggested that thin-layer chromatography might serve the double purpose of separating the oxygen analog from dimethoate insecticide and, at the same time, provide a potent means of removing interfering extractives. The nonspecific molybdenum blue colorimetric microphosphorus method (9, 10) was chosen in preference to more specific detection methods in order that the general approach might be useful for a number of organophosphorus insecticides. The modified microphosphorus procedure described here is more sensitive, and we find it more reliable, than previously reported methods. However, the procedure employs digestion with anhydrous perchloric acid at elevated temperatures and is attended by serious explosive hazards unless proper precautions, discussed below, are observed. Its useful range is 1–8 μg of phosphorus or approximately 8–60 μg of dimethoate insecticide or its oxygen analog.

## METHOD

### Reagents

*Solvents.*—Acetone, hexane, chloroform; all reagent grade.

*Sodium sulfate.*—Decahydrate, reagent grade.

*Diatomaceous earth.* — Hyflo Super-Cel (Johns-Manville).

*Activated vegetable carbon, unwashed.* — Nuchar C-190-N (Industrial Chemical Sales, Division West Virginia Pulp & Paper Co., Covington, Va.)

*Palladium(ous) chloride reagent.* — Mix 5 ml 5% solution (reagent grade) with 1 ml coned hydrochloric acid, and dilute to 100 ml with 95% ethyl alcohol.

*Silica Gels G and HF.*—(Brinkmann).

*Perchloric acid.*—70–72%, ACS reagent grade.

*Acids.*—8*N* and 1*N* nitric acid; 6*N* and 1*N* sulfuric acid.

*Propylene glycol.*—(USP).

*Ammonium molybdate.*—4% aqueous solution. Dissolve 4 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in water and dilute to 100 ml.

*ANSA reagent* (also known as Fiske-Subbarow Reagent).—Add 0.5 g 1-amino-2-naphthol-4-sulfonic acid (Distillation Products) with mechanical stirring to 200 ml freshly prepared 15% sodium bisulfite (anhydrous), and then add 1.0 g anhydrous sodium sulfite. Filter, store at room temperature in a brown bottle, and prepare fresh every 5 days.

*Standard sample solutions.* — Weigh accurately 16.00 mg dimethoate and oxygen analog (available from American Cyanamid Co., Princeton, N.J.) and dissolve each in 50 ml acetone (conen = 0.32 μg/μl). Stored in a refrigerator, these solutions are stable for at least 3 months.

*Standard phosphorus solution.* — Dissolve 4.580 mg anhydrous dibasic sodium phosphate (ACS grade, dried to constant weight at 105°C) in 500 ml distilled water. The phosphorus concentration in this solution is 2 μg/ml.

### Apparatus

*Homogenizing apparatus.* — Servall Omnimixer or equivalent.

*Büchner funnels.*—With fritted disc: medium porosity, 350 ml, and medium porosity, 15 ml.

*Separatory funnels.*—500 ml, with Teflon valve.

*Rinco rotating evaporator or equivalent.*—Connected to a mechanical vacuum pump.

*Acetylization flask.*—100 ml.

*Thin-layer apparatus.* — (May be obtained from Brinkmann Instruments, Inc., Great Neck, N.Y.) *1.* Adjustable thin-plate applicator, Model 25 00 12, template and measuring template. *2.* 2 × 8″ thin-layer plates and 2½ × 10″ cylindrical development chambers. Prepare plates as follows:

Coat 2 × 8″ thin-layer plates to a thickness of 0.5 mm with a 1+1 mixture of Silica Gel G and Silica Gel HF (mixed in the dry state). Slurry the mixture vigorously for 1 minute with a volume of water equal to twice the total weight of silica gel, and apply to the support plates immediately. Let the prepared plates dry overnight at room temperature. Prewash the plates by developing with acetone 2 cm past the intended final solvent front. Using a sharp pencil and the measuring template as a guide, score lines across the plate at the intended front 13 cm from the bottom edge and along the sides 2–3 mm from each edge.

*John spray bottle.*—A. H. Thomas 9186-R2.

*Spectrophotometer* (Bausch and Lomb Spectronic 20, or equivalent).—With ½″ absorption cells.

*Test tubes.*—18 × 150 mm, borosilicate glass with lip, with two lines scratched 180° apart to indicate the 10.0 ml mark.

*Aluminum heating block.*—Fashioned from 1″ stock which is 6″ square, to fit on standard

laboratory hot plate. (See photograph of digestion block, Fig. 1.) Holes are drilled 1″ in diameter and 3″ deep to accommodate the 18 × 150 mm test tubes (11 per block). The thermometer well is drilled 5/16 × 3″. A reflector collar 2½″ high from thin aluminum sheet sits on top of the block. It raises the temperature of the upper portion of the test tubes and speeds the volatilization of water and nitric acid from the mouths of the tubes. Nitric acid, if not all volatilized, will cause the molybdenum blue color to fade.

### *Preparation of Calibration Curve*

Pipet 25, 50, 100, 150 µl of the acetone standard solutions of dimethoate and oxygen analog into appropriately marked test tubes and dilute each to 3.5 ml with water. Add 0.5 ml 8*N* $HNO_3$ and 1 drop propylene glycol, chill momentarily in an ice bath, add 0.5 ml 70% $HClO_4$, and set the tubes in the back of a hood for 5 minutes to warm to room temperature. Place the tubes in the aluminum heating block on a *cold* hot plate, and raise the temperature slowly until the dense white fumes of anhydrous perchloric acid rise about halfway up the tube and persist there (typically, about 60–80 minutes from room temperature to 225°C, followed by about 20–30 minutes at a block temperature of 225–245°C).

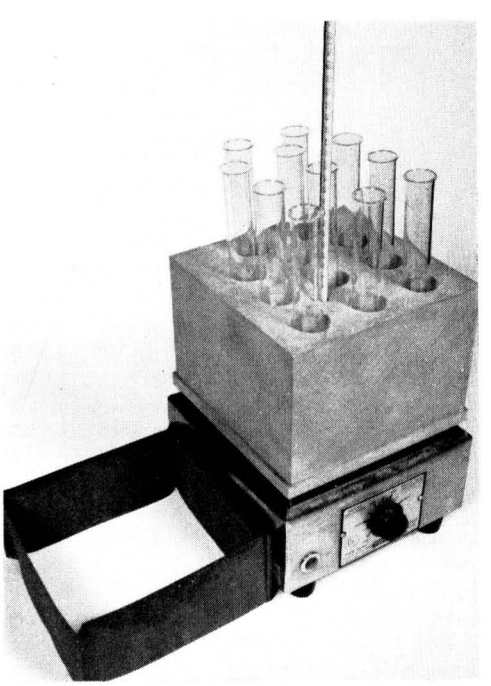

*Fig. 1*—Aluminum heating block on standard laboratory hot plate with aluminum reflector collar on left.

Turn off the hot plate and let the block cool below 150°C before removing the tubes with *clean* tongs. (See discussion on possible hazards of anhydrous perchloric acid.)

Rinse down the sides of the cooled tubes with 4–5 ml water, add (in the following order) 1 ml 6*N* $H_2SO_4$, 0.4 ml 4% ammonium molybdate, and 0.4 ml ANSA reagent, dilute to between 9.8 and 10.0 ml with water, and mix. Heat the tubes 12 ± 2 minutes in a boiling water bath (the heating period must be controlled for uniform color development), and immediately transfer them to a cold water bath to cool to room temperature. Adjust the volumes, at room temperature, exactly to the 10.0 ml mark by adding water, and mix. Transfer a suitable portion of each solution to a ½″ colorimetric tube and measure the absorbance at 820 mµ with water as a reference solution. Correct the absorbances for a similarly run reagent blank and plot the corrected absorbances for each compound. The absorbance for equivalent microgram quantities of oxygen analog is 8% higher than those for dimethoate, since its phosphorus content is correspondingly higher.

The plots obey Beer's Law from 8 to 60 µg of either compound with a slope of 0.100 absorbance units per µg of phosphorus or approximately 7 µg of either compound. Alternatively, a calibration curve can be prepared by developing color directly on the aqueous $Na_2HPO_4$ standard for 0–4 ml portions, beginning with the addition of 1 ml 6*N* $H_2SO_4$. To convert µg phosphorus to µg dimethoate or oxygen analog, multiply respectively by 7.40 or 6.90.

### *Extraction of Pesticides*

*Apples and green tomatoes.*—Macerate a representative 100 g crop sample with 100 ml acetone. Filter the resulting extract with vacuum through a medium-porosity fritted disc covered with ¼″ of diatomaceous earth. Wash the pulp with 250 ml acetone. Concentrate the combined filtrate and washings in a 1 L round-bottom flask on a rotating film evaporator at 25°C until all the acetone has been removed. It is important to remove all the acetone at this point to insure efficient extraction of waxy plant materials into the hexane. Transfer the water concentrate (75–80 ml) to a 500 ml separatory funnel and tumble gently with 80 ml hexane. Discard the hexane phase, being careful not to discard any of the resultant interface, and continue to extract the water phase with 80 ml portions of hexane until the

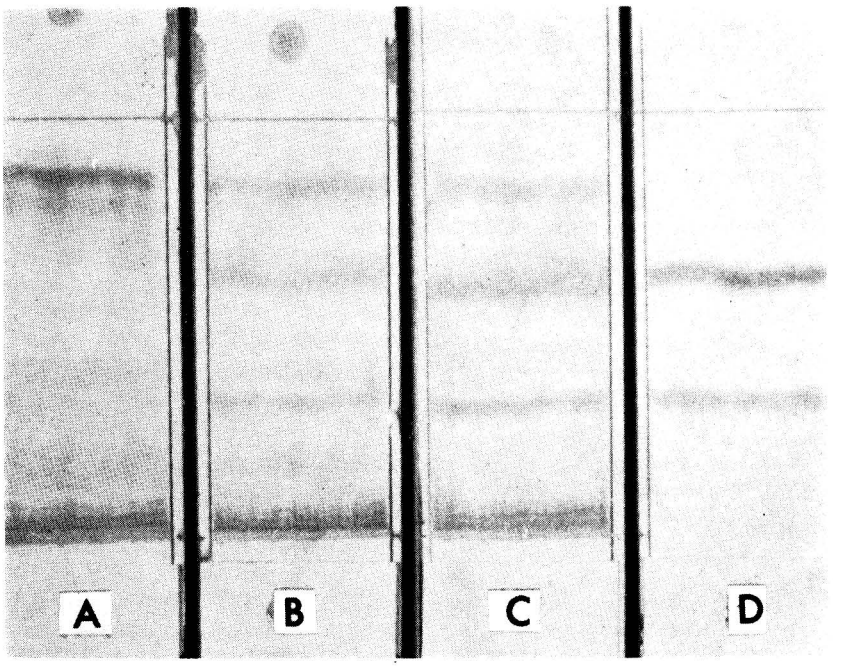


*Fig. 2*—Four sprayed 2 × 8″ thin-layer plates illustrating: *A*, 100 g green tomato control; *B*, 0.16 ppm green tomato recovery; *C*, 0.32 ppm green tomato recovery; *D*, 32 μg of dimethoate and oxygen analog.

hexane is clear and colorless (usually 5 or 6 hexane extractions are necessary). (To avoid emulsion formation, it is very important to agitate the two phases gently by carefully inverting the separatory funnel several times for at least the first two hexane extractions. The separatory funnel should not be shaken vigorously until the third hexane extraction and then only for 20–30 seconds.)

Transfer the water phase to a 1 L round-bottom flask and remove traces of dissolved hexane on a rotating evaporator with a good vacuum source until bubbles no longer persist. The volume of water at this point should be 75–80 ml; to this add 40 g sodium sulfate decahydrate. Heat gently to aid solution of the sodium sulfate and cool to room temperature before transferring to a clean 500 ml separatory funnel. Extract the water phase with three 100 ml portions of chloroform, shaking for 30 seconds each time. (Occasionally, emulsions are encountered in the chloroform phase, but stirring with a glass rod helps break them. It is important not to drain off, with the chloroform, any of the emulsion at the interface which usually persists at this point.) Concentrate the combined chloroform phases to 50 ml in a 1 L round-bottom flask on a rotating evaporator. Transfer this concentrate with a pipet to a 100 ml acetylization flask and concentrate to 100–200 μl, being careful that the residue does not go to dryness. Using a 100 μl micropipet and a suitable pipet control, apply the entire concentrate as a row of small overlapping spots to form a band less than 0.5 cm wide and 3 cm from the end of a previously prepared 2 × 8″ thin-layer plate. Place a paper curtain wet with solvent and approximately 30 ml acetone-chloroform (75/25) into the development chamber at least 15 minutes prior to development to insure chamber saturation with solvent vapors. Develop the plate with the acetone-chloroform solvent to the previously scored front 10 cm past the origin. Remove the plate from the solvent chamber and let it dry at room temperature. Spray the plate rather heavily with alcoholic palladium chloride (until the fluorescent indicator is just barely visible under a short-wave ultraviolet light), and let it air-dry and stand overnight for good color development of the oxygen analog band. (Although the colored band due to the presence of dimethoate appears immediately upon spraying, the oxygen analog appears very slowly over a period of several hours. Heating for 10 minutes at 70° C causes immediate formation of the color due to the presence of the oxygen analog, but re-

sults in small losses of both compounds as subsequently measured by the total phosphorus determination.)

Scrape the respective bands for dimethoate (5.0–6.5 cm from the origin) and oxygen analog (2.5–4.0 cm from the origin) onto 15 ml medium-porosity Büchner funnels set up to draw the eluted material directly into 18 × 150 mm borosilicate test tubes. Elute each compound from its corresponding silica gel band by adding 2.0 ml 1*N* nitric acid to each fritted funnel, swirling the funnel gently, and letting it stand 2 minutes. Apply vacuum and draw the nitric acid into the test tube. Wash the silica gel with two additional 1 ml portions of the nitric acid, letting each remain in contact with the silica for 2 minutes before drawing it off. To the combined nitric acid eluates (4 ml total) add 1 drop propylene glycol; then proceed from that point in the calibration curve preparation, beginning "Chill momentarily in an ice bath, add 0.5 ml 70–72% $HClO_4$," and continue through measurement of the absorbances of each solution at 820 mμ.

Correct the $A_{820}$ of the samples for the value obtained on an untreated crop sample carried through the entire procedure, including scraping and extracting areas from the thin-layer plate corresponding to the position of the dimethoate and oxygen analog spots. Read μg dimethoate or oxygen analog from the corresponding previously prepared calibration curves.

*Alfalfa.*—Macerate a representative 100 g crop sample with 250 ml chloroform, add 40 g sodium sulfate decahydrate, and remacerate. Let the macerate stand 30 minutes with occasional agitation. Filter the resulting extract with vacuum through a medium-porosity fritted disc covered with ¼″ of diatomaceous earth and measure the volume of chloroform recovered (usually 85–90%). Separate and discard the few ml of water usually remaining with the chloroform phase, using a separatory funnel. Add 2 g Nuchar C-190-N to the chloroform phase and stir occasionally for 15 minutes. Filter the chloroform through a layer of diatomaceous earth on a fritted funnel. Concentrate the chloroform phase in a 1 L round-bottom flask on a rotating evaporator at room temperature to near dryness (approximately 1 ml). Dissolve the residue in 50 ml *n*-hexane and transfer to a 500 ml separatory funnel. Wash the flask with several small portions of *n*-hexane, being careful that the combined volume of the hexane does not exceed 75 ml. Extract the hexane with 75 ml water by shaking 1 minute. Let the phases separate and draw off the water phase into a 250 ml beaker. Re-extract the hexane with 50 ml water, combine the water phases, and add 50 g $Na_2SO_4.10H_2O$. Proceed from that point in the extraction method for apples and green tomatoes, beginning "Heat gently to aid solution of the sodium sulfate," and continue through the procedure as described, except extract the aqueous sodium sulfate solution with three 150 ml portions of chloroform instead of the specified three 100 ml portions. Correct μg of both compounds measured for % chloroform recovered after the initial filtration step.

## Results and Discussion

The apparent values for both compounds in untreated samples obtained for the two crops analyzed were very low (Table 1) and, in fact, the only area of the developed thin-layer plates that contained any measurable amount of phosphorus was at the origin. This includes phosphorus-containing plant material as well as trace phosphorus-containing impurities found in occasional batches of chloroform. The recovery values in Table 2 were obtained by adding the compounds as an acetone solution to the respective crops before maceration. (See also Fig. 2.)

The recovery values reported for apples

**Table 1. Apparent values for dimethoate insecticide and its oxygen analog in untreated crops (100 g sample analyzed)**

| Crop | Apparent ppm Dimethoate | Apparent ppm Oxygen Analog |
|---|---|---|
| Apples | 0.018[a] | 0.017[a] |
| | 0.027[a] | 0.023[a] |
| | 0.022[a] | 0.022[a] |
| | 0.034 | 0.039 |
| | 0.033 | 0.020 |
| | 0.034 | 0.032 |
| Green tomatoes | 0.031 | 0.029 |
| | 0.028 | 0.041 |
| | 0.020 | 0.024 |
| Alfalfa | 0.025 | 0.039 |
| | 0.022 | 0.038 |
| | 0.027 | 0.019 |
| | 0.027 | 0.038 |
| | 0.030 | 0.031 |

[a] Silica gel band eluted with acetone/water (90/10) instead of 1*N* $HNO_3$.

**Table 2. Recovery values for dimethoate insecticide and its oxygen analog**

| Insecticide | ppm Added | ppm Recovered | % Recovery |
|---|---|---|---|
| Apples (100 g samples) | | | |
| Dimethoate[a] | 0.40 | 0.24 | 60 |
| | 0.40 | 0.23 | 58 |
| | 0.64 | 0.37 | 58 |
| | 0.64 | 0.36 | 56 |
| | 0.80 | 0.51 | 64 |
| Oxygen analog[a] | 0.40 | 0.23 | 58 |
| | 0.40 | 0.25 | 62 |
| | 0.64 | 0.35 | 55 |
| | 0.80 | 0.55 | 69 |
| | 0.80 | 0.58 | 72 |
| Green Tomatoes (100 g samples) | | | |
| Dimethoate | 0.16 | 0.11 | 69 |
| | 0.16 | 0.12 | 75 |
| | 0.32 | 0.21 | 66 |
| | 0.32 | 0.23 | 72 |
| Oxygen analog | 0.16 | 0.14 | 88 |
| | 0.16 | 0.15 | 94 |
| | 0.32 | 0.26 | 81 |
| | 0.32 | 0.30 | 94 |
| Alfalfa (100 g samples) | | | |
| Dimethoate | 0.19 | 0.16 | 84 |
| | 0.19 | 0.15 | 79 |
| | 0.32 | 0.25 | 78 |
| | 0.32 | 0.24 | 75 |
| | 0.34 | 0.22 | 65 |
| | 0.34 | 0.25 | 74 |
| | 0.34 | 0.21 | 62 |
| Oxygen analog | 0.16 | 0.14 | 87 |
| | 0.16 | 0.14 | 87 |
| | 0.32 | 0.23 | 72 |
| | 0.32 | 0.25 | 78 |
| | 0.32 | 0.24 | 75 |
| | 0.40 | 0.30 | 75 |
| | 0.40 | 0.29 | 72 |

[a] Silica gel eluted with acetone/water (90/10).

were obtained by using acetone/water (90/10) to elute the compounds from the silica gel. Critical evaluation of the various steps in the procedure showed that elution with this solvent resulted in a loss of approximately 10%. Elution with 1*N* $HNO_3$ followed by direct application of the perchloric acid digestion resulted in essentially complete recovery of both compounds from the thin-layer plates. Subsequent analysis of untreated apples for apparent dimethoate and oxygen analog content by utilizing the 1*N* $HNO_3$ elutant showed control values as low as those obtained with acetone/water. This modification should result in recoveries from apples about 10% higher than those reported in Table 1.

Although no effort was made to determine the lower limit of sensitivity for this method, the low control values (apparent value of 0.03 ppm; Table 1) indicate that residues as low as 0.06 ppm would be easily determinable. However, the palladium spray (modified from that of Bäumler and Rippstein (8)) is not sensitive enough for visual detection below approximately 12 $\mu$g per 100 g sample. In our laboratories, the reproducibility of $R_f$'s of both compounds from plate to plate was excellent, and we consider visual detection on the sample plate unnecessary provided standards are run on a separate plate from the same batch. This is particularly true since the specified band width to be scraped (1.5 cm) includes an area 4 mm wide on either side of the visible band and should be adequate to allow for minor band distortion.

An alternative procedure involves incorporation of internal standards. Just prior to application to the chromatographic plate, the concentrate is accurately divided into two portions, and known amounts of each compound are added to one portion. The two portions are then quantitatively streaked and developed side-by-side on a chromatographic plate prepared as previously described, except that the silica gel layer is divided lengthwise into two strips by a central score mark through the silica gel. This procedure results in a two-fold loss in sensitivity but permits location and scraping of desired bands with certainty when the amount of compounds present in the sample is less than that amount detectable by the palladium spray.

With reference to the perchloric acid digestion, it should be remembered that although the aqueous commercial material

(70–72% $HClO_4$) is as safe to handle as any strong acid, the anhydrous acid, on the other hand, which is formed on heating in the range of 180–220°C, will produce violent explosions when brought into contact with organic or other oxidizable materials. Even the vapors, at elevated temperatures, will produce violent explosions on coming into momentary contact with such substances as rubber or cork stoppers, rubber or cotton gloves, organic spills, or residues on or in hood floors and hood ducts, or organic spills on dirty beakers or test tube tongs. Digestions should be performed behind a safety shield and in a clean, well-maintained hood; the operator should wear safety glasses. On the other hand, anhydrous perchloric acid is perfectly stable in the absence of reducing materials, and the procedure when properly performed is not dangerous. See, e.g., Sterges (11) on the perchloric acid digestion of raw plant tissue: "The prescribed prior saturation of the charge of plant tissue (or other organic material) with nitric acid and the low initial temperature of the digestion have led to no explosions in the making of more than 3,800 such digestions in this laboratory." The degree of hazard involved is primarily a function of the care and cleanliness maintained by the individual operator.

The sample of oxygen analog utilized for this work was prepared chromatographically pure by the procedure of Patchett and Batchelder (12). The chromatographic column was prepared by slurrying 50 g of 28–200 mesh silica gel (Fisher) with 150 ml of chloroform and pouring the slurry into a 24 × 1″ column fitted with a sintered glass disc covered with a plug of sodium sulfate. After excess chloroform was drained off, 3 g of the crude sample in 50 g of chloroform was added to the top of the column. Chloroform, ethyl acetate, and acetone were used in the same respective ratios as those listed for hexane, chloroform, and acetone in the Patchett article through the first fifteen fractions (75 ml of each collected). Fraction 16 consisted of 75 ml of ethyl acetate/acetone (30/70) and fractions 17–19, 75 ml of acetone. Thin-layer chromatographic examination of the eluate revealed that fractions 16, 17, and 18 contained pure oxygen analog and totaled 2.1 g. Fractions 15 and 19 contained the bulk of the impurities in addition to quantities of the oxygen analog.

The high degree of specificity afforded by thin-layer chromatography, coupled with the nonspecific microphosphorus method, has proved a particularly valuable tool where simultaneous determination of parent compound and metabolites is required. The extensive data on thin-layer chromatography of pesticides published by Walker and Beroza (7) provides an excellent starting point for the development of specific procedures of this type. The approach described here should be applicable with minor modifications to the determination of microgram quantities of other organophosphorus compounds in a variety of crops.

**Acknowledgment**

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# Determination of Chlorinated Pesticide Residues in Fat by Electron Capture Gas Chromatography

By K. A. McCULLY and W. P. McKINLEY (Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada)

**A general screening method has been developed for the cleanup and estimation of chlorinated pesticide residues in fats and oils. The fats were dissolved in benzene-acetone (1 + 19) and then precipitated at −70°C. The precipitated fat was removed by filtration through a column of Darco G-60 and Solka Floc in a jacketed filtering funnel. The filtrate was concentrated to a standard volume and an aliquot was analyzed by electron capture gas chromatography. With the exception of a few pesticides in butterfat and aldrin in beef fat, recoveries were 81 to 112% after a mixture of 12 chlorinated pesticides had been added to the fats prior to precipitation.**

**A Pyrex gas chromatographic column containing a 10% stationary phase of mixed silicones (4% SE-30 methyl silicone + 6% QF-1 fluoro silicone) was used to resolve this pesticide mixture. The compounds eluted from the column in the following order: lindane, heptachlor, aldrin, Telodrin, heptachlor epoxide, *p,p'*-DDE, dieldrin, *o,p'*-DDT, Rhothane, *p,p'*-DDT, endrin, and methoxychlor.**

The determination of chlorinated pesticide residues in fats and oils is complicated in that the plant or animal may have been subjected to several pesticides. This is especially true of fats from animals that have ingested a food supply that may have come from many sources and may consist of a variety of vegetation. Therefore, a screening method must be able to distinguish and estimate a variety of chlorinated pesticides.

Recent important advances in pesticide residue analysis have been made by using gas-liquid chromatography with electron capture ionization detection as developed by Lovelock and Lipsky (1) and by Lovelock (2). Lovelock (3) has described the nature and the cause of some of the erroneous and anomalous responses that the electron capture detector may generate.

Since the first investigations by Goodwin, *et al.* (4, 5) of the use of electron capture gas chromatography to determine chlorinated pesticide residues, other workers (6–16) have applied the technique to a variety of pesticide residue problems. These investigations have been limited by incomplete separation of some chlorinated pesticides on the stationary phases used in the gas chromatographic columns. For example, Watts and Klein (6) reported that *p,p'*-DDE and dieldrin overlap, as do *o,p'*-DDT and DDD. Some recent work (15, 17) on the separation and identification of chlorinated pesticides has been carried out with stationary phases of different characteristics.

The preparation and cleanup of sample extracts prior to gas chromatography has ranged from preparing simple hexane solutions from acetone-water extracts of plant tissues to partitioning and column chromatography on Florisil for fats and oils. The Florisil column procedure is complicated because of the difficulties in standardizing the Florisil and eluting all the pesticides off the column without also removing impurities that may interfere with the final determination. Saponification is useful as a cleanup procedure in specific cases. However, not all pesticides are stable in alkali and the true residue picture may not be obtained.

In this laboratory considerable success has been achieved with the acetone precipitation of fat at −70°C as a cleanup procedure (18). Successful gas chromatograms were obtained from butterfat extracts cleaned up by the cold column procedure for

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dieldrin (19). This method was designed for dieldrin analyses only, and is rather laborious. In view of the promising results obtained, attempts were made to simplify and modify the procedure to make it applicable to as wide a range of fats and to as great a number of pesticides as possible.

A gas chromatographic column that will separate 12 of the common chlorinated pesticides and a cleanup procedure that is applicable to the determination of residues of these pesticides in fats and oils is described in this paper.

## METHOD

### *Apparatus*

(a) *Gas chromatograph.* — A Jarrell-Ash Model 700 Universal Chromatograph equipped with an electron affinity detector was used.

*Columns:* U-shaped Pyrex glass, 8 mm o.d. × 6 mm i.d. × 4 ft long, packed with 10% stationary phase (4% G.E. SE-30 methyl silicone + 6% D.C. QF-1 (FS-1265) fluoro silicone) on 60/80 mesh acid-washed Chromosorb W. To prepare packing, dissolve in ethyl acetate the appropriate quantities of SE-30 silicone and QF-1 silicone to give 4% by weight of SE-30 silicone and 6% by weight of QF-1 silicone on Chromosorb W. Add the Chromosorb W to the dissolved silicones to form a slurry, and remove the solvent under a stream of nitrogen with continuous stirring during the evaporation. After packing, precondition the column for at least 60 hr at 225°C with a small flow of nitrogen through the column. *Operating parameters:* column temp., 175°C; detector temp., 200°C; injector flash heater, 220°C; detector voltage, 17.5 v. Carrier gas, pre-purified nitrogen; flow rate, approximately 180 ml/min.

(b) *Cold bath.*—See Fig. 1. Tank, 7 × 36 × 12″ deep, made of plywood and insulated with sheet cork; lined, both inside and outside, with sheet stainless steel. Circulating pump (Wilkens-Anderson Co., Chicago) attached to cover at one end of box with motor above and pump below cover to keep solvent vapors away from motor. Pump outlet connected to first filtering funnel with tygon tubing, also used to connect 5 filtering funnels and for return to bath. Methanol was used as circulating cooling liquid instead of acetone, which attacks most types of pumps more readily than methanol. Bath filled about 8″ deep with methanol and cooled to between −70° and −78°C by adding Dry Ice directly to the methanol. Excess of solid chunks of Dry Ice was left in tank during operation. Frame around outside of bath holds stirring motor and the stirrers. Stainless steel stirrers, belt driven by motor attached to one end of frame. Clamps attached to frame to hold 1000 ml wide-mouth Erlenmeyer flasks.

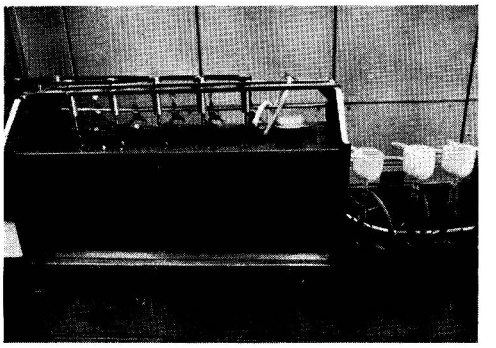

*Fig. 1*—Cold bath with stirring assembly, pump, and wooden inset rack.

(c) *Filtering funnels.*—Jacketed, 150 ml with medium porosity fritted discs; fitted with ⅌ vacuum adapters.

(d) *Flash evaporator.*—(Buchler Instruments, N.Y.)

### *Reagents*

(a) *Benzene-acetone (1 + 19).*—Dilute 1 ml redistilled benzene to 20 ml with redistilled acetone.

(b) *Wood cellulose.*—Shake Solka Floc BW 40 (Brown Co., Boston, Mass.) for 8 hr on mechanical shaker with excess of redistilled acetone. Pour off excess acetone and re-extract residue twice, once with excess of redistilled acetone and once with excess of the benzene-acetone (1 + 19). Dry residue in open pan in hood.

(c) *Charcoal.*—Darco G-60 (distributed by Brickman and Co., Montreal, and manufactured by Atlas Powder Co., Wilmington, Del.); purify like Solka Floc. After drying, heat overnight at 285°C.

(d) *Standard solution.*—Acetone solution of lindane, 0.2 μg/ml; heptachlor, 0.2 μg/ml; aldrin, 0.2 μg/ml; Telodrin,[1] 0.2 μg/ml; heptachlor epoxide, 0.2 μg/ml; *p,p'*-DDE, 0.2 μg/ml; dieldrin, 0.2 μg/ml; Rhothane (DDD, TDE), 1.0 μg/ml; *o,p'*-DDT, 2.0 μg/ml; *p,p'*-DDT, 2.0 μg/ml; endrin, 2.0 μg/ml; and methoxychlor, 6.0 μg/ml.

[1] Telodrin is the Shell trade mark name for 1,3,4,5,6,7,8,8-octachloro-1,3,3a,4,7,7a-hexahydro-4,7-methanoisobenzofuran.

### *Sample Preparation*

(a) *Vegetable oils.*—Dissolve 5 g oil in 250 ml benzene-acetone (1 + 19).

(b) *Butter.*—Cut butter sample into cubes and place in beaker in oven at 60°C. As soon as fat melts and separates into layers, decant off oily layer and filter through Whatman No. 1 filter paper. Leave sample in oven during filtration to hasten filtration rate. Dissolve 5 g of the butter oil in 250 ml benzene-acetone (1 + 19).

(c) *Animal fats.*—Blend 5 g fat, 30 g anhydrous sodium sulfate, and 75 ml benzene-acetone (1 + 19) for about 3 min. in a Waring Blendor. Filter mixture through jacketed filtering funnel with medium porosity fritted disc. Circulate hot tap water through funnel during filtration to keep fat in solution. Wash Blendor and filtering funnel with benzene-acetone (1 + 19) until about 250 ml filtrate has been collected.

### *Precipitation*

Cool cold bath to between −70° and −78°C with Dry Ice. Slurry 2 g Darco G-60 and 10 g Solka Floc with benzene-acetone (1 + 19) for each of the filtering funnels, and transfer to the funnels. Circulate cold methanol from the bath through the jacketed funnels. Place Erlenmeyer flasks containing dissolved fat samples in cold bath and adjust stirrers in place. Stir mixtures continuously during cooling and precipitation of fat. After temperature of contents reaches −70°C, leave flasks in bath for additional 20 min. Filter mixture through Darco G-60/Solka Floc in jacketed funnels. Wash flask once and precipitate twice with precooled benzene-acetone (1 + 19). Let filtrate dry over anhydrous sodium sulfate, and evaporate solvent to 1–2 ml in a flash evaporator at 38°C. Transfer this solution to a graduated flask with five 3 ml portions of acetone and reduce volume to 2 ml under a stream of nitrogen.

### *Gas Chromatography*

Inject a 0.004 ml aliquot of the sample extract into the gas chromatograph. Compare the resulting chromatogram with standards that were run on the same day.

In this work, peak heights were used for quantitative measurements of the pesticides. An aliquot of the standard solution was injected into the gas chromatograph several times each day to check retention times and to check the peak response for the pesticides.

### *Quantitative Recoveries*

Recovery experiments were carried out by adding 2 ml of the standard solution to the

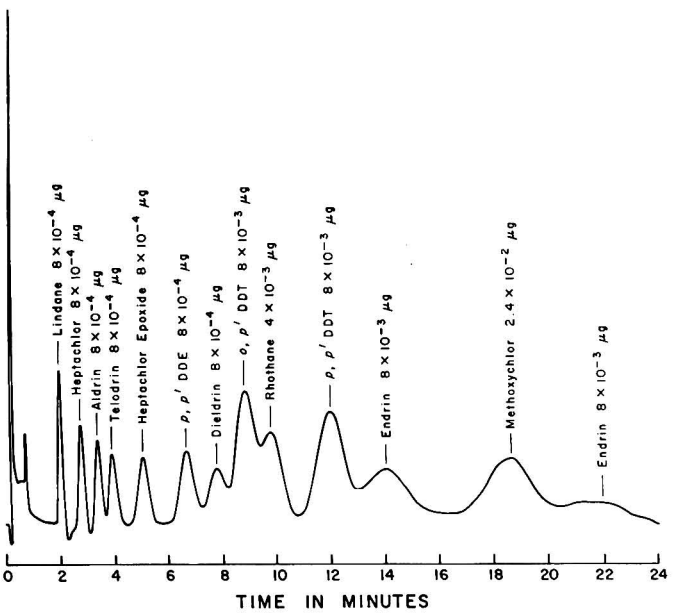


*Fig. 2*—Gas chromatogram of a 12-component chlorinated pesticide mixture. A 0.004 ml aliquot of the standard solution was chromatographed. Column: 10% stationary phase (4% SE-30 + 6% QF-1) on 60/80 mesh acid-washed Chromosorb W. Column temperature: 175°C. Detector temperature: 200°C. Injector flash heater temperature: 220°C. Nitrogen flow: 180 ml/min.

fat solution before precipitation of the fat. In the case of the animal fats the standard solution was added to the fat sample in the Waring Blendor prior to blending.

### Results and Discussion

A typical gas chromatogram of the standard solution, Fig. 2, shows the resolution of 12 common chlorinated pesticides on the 4% SE-30 + 6% QF-1 column. A 0.004 ml aliquot of the standard solution was chromatographed. Under the conditions of gas chromatography employed, endrin isomerized on the column to give two peaks, a result that is in agreement with previous work (10, 20). The relative retention times of some of the chlorinated pesticides, calculated relative to aldrin as unity, are given in Table 1. Some compounds were included in this list for reference but were not studied further.

After experiments with other stationary phases, 4% SE-30 + 6% QF-1 on Chromosorb W was selected as column packing. This packing gave better resolution of *p,p'*-DDE and dieldrin and of *o,p'*-DDT and Rhothane than did the 5% SE-30 + 5% QF-1 column. Although the 3% SE-30 + 7% QF-1 column gave even better resolution of these two pairs of compounds than the other two columns, it yielded poor resolution of dieldrin and *o,p'*-DDT. The 4% SE-30 + 6% QF-1 column gave the best resolution that we have achieved, although it did not completely separate all the chlorinated pesticides.

**Table 1. Relative retention times of chlorinated pesticides on 4% SE-30 silicone + 6% QF-1 silicone columns**

| Pesticide | Relative Retention Time |
|---|---|
| BHC | 0.45, 0.57, 0.68 |
| Lindane | 0.57 |
| Heptachlor | 0.81 |
| Aldrin | 1.00 |
| Telodrin | 1.15 |
| Kelthane | 1.38 |
| Heptachlor epoxide | 1.50 |
| Thiodan | 1.92, 3.07 |
| *p,p'*-DDE | 1.98 |
| Perthane | 2.13 |
| Dieldrin | 2.31 |
| *o,p'*-DDT | 2.62 |
| Rhothane | 2.89 |
| *p,p'*-DDT | 3.54 |
| Endrin | 4.13, 6.38 |
| Methoxychlor | 5.44 |
| Tedion | 9.79 |

A few reports have appeared in the literature on the presence of breakdown products and metabolites of chlorinated pesticides. Cueto and Hayes (21) have shown the existence of dieldrin metabolites in human urine. Korte, *et al.* (22) have shown that aldrin and dieldrin are metabolized by mosquito larvae, by cultures of some microorganisms, and by rats to give up to four transformation products. Gas chromatographic analysis of samples of dieldrin-treated herbage has revealed the presence of an unknown peak in addition to that of dieldrin (23). A similar substance was formed when aldrin and dieldrin were subjected to ultraviolet light. Ultraviolet light also converted endrin, *p,p'*-DDE, *p,p'*-DDT, and *p,p'*-TDE to various reaction products. Although the behavior of metabolites on the 4% SE-30 + 6% QF-1 column is not known, it should be emphasized that extreme care must be taken in interpreting gas chromatograms from samples of unknown spray history and from samples in which the presence of breakdown products or metabolites cannot be excluded.

When this work was initiated, we attempted to precipitate the fat from acetone solution in the cold and to remove the precipitated fat on the jacketed funnels without any column material. Corn oil and butterfat were used for the preliminary work. Relatively good cleanup was achieved without any column material on the filter. The purification was approximately 99% for corn oil and 95–96% for butterfat. However, enough material remained in the sample extract to contaminate the electron capture detector and result in a rapid loss of sensitivity.

Darco G-60/Solka Floc columns have been used successfully in this laboratory for the analysis of butterfat for dieldrin residues (19). It was found that a column of 2 g Darco G-60 and 10 g Solka Floc on the jacketed funnel was sufficient to give good cleanup of a 5 g sample of fat. When acetone was used as the solvent and the Darco

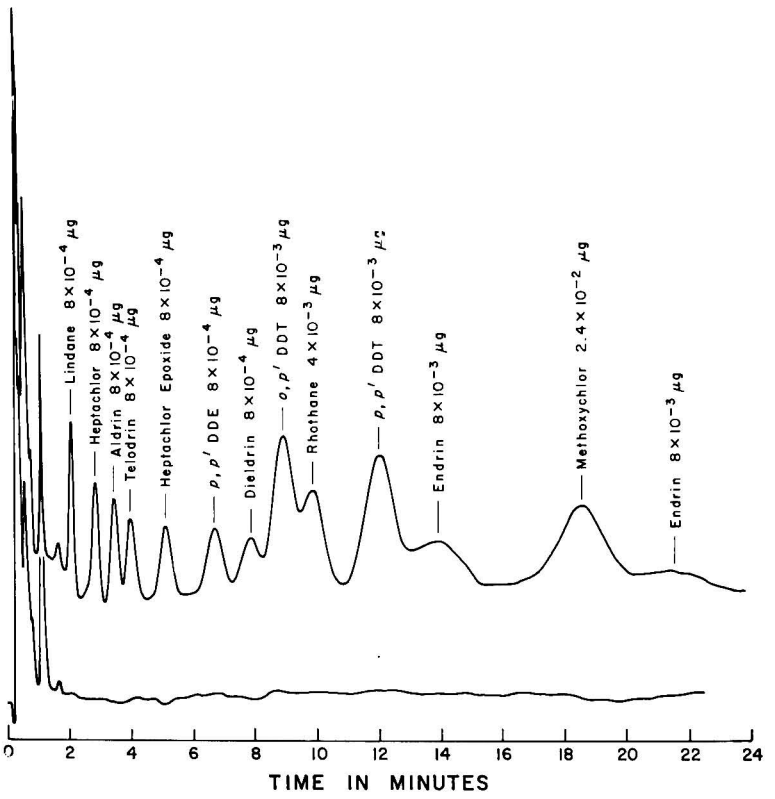


*Fig. 3*—Gas chromatograms of unfortified (lower) and fortified (upper) samples of corn oil. In both cases the extract from a 5 g sample of oil was made to 2 ml and a 0.004 ml aliquot was analyzed. Column and other conditions are the same as in Fig. 2.

G-60/Solka Floc column was used, excellent purification (99.97%) of the corn oil was obtained. These extracts did not contaminate the electron capture detector. However, recoveries for fortified samples were low and ranged from 50% for lindane to 92% for Rhothane.

Good recoveries of pesticides added to corn oil were obtained when a hexane-acetone (1 + 19) mixture was used. The recoveries ranged from 78% for lindane to 98% for Rhothane. The purification decreased slightly to 99%. However, enough material remained in the extract to cause detector contamination and loss of sensitivity.

Benzene-acetone mixtures (1 + 9) and (1 + 19) were tried and gave successful results. Good cleanup was achieved and the detector did not become contaminated. Benzene-acetone (1 + 19) was found to be better (equal recoveries and slightly better purification) than the benzene-acetone (1 + 9).

**Table 2. Percentage[a] purification of fats and oils**

| Fat or Oil | Percentage Purification |
|---|---|
| Corn oil | 99.93 (10)[b] |
| Cottonseed oil | 99.88 (4) |
| Rapeseed oil | 99.94 (7) |
| Butterfat | 97.43 (11) |
| Margarine | 99.87 (4) |
| Beef fat | 99.93 (5) |
| Mutton fat | 99.90 (10) |
| Pork fat | 99.91 (1) |
| Chicken fat | 99.60 (1) |
| Turkey fat | 99.96 (1) |
| "Commercial" shortening | 99.10 (1) |
| "Commercial" lard | 99.96 (1) |

[a] Percentage = 100 − (wt residue/wt sample) × 100.
[b] The numbers in parentheses refer to the number of determinations.

After experiments with different volumes of solvent we found that 250 ml of solvent was the most useful volume for cleanup and recovery of the pesticides. In this work 5 g samples of fat were dissolved in 250 ml of benzene-acetone (1 + 19) and the precipitated fat was removed on a column of 2 g of Darco G-60 and 10 g of Solka Floc.

The chromatograms of an unfortified and a fortified sample of corn oil are shown in Fig. 3. The lower chromatogram from the unfortified sample shows a constant baseline free from any interfering peaks. This indicates that substances which may interfere with the gas chromatographic analysis have been removed by the cleanup procedure used. The upper chromatogram (Fig. 3) is the curve obtained from the sample that was fortified with 2 ml of the standard solution prior to precipitation of the fat. In both cases the extract from 5 g of fat was made to 2 ml and a 0.004 ml aliquot was injected into the gas chromatograph. The separation of the pesticides and the peak response of the individual pesticides in the upper chromatogram of Fig. 3 are the same as those shown in the chromatogram of the standard solution (Fig. 2). These results show that the 12 chlorinated pesticides of the standard solution, added to the fat sample prior to precipitation, are recovered quantitatively and unchanged.

The precipitation of fat in the cold gave good cleanup of all the fats and oils investigated in this work. The percentage purifications obtained for these fats and oils are given in Table 2. The purifications were all above 99%, except butterfat, which was 97.43%. The poorer cleanup of butterfat was probably due to the low molecular weight glycerides that were not removed by cold precipitation.

On the basis of the 5 g sample of corn oil used (Fig. 3) the concentrations of pesticides present in the fortified oil were: lindane, 0.08 ppm; heptachlor, 0.08 ppm; aldrin, 0.08 ppm; Telodrin, 0.08 ppm; heptachlor epoxide, 0.08 ppm; *p,p'*-DDE, 0.08 ppm; dieldrin, 0.08 ppm; Rhothane, 0.4 ppm; *o,p'*-DDT, 0.8 ppm; *p,p'*-DDT, 0.8 ppm; endrin, 0.8 ppm; methoxychlor, 2.4 ppm.

The recoveries obtained for the 12 chlorinated pesticides added to fats and oils are shown in Table 3. Correction was made for any identifiable individual pesticides that were detected before fortification. The recoveries of the added pesticides ranged from 87 to 101% for corn oil; 88 to 105% for cottonseed oil; 83 to 98% for rapeseed oil; 51 to 91% for butterfat; 86 to 100% for margarine (a vegetable and marine oil margarine); 73 to 103% for beef fat; and 82 to 112% for mutton fat. In general the recoveries of the pesticides added to butterfat

**Table 3. Percentage recovery of pesticides added to fats and oils**

| Pesticide | Corn oil (10)[a] | Cottonseed oil (2) | Rapeseed oil (3) | Butterfat (3) | Margarine (2) | Beef fat (2) | Mutton fat (2) |
|---|---|---|---|---|---|---|---|
| Lindane | 88 | 89 | 83 | 62 | 87 | 81 | 82 |
| Heptachlor | 90 | 98 | 95 | 91 | 98 | 89 | 87 |
| Aldrin | 87 | 93 | 85 | 74 | 92 | 73 | 84 |
| Telodrin | 89 | 96 | 88 | 88 | 94 | 83 | 89 |
| Heptachlor epoxide | 87 | 98 | 85 | 83 | 94 | 86 | 88 |
| *p,p'*–DDE | 89 | 93 | 86 | 53 | 86 | 92 | 95 |
| Dieldrin | 92 | 97 | 90 | 72 | 92 | 101 | 99 |
| *o,p'*–DDT | 98 | 100 | 96 | 79 | 92 | 96 | 112 |
| Rhothane | 94 | 100 | 89 | 76 | 97 | 96 | 98 |
| *p,p'*–DDT | 101 | 105 | 96 | 79 | 92 | 103 | 102 |
| Endrin I[b] | 93 | 88 | 91 | 84 | 87 | 90 | 89 |
| Methoxychlor | 100 | 101 | 98 | 51 | 87 | 95 | — |
| Endrin II[b] | 97 | 93 | 90 | 86 | 100 | 92 | 96 |

[a] The numbers in parentheses refer to the number of determinations.
[b] Under the conditions of gas chromatography employed, endrin isomerizes on the column to give two peaks which are described here as endrin I and endrin II.

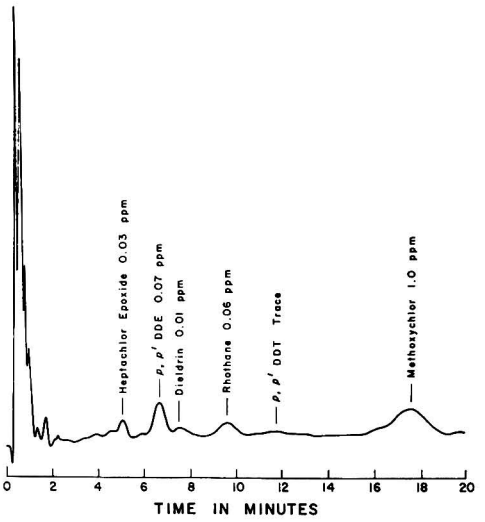


*Fig. 4*—Gas chromatogram of a butter sample. Column and other conditions are the same as in Fig. 2.

were somewhat lower than the recoveries from the other fats and oils. The lowest recoveries were for methoxychlor, *p,p'*-DDE, and lindane. No value is given for the recovery of methoxychlor from mutton fat because an unidentified substance or an artifact was present that had approximately the same retention time as methoxychlor. The unfortified mutton fat did not show this unknown peak. Further work is necessary in order to clarify the result.

Figures 4 and 5 demonstrate the application of this method to "unknown" samples. Figure 4 is the chromatogram obtained from a sample of butterfat and shows a very good baseline despite the poorer cleanup (97.43%) in comparison to the other fats and oils investigated. Figure 5 is the chromatogram obtained from a sample of turkey fat in which 7 pesticides were identified by electron capture gas chromatography.

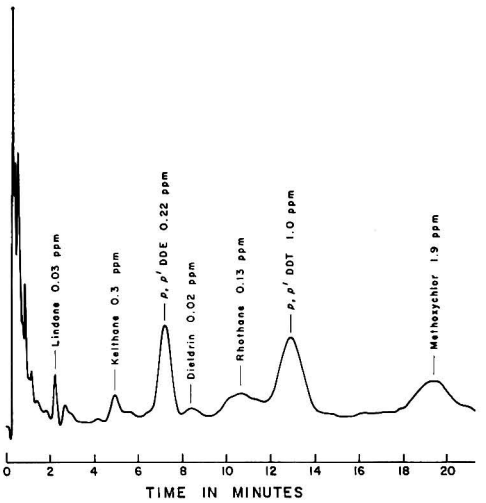


*Fig. 5*—Gas chromatogram of a sample of turkey fat. Column and other conditions are the same as in Fig. 2.

Further work is in progress to determine recoveries from fats and oils not included in this report. The behavior of breakdown products and metabolites of chlorinated pesticides on the 4% SE-30 + 6% QF-1 column will be studied also.

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# Cleanup Method for Electron Capture Determination of Endrin in Fatty Vegetables

By RICHARD A. ALBERT (Pattison's Laboratories, Inc., Harlingen, Texas)

**A method has been developed in which a KOH-Celite column is used instead of the longer saponification method for cleanup of vegetables, reducing the time of analysis by about 2 hours. The method involves fewer sample transfers, thereby increasing the per cent recovery. Good results were obtained with samples of carrots, onions, collards, and broccoli.**

The determination of endrin in carrots by electron capture determination involves certain fats that have the same retention time as endrin. The presence of large quantities of these fats masks both of the endrin peaks. When the concentration of endrin is less than 0.05 ppm it goes completely undetected. Because the usual method of saponification and elution through MgO-Celite mixture (1) is time consuming, a more rapid method of cleanup is needed when many samples are to be determined at one time.

It was found that KOH-Celite mixture sandwiched between MgO-Celite mixture gave satisfactory cleanup of these fats.

## METHOD

### *Reagents for Cleanup*

(a) *KOH-Celite mixture* (1 + 1).—Prepare enough for 1 day only. Pulverize KOH to about 60-mesh by a hammering action, rather than a grinding action, under conditions of low relative humidity. Transfer KOH and equal weight of Celite 545 to a large-mouth jar. Add 16%, by weight, of water as a fine mist, distributing it as evenly as possible. Close jar and mix by vigorous shaking. It is important that the moisture content does not fall below 14% or go over 17%. Under these mixing conditions, the carbon dioxide content will not exceed 1.50%.

(b) *MgO-Celite mixture.*—Place 200 g MgO Sea Sorb 43 in a large beaker and make a thin slurry. Heat this on a steam bath for 30 minutes. Filter with suction and dry overnight at 105°C. Pulverize to pass a 60-mesh screen. Mix MgO by equal weight with Celite 545.

(c) *Sodium sulfate.*—Anhydrous.

(d) *Petroleum ether.*—Redistilled.

### *Apparatus*

(a) *Chromatographic column.*—20 mm i.d. × 300 mm including 250 ml well at top; fitted with T-joint at bottom to receive Kuderna-Danish evaporator.

(b) *Chromatographic tube.*—Fischer-Porter No. 274-555, 20 mm i.d. × 400 mm, with Teflon stopcock.

(c) *Kuderna-Danish concentrator.*—Equipped with 15 ml receiving tube.

### *Determination*

(The method of extracting dieldrin and endrin from plant material is essentially the same as outlined by Johnson (2) except that 15 g Celite 545 is added at time of blending, and the mixture is filtered instead of centrifuged.)

Place 100 g plant material in Waring Blendor, add 15 g Celite 545 and 150 ml acetonitrile, and blend for 2 minutes at high speed. Filter through Buchner funnel. Rinse the material remaining in the blender into the Buchner funnel with an additional 30 ml acetonitrile. Press the pulp down with a stainless steel tamper. Release the vacuum and add an additional 70 ml acetonitrile. Allow about 1 minute for acetonitrile to penetrate pulp, again apply suction, and press with tamper until pulp appears dry.

Transfer the acetonitrile extract to a 1 L separatory funnel and rinse the suction flask

twice with 25 ml portions of petroleum ether. Add these rinsings to the separatory funnel together with an additional 50 ml petroleum ether. Shake the separatory funnel vigorously for 1 minute. Add 50 ml of a 30% sodium sulfate solution and 500 ml water. Mix this by shaking for 1 minute and let phases separate. Drain off the bottom layer and discard. Wash the petroleum ether layer with 2 more 200 ml portions of water. Transfer the ether layer quantitatively to a 250 ml beaker and concentrate almost to dryness. Add 1 g anhydrous sodium sulfate and 10 ml petroleum ether.

Prepare the chromatographic column (a) for elution of sample by adding 5 inches of activated Florisil and 1 inch of sodium sulfate. Pre-wet the column with 40 ml petroleum ether. When ether is about ½ inch from sodium sulfate, attach the Kuderna-Danish concentrator. Transfer entire sample to column and rinse beaker with small portions of petroleum ether. Let the sample just sink into the column; then add 200 ml 6% ethyl ether in petroleum ether. When the 6% ether reaches the top of the column, change receivers and add 200 ml 15% ethyl ether in petroleum ether. This fraction contains the dieldrin and endrin.

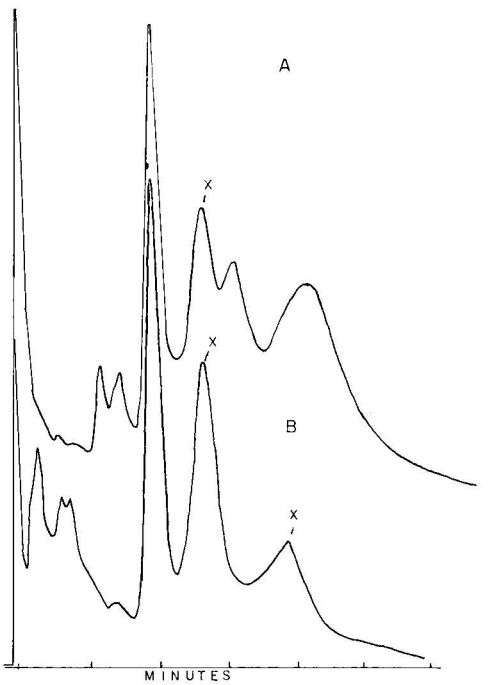


*Fig. 1*—Analysis of duplicate samples by A, elution through MgO-Celite, and B, elution through KOH-Celite. Endrin concentration, 0.07 ppm.

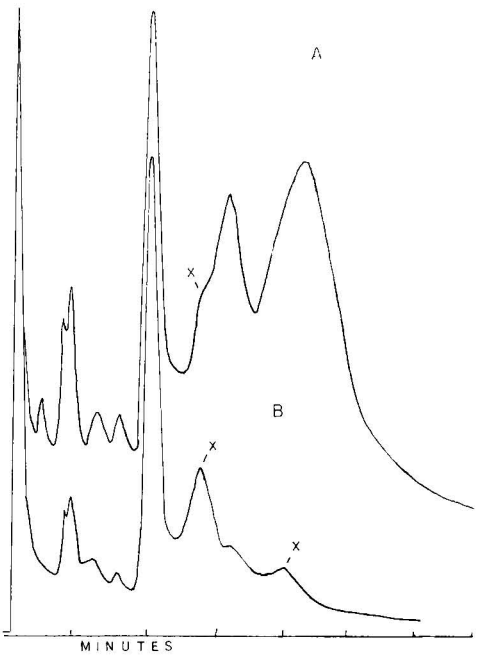


*Fig. 2*—Analysis of duplicate samples by A, elution through MgO-Celite, and B, elution through KOH-Celite. Endrin concentration, 0.025 ppm.

Concentrate this fraction to 5 ml, remove from steam bath, and let all condensate from the Snyder column and bulb drain into the receiving tube. The final volume will be about 15 ml.

Prepare cleanup chromatographic column (b) as follows: pour in enough MgO-Celite mixture to make 1 inch after packing, apply suction, and pack lightly; add ½ inch of anhydrous sodium sulfate and release vacuum, add enough KOH-Celite mixture to the column to make a 1½ inch packed column, apply vacuum, and pack firmly; add ½ inch of anhydrous sodium sulfate on top of this mixture and release vacuum. Place enough MgO-Celite on top of this to make 1½ inches after packing, apply vacuum, and pack lightly; then top

**Table 1. Total residue left after elution by two methods**

| Sample | KOH-Celite Column, mg | Saponification, mg |
|---|---|---|
| Carrots | 1.6 | 1.5 |
| Onions | 1.6 | 1.5 |
| Collards | 0.9 | 0.9 |
| Broccoli | 1.0 | 0.9 |

**Table 2. Recovery results for endrin by two methods[a]**

| Variety | Crop Blank, ppm | Endrin added, ppm | Endrin Recovered[b] KOH-Celite Column ppm | % | Saponification ppm | % |
|---|---|---|---|---|---|---|
| Carrots | 0.003 | 0.05 | 0.050 | 100 | 0.048 | 96.0 |
| Onions | 0.008 | 0.05 | 0.053 | 106 | 0.047 | 94.4 |
| Collards | 0.005 | 0.05 | 0.051 | 102 | 0.049 | 98.0 |
| Broccoli | 0.002 | 0.05 | 0.051 | 102 | 0.048 | 96.0 |

[a] Figures represent average results calculated from triplicates.
[b] Corrected for crop blank.

this with ½ inch of sodium sulfate. Pre-wet the column with 40 ml petroleum ether, using suction. Close the stopcock and place a Kuderna-Danish concentrator under the column.

Transfer sample to column and wash down sides of column with a small amount of petroleum ether. Open stopcock and apply 2½ lb of pressure to the column. As soon as the sample has sunk into the column, elute the column with 200 ml petroleum ether, using 2½ lb pressure.

Make a preliminary determination of how much ether is required to elute sample from column. The tests reported here required 150 ml to elute endrin; 200 ml was used to compensate for slight variations in column length.

Concentrate this fraction to exactly 5 ml and inject 1 $\mu$l directly into chromatograph from this tube.

In this work a Barber-Colman instrument was used. The column is 3 feet long packed with 2.5% S.F. 96. The detector is a 4072 radium diode. The operating conditions are as follows: Column temp., 215°C; injection port, 250°C; detector, 225°C; voltage, 16 volts; nitrogen, 25 pounds; chart speed, 1 inch per minute; sensitivity, 30; attenuation, 2×.

The area of the sample is related to the area of a nanogram standard. ppm Endrin = ng/mg sample per $\mu$l.

## Results

Figure 1 shows the results of analysis of duplicate samples. Chromatogram A is the result of elution through MgO-Celite mixture and chromatogram B is the result of elution through both MgO-Celite and KOH-Celite columns. The concentration of endrin in this sample was 0.07 ppm. Chromatogram B shows a well-defined endrin curve. All of the fats have been eliminated.

Figure 2 is a duplicate of the sample from Fig. 1, except that the endrin concentration is 0.025 ppm. The endrin peak in A is scarcely visible, whereas the endrin peak in B is very clear and definite. Both figures are actual chromatograms from samples of carrots that were not fortified with endrin.

Table 1 shows that the amount of residue left after elution through the KOH-Celite is essentially the same as that left by the saponification method. The results from Table 1 indicate that the KOH-Celite column will be satisfactory for paper chromatography as well as electron capture gas chromatography.

Table 2 presents the results obtained when samples of vegetables were fortified with 0.05 ppm endrin and the per cent of insecticide residue was determined by both the column and the saponification methods. The recovery is better by the column method, probably because the procedure involves fewer manipulations.

It was found through further tests that when the KOH-Celite mixture contained 19% water, some of the fats were allowed to pass through, so that the large endrin curve was slightly disfigured. When the water content was increased to 25%, the column became useless and the results were the same as when the KOH-Celite mixture was used dry. The water content is thus a critical factor in the use of this material.

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# Oxidative Metabolism and Persistence of Trithion on Field-Sprayed Lettuce

By D. E. COFFIN (Food and Drug Laboratories, Tunney's Pasture, Ottawa, Ontario, Canada)

**Trithion was applied to field-growing lettuce, and samples were removed at various times after spraying and analyzed for Trithion and its oxidative metabolites. Trithion persisted in growing lettuce for 21 days. Five oxidation products of Trithion appeared within 4 hours of spraying and persisted for up to 21 days. The results indicate that the principal route of oxidation of Trithion involves thioether oxidation to form the sulfoxide and then the sulfone, followed by phosphorothionate oxidation to form the thiolphosphate sulfone.**

Trithion, O,O-diethyl S-(*p*-chlorophenylthio) methyl phosphorodithioate, is an insecticide that exhibits moderately long residual activity. Trithion has five possible oxidation products, some or all of which might appear in growing plants as the result of Trithion application. These possible oxidation products (see Fig. 1) are O,O-diethyl S-(*p*-chlorophenylsulfinyl) methyl phosphorodithioate, O,O-diethyl S-(*p*-chlorophenylsulfonyl) methyl phosphorodithioate, O,O-diethyl S-(*p*-chlorophenylthio) methyl phosphorothioate, O,O-diethyl S-(*p*-chlorophenylsulfinyl) methyl phosphorothioate, and O,O-diethyl S-(*p*-chlorophenylsulfonyl) methyl phosphorothioate. In this paper these compounds will be referred to by the Stauffer Chemical Company designations of R 1988, R 1776, R 1472, R 1990, and R 1777, respectively.

The present work was undertaken, with lettuce growing under field conditions, to (*1*) develop procedures applicable to the determination of residues of Trithion and its oxidation products, (*2*) determine the extent of oxidation of Trithion on or in growing plants, and (*3*) measure the persistence of Trithion and its oxidation products. No attempts have been made to measure any of the hydrolytic degradation products of Trithion or its oxidation products.

## METHOD

### *Reagents*

(Redistill all solvents before use.)

(a) *Chromatographic system.*—Whatman No. 1 paper, 20 × 7″. Immobile phase: 4% v/v light mineral oil in peroxide-free ether. Mobile phase: acetonitrile + water (4 + 6, v/v).

(b) *Polyethylene coated alumina.*—(Kensington Scientific Corp., Berkeley, Calif.)

(c) *Magnesol.*—Industrial regular (Canada Colors and Chemicals Ltd., Toronto, Ont.).

(d) *Beef liver homogenate* (1).—Homogenize 1 g beef liver with 9 ml water. Dilute with 30 ml water.

(e) *Chromogenic reagent* (1).—Prepare just prior to use. (*1*) Dissolve 25 mg 1-naphthylacetate in 10 ml absolute ethanol. (*2*) Dissolve 50 mg Azoene Fast Blue RR salt in 20 ml water and add 20 ml BDH buffer (pH 7.0). Add solution (*1*) to solution (*2*).

(f) *Isobutyl-benzene.*—Mix equal volumes of isobutyl alcohol and thiophene-free benzene.

(g) *Molybdate reagent.*—Dissolve 25 g ammonium molybdate in 200 ml 10*N* $H_2SO_4$ and dilute to 500 ml with water.

(h) *Ethanolic sulfuric acid.*—Dissolve 20 ml concentrated $H_2SO_4$ in 480 ml absolute ethanol.

(i) *Stannous chloride.*—Dissolve 10 g $SnCl_2 \cdot 2H_2O$ in 25 ml concentrated HCl and store in a glass-stoppered brown bottle. Dilute 1 ml of this stock solution to 200 ml with 1.0*N* $H_2SO_4$ solution immediately prior to use.

(j) *p-Nitrophenol indicator.*—Dissolve 25 mg *p*-nitrophenol in 25 ml water.

(k) *Mercury-zinc amalgam* (2).

### *Apparatus*

(a) *Flash evaporator.*—(Buchler Instruments, N.Y.).

(b) *Chromatographic columns* (3).

(c) *Oxygen-flask ignitor.*—(Arthur H. Thomas Co., Philadelphia, Pa.).

### *Standards*

(a) *Organophosphates.*—0.01*M* solution of

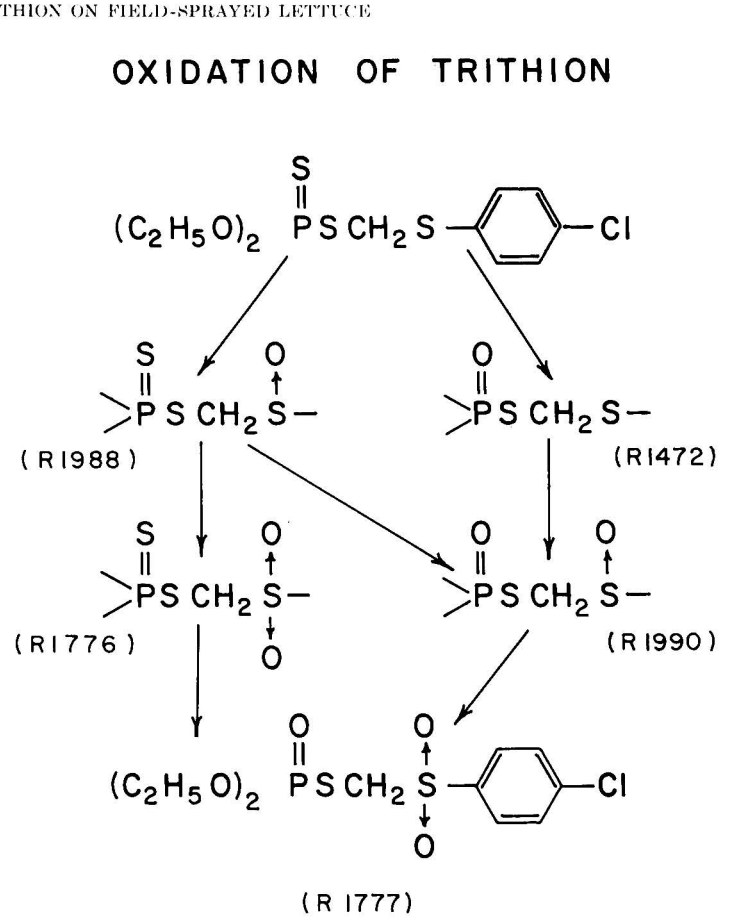


*Fig. 1*—The possible oxidation products of Trithion.

Trithion in chloroform and 1 mg/ml solutions of R 1472, R 1776, R 1777, R 1988, and R 1990 in benzene.

(b) *Phosphorus.*—Prepare from oven-dried $KH_2PO_4$ and dilute to give 1 μg phosphorus/ml.

### *Field Plots*

One plot of lettuce was sprayed with Trithion emulsifiable concentrate at the rate of 400 g active ingredient/acre. Representative samples were removed from the treated plot and a control plot at 4 hours and 1, 2, 3, 7, 10, 14, 21, and 28 days after spraying. These samples were stored at −20°C until the analyses were performed.

### *Extraction, Cleanup, and Determination*

Extract and clean up 50 g samples of lettuce by the procedure described by Coffin and McKinley (3). (This involves acetonitrile extraction, elution from polyethylene-coated alumina with 40% acetonitrile, partitioning from water into chloroform, and successive elution from magnesol with chloroform, acetone, and methanol.)

Concentrate suitable aliquots of the chloroform and methanol eluates and transfer to paper chromatograms. Develop the chromatograms until the solvent front advances 15″ from the point of sample application, dry, and use the enzymatic detection technique as described by McKinley and Johal (1) for identification of the organophosphate compounds. Cut areas corresponding to the organophosphates out of unsprayed chromatograms and estimate the amount of the organophosphate by determination of total phosphorus (3).

### *Reduction*

Since R 1472 and R 1988 were not separated by the paper chromatographic system, it was necessary to calculate the R 1988 content by difference after it was removed from solution (Table 2) by the following reduction technique:

In a small test tube evaporate an aliquot of

**Table 1. Recovery of standards of Trithion and its oxidation products after cleanup and paper chromatographic separation**

| Compound | Added (μg) | Recovered[a] | | | |
|---|---|---|---|---|---|
| | | From Solution | | From Lettuce | |
| | | μg | % | μg | % |
| Trithion | 58.6 | 47.2 | 81 | 47.3 | 81 |
| R 1472 | 34.4 | 29.9 | 87 | 29.7 | 86 |
| R 1988 | 49.0 | 49.7 | 102 | 48.5 | 99 |
| R 1990 | 35.9 | 37.6 | 105 | 34.8 | 97 |
| R 1776 | 30.0 | 31.4 | 105 | 29.9 | 100 |
| R 1777 | 62.3 | 61.6 | 99 | 60.2 | 96 |

[a] Each value is the average of 4 determinations.

the cleaned up chloroform extract to dryness. Dissolve the residue in 2 drops of glacial acetic acid. Add 1.5 ml water and transfer to a 0.5″ column containing a 6″ layer of mercury-zinc amalgam in 1*N* acetic acid. Rinse the tube twice with 2 ml portions of 1*N* acetic acid and transfer the washings to the column. Elute the sample through the column with 50 ml 1*N* acetic acid. Extract the eluate with four 20 ml portions of chloroform. Filter the chloroform extracts through anhydrous sodium sulfate and evaporate the chloroform on the flash evaporator at 35°C to 1–2 ml. Transfer the residue and dilute to 10 ml with chloroform. Concentrate an aliquot equivalent to that previously spotted and develop the paper chromatogram. After measuring the R 1472 content by determining total phosphorus on the appropriate spot, calculate the R 1988 by the difference between the value on the unreduced extract and this reduced extract.

## Results and Discussion

The possible oxidation products of Trithion are shown in Fig. 1. This figure illustrates that there are two separate sites in the molecule at which oxidation may take place to form as many as five oxidation products.

Known amounts of Trithion and its five possible oxidation products were subjected individually to the cleanup and separation procedures. The recoveries after cleanup and separation and the recoveries of these compounds from lettuce are shown in Table 1. These results demonstrate that the six compounds are recovered after cleanup in an unaltered form and that they are quantitatively recovered from lettuce by the procedures employed. Trithion and 4 of the 5 oxidation products were recovered in the chloroform eluate, and R 1990 was recovered in the methanol eluate for the magnesol column.

Known amounts of Trithion, R 1472, R 1776, R 1777, and R 1988 were subjected individually to the reduction procedure. The recoveries of these compounds after reduction and paper chromatography are shown in Table 2. This table shows that R 1472, R 1776, and R 1777 are recovered unaltered from the reduction procedure. Only small amounts of Trithion were recovered, apparently because of the insolubility of Trithion in the acetic acid solution; Trithion could be recovered quantitatively by washing the amalgam with acetone. R 1988 was not recovered after reduction, but an amount of Trithion equivalent to the R 1988 could be recovered. The R 1988 was quantitatively reduced to Trithion by mercury-zinc amalgam in 1*N* acetic acid.

The paper chromatographic separation of Trithion and its oxidation products is shown in Fig. 2 (A–D). This system provided adequate separation of Trithion, R 1776, and R 1777. R 1472 and R 1988 were separated from the other compounds but

**Table 2. Recovery of standards of Trithion and its oxidation products after reduction on a column of mercury-zinc amalgam**

| Compound | Added (μg) | Recovered[a] | |
|---|---|---|---|
| | | μg | % |
| R 1472 | 1.38 | 1.30 | 94 |
| | 3.44 | 3.26 | 95 |
| | 6.9 | 6.7 | 97 |
| | 17.2 | 16.8 | 98 |
| | 34.4 | 34.0 | 99 |
| R 1776 | 30.0 | 29.1 | 97 |
| R 1777 | 31.2 | 30.6 | 98 |
| R 1988 | 9.8 | 0.0 | 0 |
| | 49.0 | 0.0 | 0 |
| Trithion | 58.6 | Low and inconsistent (10–25%) | |

[a] Each value is the average of 4 determinations.

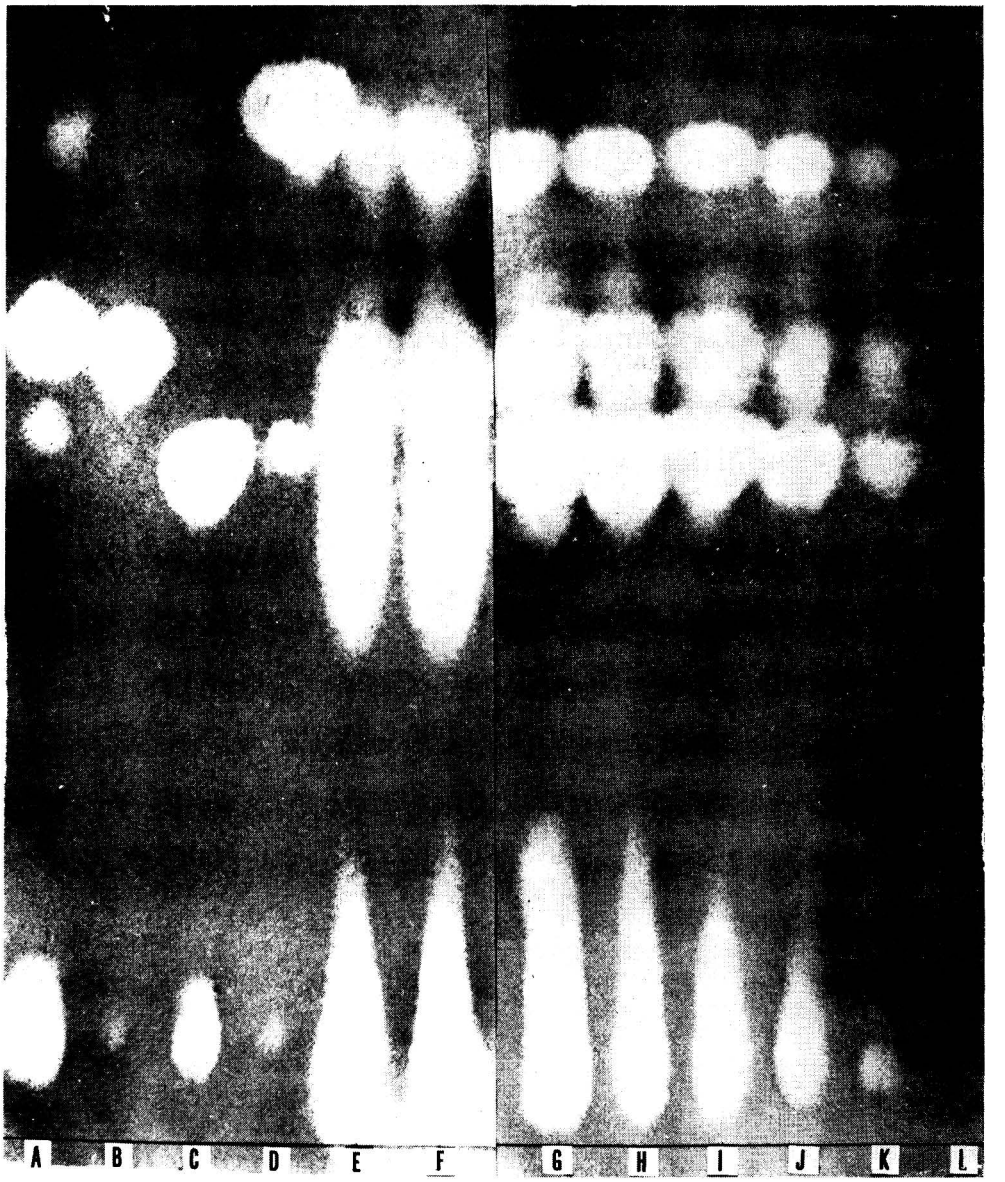


*Fig.* 2—Paper chromatograms showing the separation of Trithion and its oxidation products (A–D) and the Trithion and oxidation products obtained from 2.5 g samples of lettuce at various times after spraying with Trithion (E–L). A, 5 μg Trithion + 5 μg R 1472 (in ascending order). B, 5 μg R 1988. C, 5 μg R 1776. D, 5 μg R 1777. E, 4 hours. F, 1 day. G, 3 days. H, 7 days. I, 10 days. J, 14 days. K, 21 days. L, control.

not from each other. Similarly R 1777 and R 1990 were not separated by the paper chromatographic procedure, but R 1990 had previously been separated from the other compounds during cleanup. Figure 2 (E–L) shows the chromatographic patterns obtained from the chloroform eluates from samples taken at intervals from 4 hours to 21 days after spraying with Trithion. Trithion, R 1472 and/or R 1988, R 1776, and R 1777 were present in all of these samples, but none of these compounds could be detected in samples taken 28 days after spraying. R 1990, which appeared in the methanol

eluates and is not shown in Fig. 2, was present in detectable quantities up to 14 days, but could not be detected at 21 days.

Although only one sample of the control crop is shown in the chromatogram, it is representative of the control crop at all stages of this investigation. No evidence of esterase-inhibiting compounds was obtained from any of the control crop samples. Phosphorus could not be detected in the cleaned up extracts from control samples.

The amounts of Trithion, R 1776, R 1777, and R 1990 were determined after paper chromatographic separation. The total phosphorus content of R 1472 and R 1988, which were not separated in this system, was determined. The amount of R 1472 was determined from chromatograms developed after the reduction of R 1988 to Trithion by mercury-zinc amalgam, and the amount of R 1988 was then calculated by difference.

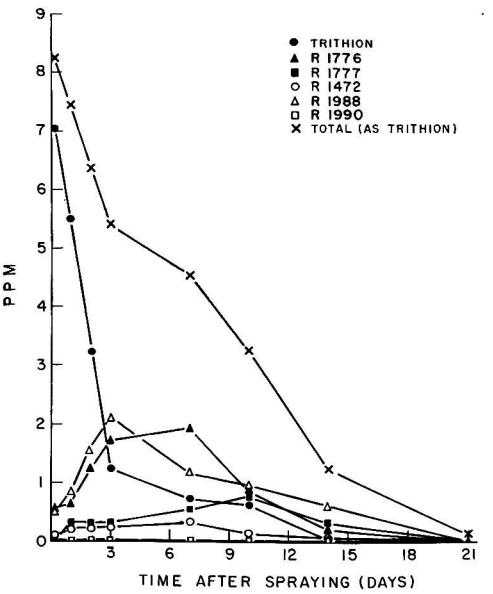


*Fig. 3*—**The quantities of Trithion and its oxidation products recovered from samples of lettuce after spraying with Trithion.**

The amounts of Trithion and its oxidation products obtained from lettuce at intervals after application of Trithion are shown in Fig. 3. The concentration of Trithion decreased rapidly from 7.0 ppm to 1.2 ppm in the first 3 days and then slowly decreased to less than 0.1 ppm at 21 days. R 1472 was present in all samples from 4 hours to 21 days with a maximum of 0.3 ppm at 7 days. R 1990 was present at a concentration of less than 0.1 ppm in samples from 4 hours to 14 days. The R 1988 content increased from 0.5 ppm at 4 hours to 2.1 ppm at 3 days and then steadily decreased to less than 0.1 ppm at 21 days. Similarly, the R 1776 content increased from 0.5 ppm at 4 hours to a maximum of 1.9 ppm at 7 days and decreased to less than 0.1 ppm at 21 days. R 1777 increased from less than 0.1 ppm at 4 hours to 0.8 ppm at 10 days and then decreased to less than 0.1 ppm at 21 days. The total phosphorus content of the cleaned up extracts agreed within experimental error with the total phosphorus content of the separate components on the chromatograms. It would appear that the 6 identified compounds were the only phosphorus-containing materials present in the cleaned up extract.

From the preceding results, it would appear that oxidation of Trithion proceeded mainly to form R 1988, then R 1776, and finally R 1777. Apparently thioether oxidation takes place to form the sulfinyl dithiophosphate and the sulfonyl dithiophosphate, which then undergoes thiolphosphate oxidation to form sulfonyl thiophosphate.

## Summary

Five oxidation products of Trithion have been isolated from lettuce that had been sprayed with Trithion. Trithion and 4 of the 5 oxidation products were found in measurable quantities and persisted in the growing crop for 21 days. Detectable levels of the other oxidation product were found in samples taken from 4 hours to 14 days after application of Trithion.

The amounts and the rates of formation of the oxidation products indicated that the principal oxidation of Trithion proceeded to form first the sulfinyl dithiophosphate, then the sulfonyl dithiophosphate, and finally the sulfonyl thiophosphate.

## Acknowledgments

The author is indebted to Dr. K. A. McCully and Dr. W. P. McKinley for constructive criticism of the manuscript, to Mr.

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# Thin-Layer Chromatography as a Rapid Screening Method for the Determination of Carbaryl and 1-Naphthol Residues Without Cleanup

By M. CHIBA[1] and H. V. MORLEY (Analytical Chemistry Research Service, Research Branch, Canada Department of Agriculture, Ottawa, Ontario, Canada)

**A rapid, sensitive, screening method has been developed in which thin-layer chromatography is used for the detection and estimation of carbaryl and 1-naphthol residues in apple, lettuce, and tomato extracts without prior cleanup. A procedure giving a convenient screening range of 0.1–30 ppm is described, but 0.02 ppm in apples and lettuce and 0.03 ppm in tomatoes may be detected without cleanup. By use of a partitioning cleanup 5 ppb of carbaryl may be detected.**

Carbaryl (1-naphthyl methylcarbamate), as a result of its broad spectrum of insecticidal activity and its low mammalian toxicity, has been extensively used in the control of pests on both animals and plants. Colorimetric procedures described for the determination of carbaryl residues and its breakdown product, 1-naphthol, have utilized aminoantipyrine (1, 2), *p*-nitrobenzenediazonium fluoborate (3–12), or diazotized sulfanilamide (13) as chromogenic reagents. The limitations of colorimetric methods have been outlined by Zweig and Archer (14). Enzymatic procedures based on the anticholinesterase activity of carbaryl (2, 14) are nonspecific, except in the absence of other cholinesterase inhibitors, and do not detect 1-naphthol if present. A paper chromatographic method described for carbaryl residues in wine (14) employed a minimum of cleanup but extension to plant or animal material would doubtless involve a more complicated cleanup procedure and thus render the method unsuitable for rapid screening of a large number of samples. Adsorption chromatography, usually on Florisil (5–8), and partitioning between solvents have been used as cleanup procedures. Absorption in the ultraviolet (15) and thin-layer chromatography (16) are other methods of analysis that have been described in the literature but have not been applied to the determination of carbaryl residues.

The authors have already shown that thin-layer chromatography may be used for the rapid screening of DDT, DDE, aldrin, and heptachlor residues in some plant extracts without a preliminary cleanup (17). This technique has now been extended to provide a specific and sensitive method for the determination of 0.1 μg of carbaryl and 1-naphthol in apple, lettuce, and tomato extracts. Without cleanup, 0.02 ppm of carbaryl and 1-naphthol may be detected in apple and lettuce extracts, and 0.03 ppm in tomato extracts. Detection of 5 ppb of these compounds in apple extracts was achieved by use of a simple cleanup described by Hardon,

[1] N. R. C. Post-doctorate Research Fellow, 1962–1964.

Contribution No. 52, Analytical Chemistry Research Service, Research Branch, Canada Agriculture, Ottawa, Canada.

*et al.* (13). The method provides a rapid screening technique for carbaryl and its metabolite, 1-naphthol, in the 0.1–30 ppm range for apple, lettuce, and tomato extracts. Comparison of the method with an AOAC collaborative colorimetric procedure (18) gave good agreement. The method has also been applied to bee extracts.

## Experimental

### *Apparatus*

(a) *Desaga applicator and equipment.*—Available from Canlab, Canada, or Brinkmann Instruments, Inc., Long Island, N.Y.

(b) *Chromatographic chamber* (Arthur H. Thomas Co., No. 3108-B05).

(c) *Liner for chromatographic tank.*—Whatman No. 1, 22 × 26 cm.

(d) *Spray bottle.*—8 oz (Arthur H. Thomas Co., No. 9186-R2).

(e) *High-speed blender.*—Such as Servall Omni-mix.

(f) *Spectrophotometer.*—Unicam SP. 500 (Canlab, Canada) was used in this work.

### *Reagents*

(a) *Methylene chloride.*—(Matheson, Coleman and Bell No. DX835).

(b) *Petroleum ether.*—B.p. 30–60° (Mallinckrodt, No. 4980), distilled over sodium-lead alloy (Baker, No. 9413).

(c) *2-Propanol.*—Analar (British Drug Houses, Canada).

(d) *Benzene.*—Analar (B.D.H., Canada).

(e) *Acetone.*—Analar (B.D.H., Canada).

(f) *Hyflo Super-Cel* (Johns-Manville Co. Ltd.).

(g) *Silica gel* (Kieselgel).—With $CaSO_4$ binder (Camag D5, Arthur H. Thomas Co.).

(h) *Calco oil blue NS.*—(Cyanamid of Canada Ltd.). 250 mg % in acetone. This material was purified by chromatography on a silica gel column. Elution with benzene removed a contaminating red dye; further elution with acetone removed the oil blue.

(i) *Carbaryl.*—(Union Carbide Chemical Co. 99.7%). Standard solutions, 50 mg % and 5 mg % in methylene chloride.

(j) *1-Naphthol.*—Analar (B.D.H., Canada), purified with charcoal and recrystallized from water. Standard solutions, 50 mg % and 5 mg % in methylene chloride.

(k) *Chromogenic agents.*—(*1*) Iodine vapor in a closed chamber; (*2*) 1.5*N* methanolic sodium hydroxide followed by *p*-nitrobenzenediazonium fluoborate (Eastman Organic Chemicals), 10 mg % in diethyl ether:methanol (1:1).

### *Determination*

*Extractions.*—Chopped plant material (50 g) was transferred to a 400 ml stainless steel chamber, and 100 ml methylene chloride and 100 g anhydrous sodium sulfate were added. After blending at high speed for 3 min. the slurry was allowed to settle, and the methylene chloride layer was filtered with suction through a Whatman No. 2 filter paper layered with a 6 mm pad of Super-Cel previously wetted with solvent. The residue in the chamber was re-extracted twice more with two 100 ml portions of methylene chloride. The combined filtrates were concentrated by evaporation under reduced pressure to a convenient volume (1 ml = 1 g of extract).

*Preparation of Chromatographic Plates.*—This was carried out as described previously (17), but a 0.25 mm layer of silica gel slurry was applied instead of a 0.2 mm layer.

*Application of Samples.*—The technique used was a slight modification of that described previously (17). Prior to spotting, holes 1.5 mm in diameter were made in the silica gel at the spotting positions. An aliquot of the methylene chloride extract (1 ml) was transferred to a small test tube and evaporated just to dryness in a hood under a stream of filtered air. The residue was dissolved in 0.1 ml petroleum ether and spotted onto the plate from a 2 µl pipet. The spotting procedure took approximately 3 min. and the spot diameter did not exceed 8 mm. Two µl (5 µg) of the oil blue solution was spotted on each plate to act as a reference point and to check the activity of the silica gel.

Spotting the samples into the previously marked holes served to confine the area of the spot and to prevent the flaking sometimes observed when relatively large volumes of extract are applied. The spot shape did not appear to be affected by this technique (Fig. 1).

*Development.*—After drying, the plate was developed in a closed rectangular tank fitted with a paper liner (22 × 26 cm) and containing a mixture of 5% acetone in benzene (v/v) to a depth of 1.0 cm. The plate was removed after the solvent front had reached a previously marked line 10 cm from the origin. Development time was 35–40 min.

*Detection.*—The plate was dried at room temperature and then sprayed with methanolic sodium hydroxide (1.5*N*), followed, while the plate was still damp, by the fluoborate

reagent. 1-Naphthol gave a purple spot and carbaryl a brilliant blue spot which gradually changed during 10 min. to the 1-naphthol color. The lower limit of detectability for both compounds was 0.1 μg. These reagents were slight modifications of those used by Zweig and Archer for their paper chromatographic method (14). The procedure described above gave a five-fold increase in sensitivity over the paper chromatographic method.

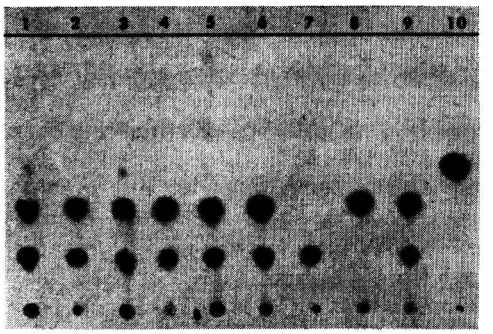


*Fig. 1*—**Standard amounts of carbaryl (1 μg, position 7) and 1-naphthol (1 μg, position 8) and a mixture (position 9). Positions 1–6 show the background obtained from a mixture of 1 μg of each component added to plant extracts and spotted without cleanup. Positions 1, 3, and 5 represent solutions equivalent to 1 g, and positions 2, 4, and 6, 0.1 g of apple, lettuce, and tomato, respectively. Position 10 had 5 μg of oil blue applied. Solvent system benzene-acetone (19 + 1 v/v), fluoborate chromogenic spray.**

Alternatively the compounds were detected by placing the plate in a closed tank saturated with iodine vapor; brown spots on a yellow background formed with both carbaryl and 1-naphthol. The carbaryl color took 10 min. to develop and the lower limit of detectability was 3 μg. Uptake of iodine by 1-naphthol reached a maximum after 1–2 min. and the lower limit of detectability was 0.1 μg. Walker and Beroza found a lower limit of detectability of 5 μg for carbaryl after a 5 min. exposure to iodine vapor (16).

## Results

Figure 1 shows the results obtained with plant extracts spiked with carbaryl and 1-naphthol at the 1.0 and 10.0 ppm level and spotted without cleanup. The only visible background was a faint yellow resulting from the application of extract equivalent to 1 g of tomato (position 5). This slight background color did not affect the lower limits of detectability (0.1 μg). The results showed that a convenient screening range of from 1 to 10 ppm for carbaryl and its metabolite, 1-naphthol, could be achieved for apple, lettuce, and tomato extracts without a preliminary cleanup. The over-all range of the method was 0.1–3 ppm when 1 g of extract equivalent was used or 1–30 ppm when 0.1 g was used.

Methylene chloride extracts of homogenized bees were also spotted directly onto plates without cleanup. Slight interference by oil resulted in a depression of $R_f$ values but 0.1 μg of carbaryl and 1-naphthol were still detectable at the 0.2 ppm level.

Attempts to apply the technique to field-treated crops were unsuccessful, as none of the samples obtained contained detectable quantities of carbaryl or 1-naphthol. This result was confirmed by the fluoborate colorimetric procedure as used in an AOAC collaborative study of a method for carbaryl residues (18).

## Discussion

Methylene chloride has been used extensively as an extraction solvent for carbaryl. Methylene chloride solutions, however, when spotted on a plate, gave large diffuse spots, whereas petroleum ether extracts gave sharp, distinct spots. Since carbaryl is soluble in petroleum ether and 2-propanol, attempts were made initially to use this extraction mixture to avoid the subsequent transfer of

**Table 1. $R_f$ and $R_{OB}$ values of carbaryl, 1-naphthol, and oil blue spotted with 0.1–1.0 g of tomato, apple, or lettuce extracts with various solvent systems[a]**

| | Carbaryl | | 1-Naphthol | | Oil Blue |
|---|---|---|---|---|---|
| Solvent | $R_f$ | $R_{OB}$ | $R_f$ | $R_{OB}$ | $R_f$ |
| Benzene-acetone | | | | | |
| 49 + 1 | 0.07 | 0.32 | 0.20 | 0.91 | 0.22 |
| 19 + 1 | 0.17 | 0.39 | 0.33 | 0.75 | 0.44 |
| 9 + 1 | 0.32 | 0.53 | 0.50 | 0.83 | 0.60 |
| *n*-Hexane-acetone | | | | | |
| 13 + 7 | 0.35 | 0.64 | 0.48 | 0.87 | 0.55 |
| 4 + 1 | 0.16 | 0.37 | 0.32 | 0.74 | 0.43 |

[a] $R_{OB}$ = $R_f$ value of compound/$R_f$ value of oil blue.

the methylene chloride extract to petroleum ether. The two extraction methods were compared on whole apples and apple peel, with determination by the standard colorimetric procedure (18). At the 0.04 and 0.08 ppm levels, however, petroleum ether:2-propanol (3:1) extracted only 25% of the available carbaryl. Gunther, Blinn, and Carman (5) have reported that although carbaryl has a solubility in *n*-hexane of 295 μg per ml at 28°C, methylene chloride was 44% more efficient than hexane as an extraction solvent.

Although it has been reported that solutions of 1-naphthol in methylene chloride are unstable (10), we found that solutions kept at room temperature for 6 weeks showed no signs of instability.

Various solvent systems were examined for maximum separation of carbaryl and 1-naphthol from co-extractives. Of the mobile systems tested, benzene:acetone (19 + 1) was selected, as it gave the sharpest spots for both carbaryl and 1-naphthol, although other mixtures also gave good separations. $R_f$ and $R_{OB}$ values are given in Table 1.

For screening purposes cleanup was not required, since up to 5 g of apple or lettuce or 3 g of tomato extract equivalent can be spotted without undue interference from the background. For specific determination, the thin-layer chromatographic method may be extended to the ppb range for carbaryl by use of a suitable cleanup. A partitioning cleanup, such as that used by Hardon, *et al.* (13), made it possible to estimate 5 ppb of carbaryl, since extracts from 100 g of apple, spiked with 0.5 μg of carbaryl, may be applied to a plate. The cleanup employed did not completely remove co-extractives, however, and the $R_{OB}$ of carbaryl was 0.57, compared to 0.39 with the screening procedure. This cleanup was not suitable for 1-naphthol because of extensive loss of material in the partitioning.

**Acknowledgment**

We wish to thank J. H. Langevin for valuable technical assistance.

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## FRUITS AND FRUIT PRODUCTS

# Identification of Grape Varieties

By A. C. RICE (The Taylor Wine Company, Inc., Hammondsport, N.Y.)

**A series of grape juices and concentrates were diluted with MacIlvaine's buffer at pH levels ranging from 2 to 8 and analyzed spectrophotometrically. The relationship of various Concord and California grape juice samples from different geographical areas were characterized individually and in combination. The spectral characteristics of Concord and Salvador grape juice were found to be similar except at pH 7, where the Concord curve has two peaks (583 and 450 mμ) and the Salvador curve has only one. The anthocyanin components in Concord grape juice were studied chromatographically for an explanation of its unique spectral response at pH 7. Results are incomplete, and further study is recommended.**

Concord grape juice is sometimes adulterated by blending with the juice of other grape varieties. A method is therefore needed for the identification and characterization of Concord grape juice as well as other grape varieties.

Meschter (1) suggested that grape varieties might be identified by differential spectral characteristics of the anthocyanin pigments found in *Vitis labrusca* hybrids (Concords) and in *Vitis vinifera* and hybrids (California red grapes, e.g., Carignane, Alicante, and Salvador), since this approach was successful with strawberries.

This report will review briefly the spectrophotometric and chromatographic investigations of grape varieties.

### Spectrophotometric Studies

#### *Experimental*

A series of grape juice and concentrate samples (Table 1) were analyzed spectrophotometrically on a Beckman DK-1 Spectrophotometer. Immediately prior to analysis, the juice samples were diluted 1:50 and the concentrate samples 1:200 with MacIlvaine's buffer at pH levels of 2.1, 3, 4, 5, 6, 7, and 8.

The Beckman DK-1 was operated under the following conditions: reference liquid, MacIlvaine's buffer at the appropriate pH;

**Table 1. Grape juice and concentrate samples used for the spectrophotometric studies**

| Juice or Concentrate | Origin |
|---|---|
| 1961 Season | |
| Concord juice | Finger Lakes, N. Y. |
| Concord juice | Lake Erie, N. Y. |
| Concord juice | Hudson Valley, N. Y. |
| Concord juice | Finger Lakes, N. Y. |
| Concord juice | Lake Erie, N. Y. |
| Concord juice | Spartanburg, S. C. |
| Concord juice | Wash. (Expt No. 4)[a] |
| Concord juice | Wash. (Commercial No. 1)[a] |
| Concord juice | Wash. (Commercial No. 2)[a] |
| Concord juice | Wash. (Commercial No. 3)[a] |
| Salvador juice | Fresno, Calif. |
| Special red grape concentrate | Fresno, Calif. |
| Special red grape concentrate | Fresno, Calif. |
| Carignane juice | Fresno, Calif. |
| Royalty grape concentrate | Fresno, Calif. |
| Ruby red grape concentrate | Fresno, Calif. |
| 1962 Season | |
| Concord juice | Hudson Valley, N. Y. (9–22) |
| Concord juice | Hudson Valley, N. Y. (10–5) |
| Concord juice | Lake Erie, N. Y. (9–22) |
| Concord juice | Lake Erie, N. Y. (10–5) |
| Concord juice | Finger Lakes, N. Y. (9–22) |
| Concord juice | Finger Lakes, N. Y. (10–5) |
| Concord juice | Finger Lakes, N. Y.[b] |

[a] Supplied by courtesy of Dr. A. M. Neubert, Agricultural Research Service, Western Utilization Research and Development Division, U. S. Department of Agriculture, Prosser, Wash.
[b] Hot-packed.

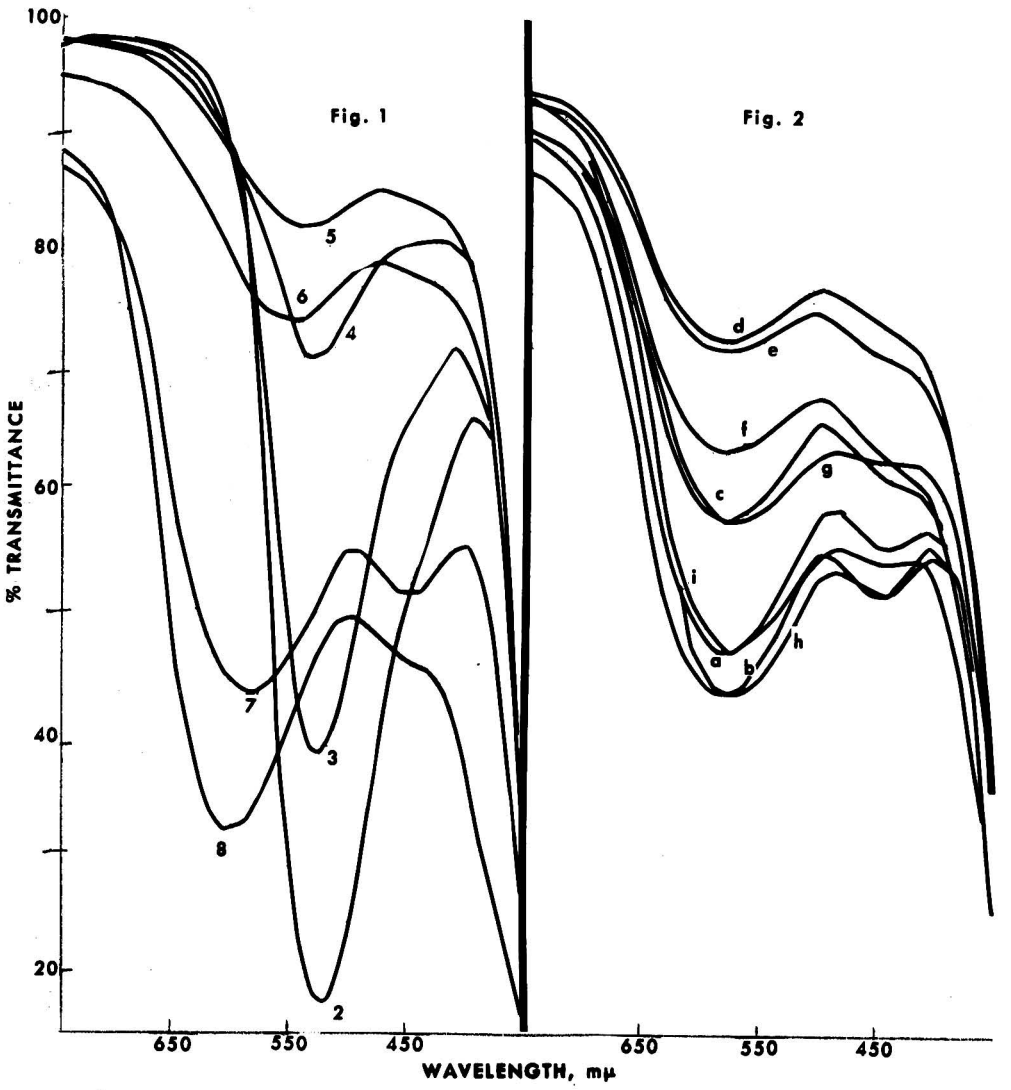


*Fig. 1*—Effect of pH on the spectral characteristics of Concord grape juice (Finger Lakes, N.Y., 1961) diluted 1:50 with MacIlvaine's buffer.

*Fig. 2*—Spectral curves for various samples of Concord grape juice at pH 7 (diluted 1:50 with MacIlvaine's buffer). *a*, Hudson Valley, N.Y., 1961. *b*, Finger Lakes, N.Y., 1961. *c*, Lake Erie, N.Y., 1961. *d*, Spartanburg, S.C., 1961. *e*, Prosser, Wash., 1961, export. *f*, Prosser, Wash., 1961, commercial. *g*, Hudson Valley, N.Y., 1962. *h*, Finger Lakes, N.Y., 1962. *i*, Lake Erie, N.Y., 1962.

silica cells; tungsten lamp; photomultiplier at 1×; chart speed, 1.5″/min.; λ drive, 10; period, 0.6 sec.; gain, 100; scale, 100% transmission; automatic scan; wavelength, 100 mμ/min.

### *Results*

Typical spectral curves are presented in Figs. 1–5. (These figures were reproduced by tracing over the original curves.)

Figures 1 and 3 illustrate the effect of pH on the spectral characteristics of Concord and Salvador grape juice. The pattern of the curves as the pH changes from 2 to 8 is similar for both juices except at pH 7. In both sets of curves, the wavelength of maximum absorptions gradually shifts from about 525 mμ at pH 2 to about 600 mμ at pH 8. The % transmittance at the wavelength of maximum absorbance increases as the pH is

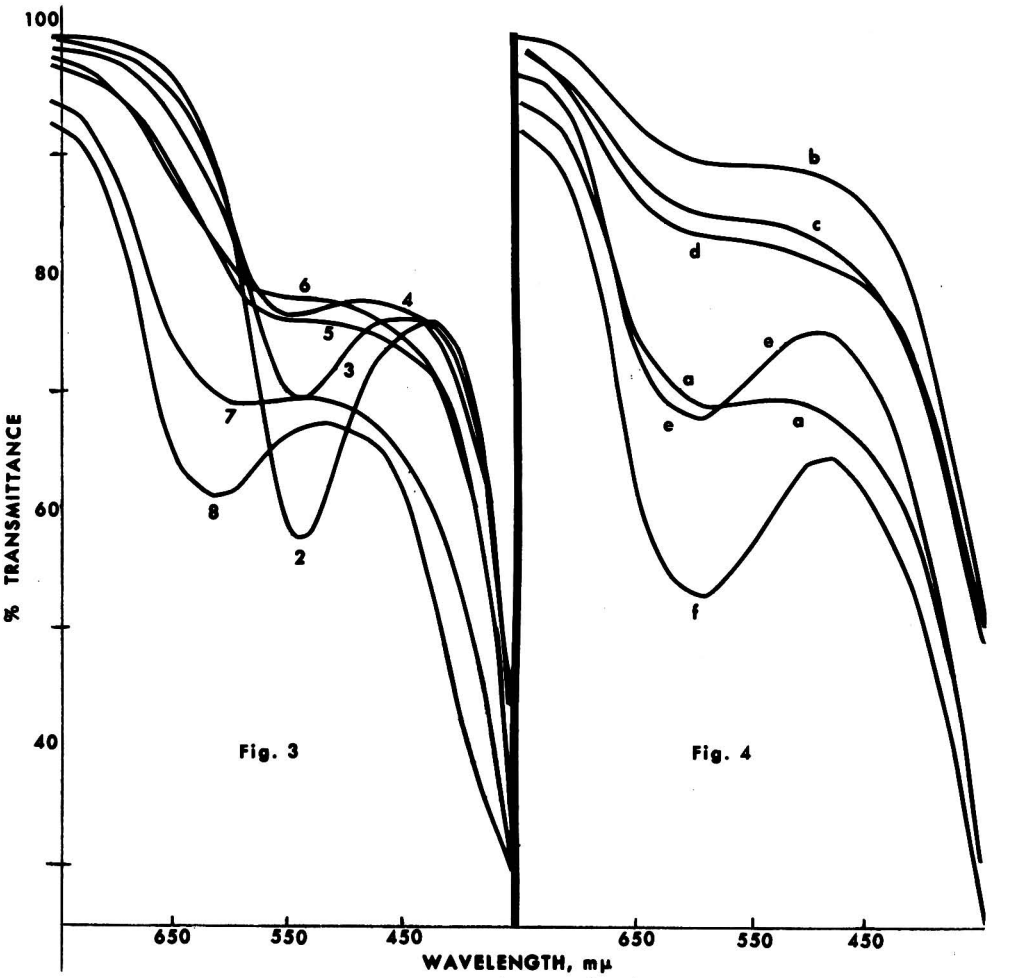


*Fig. 3*—Effect of pH on the spectral characteristics of Salvador grape juice (California, 1961) diluted 1:50 with MacIlvaine's buffer.

*Fig. 4*—Spectral characteristics of various California juices and concentrates at pH 7 (juices diluted 1:50, concentrates diluted 1:200 with MacIlvaine's buffer). *a*, Salvador juice, 1961. *b*, Carignane juice, 1961. *c*, Special red grape concentrate (20% Salvador, 80% Thompson seedless), 1961. *d*, Special red grape concentrate (similar to *c*). *e*, Royalty grape concentrate, 1961. *f*, Ruby red grape concentrate, 1961.

raised, from pH 2 to 5 for Concord and to 6 for Salvador, and then decreases to nearly the level observed at pH 2. At pH 7, the Concord curve includes two peaks (583 and 450 mμ), whereas the Salvador curve has only one peak.

Figure 2 shows the relationship among the various samples of Concord juice at pH 7. The characteristics of the curve range from those with two different peaks to those that possess only a slight shoulder on the curve at the wavelength of the secondary peak. The New York State Concord juices exhibit the most pronounced secondary peaks for both the 1961 and 1962 samples, whereas the South Carolina and Washington juices exhibit the least pronounced secondary peak.

Figure 4 indicates the relationship among the California juices and concentrates at pH 7. The characteristics of these curves range from one very distinct peak to a simple flattening of the curve at the point of maximum absorbance. There is no indication of a secondary peak at 440–450 mμ in any of these curves.

Figure 5 shows the effect on the Concord

double peak at pH 7 when Concord was blended with special red grape concentrate and ruby red concentrate (each concentrate being diluted to a 16° Brix single-strength juice prior to blending). When Concord was blended 75–25% with special red and ruby red (both at 16° Brix, v/v), the minor peak is still discernible. When blended 50–50%, the secondary peak is reduced to a shoulder;

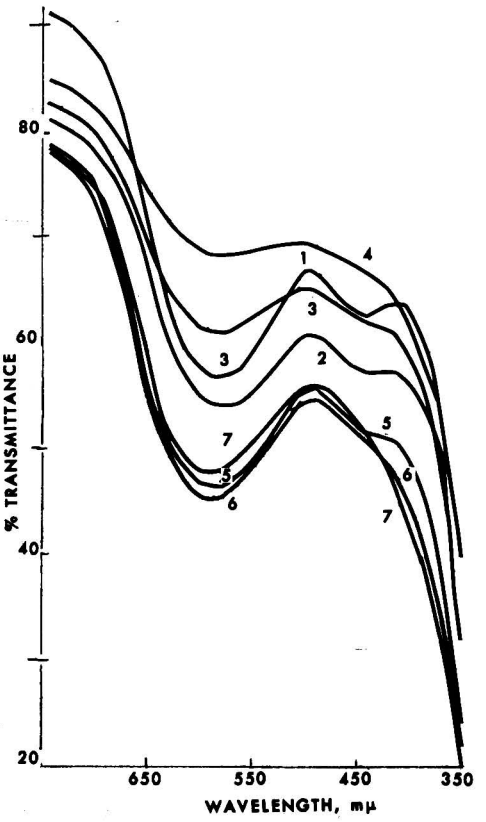


*Fig. 5*—Effect of Special red grape concentrate on Concord juice at pH 7; blends diluted 1:50 with McIlvaine's buffer. *1*, Concord juice. *2*, 75% Concord, 25% Special red grape concentrate. *3*, 50% Concord, 50% Special red grape concentrate. *4*, 25% Concord, 75% Special red grape concentrate. *5*, 75% Concord, 25% Ruby red concentrate. *6*, 50% Concord, 50% Ruby red concentrate. *7*, 25% Concord, 75% Ruby red concentrate.

when blended 25–75%, the secondary peak has disappeared and the curve assumes the shape of the California juice. Blends were not prepared with South Carolina or Washington Concord but (from the curves in Fig. 2) the shoulder would apparently disappear with a lower proportion of California juice in the blend than was required with the samples shown.

Differences in absorbances among the 1961 Concord juice are presented in Fig. 6. With the absorbance of the 1:50 dilution of New York Finger Lakes juice as standard, it required 1.2 times as much New York Hudson Valley juice, 1.6 times as much New York Lake Erie juice, 2.1 times as much Washington juice, and 2.9 times as much South Carolina juice to equal this standard. This parallels the differences in % transmittance values among the various samples noted in the spectral curves at pH 7 (Fig. 2).

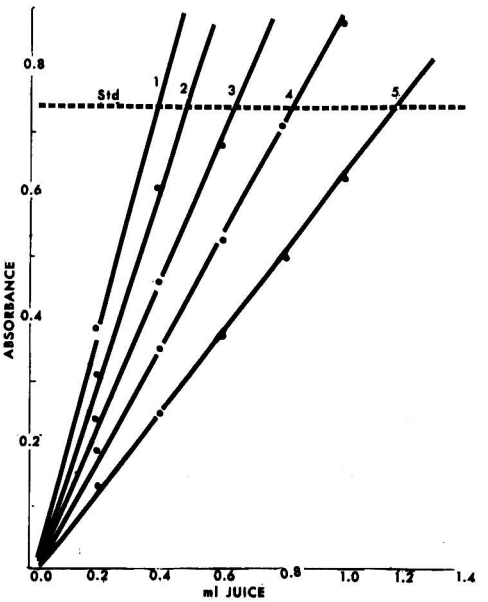


*Fig. 6*—Absorbance of Concord juices (1961) at 525 mμ. *1*, Finger Lakes, N.Y. *2*, Hudson Valley, N.Y. *3*, Lake Erie, N.Y. *4*, Prosser, Wash. *5*, Spartanburg, S.C. Dotted line is the standard—sample from the Finger Lakes, N.Y. Diluted 1:50 at pH 2.1.

A comparison of the average % transmittance values at the peak of maximum absorbance for all California samples and for all Concord samples (Fig. 7) indicates that the Concord samples have maximum transmittance at pH 5, whereas the California samples have maximum transmittance at pH 6. The shift in wavelength of maximum absorbance with pH (Fig. 7) is comparable for both groups, shifting from about 525 to 600 mμ as the pH changes from 2 to 8.

***Discussion***

The spectral analyses of the samples of California juices and concentrates and Concord juices have shown that a marked difference exists in the character of the curves at pH 7. The Concord samples reveal a secondary peak or shoulder at 440–450 mμ which is not found in the California samples. This characteristic is variable in degree among Concord juices from the several growing areas of the country but appears to be consistant in all Concord juices. Although this spectral characteristic may not in itself provide a means of differentiating *Vitis labrusca* from *Vitis vinifera* and hybrids, the component or components of the juice which produce this character may provide a basis for such differentiation.

It was with this possibility in mind that another approach to the problem was initiated.

**Chromatographic Studies**

To identify the specific pigments which produce this spectral characteristic, it was considered necessary to separate and identify the several components of the anthocyanin mixture comprising the color of Concord grape juice. Beginning in the late summer of 1962, emphasis was directed toward the identification of the anthocyanin pigments in Concord grape juice.

***Experimental***

Several procedures were tried to separate the anthocyanin pigments. Column chromatography, according to the method of Spaeth and Rosenblatt (2), was found to be unsatisfactory. One-dimensional paper chromatography, employed *n*-butanol:2*N* HCl, was tried with little success. Two-dimensional paper chromatograms were explored, using butanol:2*N* HCl (1:1, v/v) as the organic phase of the first dimension and *m*-cresol:1*N* acetic acid (1:1, v/v) for the second dimension. Although this technique was promising, the quantity of relatively pure pigments available was too small for further characterization work.

For mass extraction of pure pigments, the technique described in detail by Pascal Ribereau-Gayon (3) was employed. Concord juice was extracted with lead acetate and the resultant bright red extract was applied in streaks to 18 × 22″ sheets of filter paper by using a 1 ml pipet. Chromatograms were developed for 16 hours by the descending technique, with butanol:2*N* acetic acid as the organic phase. The chromatograms were then removed and dried, and the bands marked off under ultraviolet light. Corresponding bands were then cut off and eluted with 15% aqueous acetic acid. After reduction to near dryness (Rotovac at 55°C), pigments were dissolved in 1% HCl and applied, as above, for chromatography in the aqueous phase of acetic butanol. After drying, spot tests were made of the resulting bands and $R_f$ values were determined. Bands with comparable characteristics were combined for elution so that a reasonably concentrated extract would be obtained. The eluate from each fraction was reduced in volume to about 50 ml and two-dimensional chromatograms were made of the concentrates with the two phases of water:acetic acid:butanol as solvents. Isomerization, oxidation, and degradation occurred during the elution and concentration of the fractions. By repeating the chromatographic separation of the relatively pure bands, it was possible to separate the pigments from other components and obtain them in pure form.

***Results***

To date, five monoglucosides have been identified: malvidin, delphinidin, cyanidin,

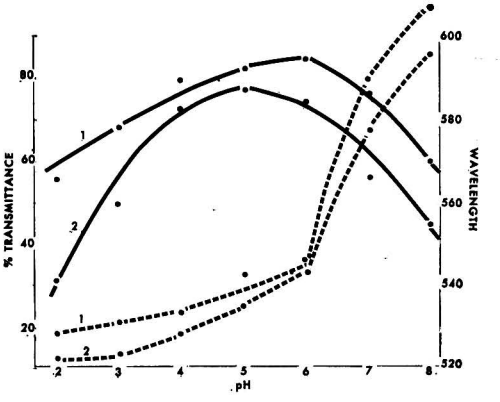


*Fig. 7*—Average % transmittance values (solid line) and average wavelength values (broken line) at maximum absorbance peak for all Concord and all California samples at pH values 2 to 8. *1*, California samples. *2*, Concord samples.

petunidin, and peonidin. Work is progressing on the diglucosides.

### *Discussion*

Considerable effort has been devoted to the determination of the anthocyanin components in Concord grape juice as a result of finding the unique spectral response of Concord juice at pH 7. Results of this study are incomplete. It is interesting to study the recent publication (4) concerning the pigments in Concord grapes and to find that the specific pigments that have been isolated and identified thus far are in agreement with the results of the Washington investigators.

It is felt that if the anthocyanin pigments in grapes are to serve as a basis for identifying grape varieties in a grape juice blend, it will first be necessary to know qualitatively and quantitatively the anthocyanins present in the several grape varieties involved.

### Recommendations

It is recommended—

(1) That study be continued on the spectral characteristics of Concord and California grape juice, and the identification of anthocyanins present in Concord grape juice.

(2) That the fluorescence patterns of the pigments in Concord and California grape juices be investigated.

(3) That attempts be made to develop a procedure to determine the presence and amounts of *Vitis vinifera*-type grape juices in combination with Concord grape juice.

### Acknowledgments

The Associate Referee wishes to thank Dr. L. R. Mattick, New York State Agricultural Experiment Station, Cornell University, Geneva, N.Y., for his spectrophotometric studies; Dr. Mattick and Mr. L. D. Weirs, of his laboratory, for their chromatographic studies; and the following collaborators:

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A. D. Webb, Department of Viticulture and Enology, University of California, Davis, Calif.

J. J. Powers, Department of Food Technology, University of Georgia, Athens, Ga.

A. M. Neubert, Agricultural Research Service, Western Utilization Research and Development Division, U.S. Department of Agriculture, Prosser, Wash.

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The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D and were accepted by the Association. See *This Journal*, **47**, 131 (1964).

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

# Determination of Hesperidin in Dry Matter of Citrus Tissues by Ultraviolet Spectrophotometry Method[1]

By R. GOREN and S. P. MONSELISE (The Hebrew University, Faculty of Agriculture, Department of Citriculture, Rehovot, Israel)

**An ultraviolet spectrophotometric method was suggested in 1959 for the determination of hesperidin in juice and fresh extract of orange peel. This method, based on the differences in absorbance units of hesperidin between 290 and 300 mμ, has been adapted to the determination of hesperidin in dry matter of citrus tissues. Hesperidin is extracted from dry matter with 0.2*N* NaOH for 22 hours at 10°C, the pH is adjusted to 3.8 ± 0.25, and plant residues are separated by centrifugation. The solution is diluted with 99% isopropanol, proteins are precipitated, and the extract is read on a spectrophotometer. Results are calculated from the difference in absorbances between 290 and 300 mμ.**

Hesperidin, 7-β-L-rhamnosido-D-glucoside of 5,7,3′-trihydroxy-4′-methoxyflavanone, is the main flavanone in orange tissues. Several methods have been suggested for the determination of hesperidin for technological purposes (1–4). In the commonly used Davis test (1) fresh material or orange juice is extracted with water or alcohol and the extracts are further treated with diethylene glycol in the presence of 4*N* NaOH. The intensity of the yellow solution obtained is measured at 420 mμ. Since this method is sensitive to many other phenols, it is not specific for the determination of hesperidin, but rather evaluates "total flavanoids." The diazo coupling test (2), based on a color reaction between *p*-dinitroaniline and phenolic compounds in the presence of sodium hydroxide to produce a yellow compound measured at 425 mμ, is also nonspecific. The cyanidin method (3, 5, 6) is based on a color reaction between a solution of the flavanoid compound and an ethanol-HCl mixture in the presence of a strip of magnesium. The resulting crimson solution is read at 560 mμ. Like the previous methods, it is also nonspecific.

Hesperidin and other flavanoids have an absorbance peak in the ultraviolet region (2, 5–9). Horowitz and Gentili (10) have suggested using spectrophotometric determination at the natural peak of hesperidin in the ultraviolet region, instead of the Davis reaction.

Hendrickson and Kesterson, who used the Davis test for a survey on the occurrence of hesperidin in Florida oranges (11), later suggested a new, specific method for the determination of hesperidin in juice and fresh extract of orange peel (9). In this method orange juice or alkaline peel extract is diluted with 99% isopropanol, and results are calculated from the difference in absorbance at 290 and at 300 mμ. For a rough estimation of hesperidin they have suggested an approximate calculation, while stating that more accurate results may be obtained with a standard curve.

In our physiological work with citrus tissues (12, 13), we have been confronted with the need for a specific, sensitive, and quantitative method to test small amounts of dry matter. The use of dry matter, which is very desirable when conducting long range surveys, is possible in the case of hesperidin, since flavanones are not degraded by fast drying in an oven nor during successive storage (5).

We have therefore adapted Hendrickson and Kesterson's method (9) for use with dry matter, developed a standard curve, and calculated the regression equation, which allows accurate hesperidin determination.

## Experimental

### *Materials*

(a) *Pure hesperidin.* — As pure hesperidin is not available in commerce, we prepared

[1] Part of the Ph.D. thesis of R. Goren, submitted to the Hebrew University, June, 1963.

it from young Shamouti orange fruits, a very rich source (11, 12), by alkaline extraction and purification with formamide (14). We obtained pure hesperidin, m.p. 261.5°C (11, 15).

(b) *Plant material.* — Freshly picked oranges were washed with detergent, tap water, and distilled water. The fruits were cut into pieces, and dried at 75°C in a ventilated oven for 24 hr, then for 6 more days at 65°C. (No loss of hesperidin was detected in preliminary experiments conducted under these conditions.) The dry material was then ground in a Wiley mill to pass a 40-mesh screen and stored in the dark in plastic bottles fitted with air-tight lids.

(c) *Hesperidin standard solution* (16).— A standard solution was prepared by dissolving 63.5 mg pure hesperidin in 254 ml dimethylformamide (0.025% hesperidin).

### *Procedure*

The plastic bottle containing dry material was dried for 24 hr at 65°C, and 100 mg dry matter was extracted with 50 ml 0.2*N* NaOH for 22 hr at 10°C. (The addition of $CuSO_4$ and the aeration needed (9) to prepare juice or fresh material for hesperidin determination have been omitted since ascorbic acid oxidation is believed to have been completed during the preparation of dry plant material; also ascorbic acid oxidase cannot be further activated after drying.) Next 0.36 ml concd HCl is added to a 25 ml aliquot of alkaline extract, and the pH of the solution is adjusted to 3.8 ± 0.25 by adding a few drops of HCl (4*N* and 2*N*, successively) under constant control by a pH meter. Residues of plant material are separated by centrifuging 10 ml of the acid solution at 2,500 rpm for 5 min., and 2.5 ml of centrifuged solution is diluted to volume in a 50 ml volumetric flask with 99% isopropanol. The proteins are allowed to precipitate for 1 hr, and the isopropanol extract is filtered through Whatman No. 1 filter paper. An aliquot of the extract is read on a Beckman Model DU spectrophotometer, in 10 ml quartz cuvettes at 290 and 300 mμ, respectively (slit width, 0.15).

Calculation of results is based on the difference in absorbances between 290 and 300 mμ ($A_{290} - A_{300}$), plotted against a standard curve or the corresponding regression equation. Results are expressed as % hesperidin in dry matter or in ppm of extract.

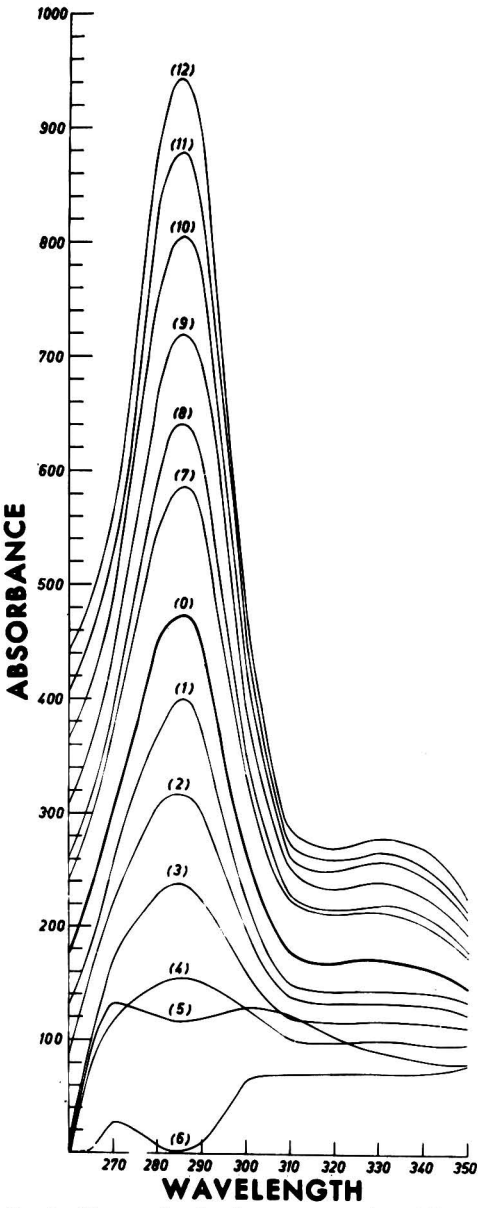


*Fig. 1*—Changes in absorbance curves when different amounts of hesperidin are added to a dry matter extract or to a blank (spectrophotometric subtraction).

### *Preparation of Standard Curve*

The isopropanol extract from the *Procedure*, which contains natural hesperidin, is used to prepare a standard curve by the

ultraviolet subtraction method. This procedure eliminates interference from the presence of other natural substances which are also extracted; in addition, the absorbance peak for hesperidin is rounded off at 286 mμ.

The amount of hesperidin in the extract is first evaluated by adding increasing quantities of hesperidin to the blank (99% isopropanol). The quantities increase at a known rate and cause $A_{290} - A_{300}$ to be reduced (spectrophotometric subtraction). When the amount of hesperidin in the blank equals that in the extract, $A_{290} - A_{300} = 0$. Adding further amounts of hesperidin to the blank yields a negative curve (Fig. 1, curve 5); adding increasing quantities of hesperidin to the extract causes the absorbance values to increase (Fig. 1, curves 7–12).

*Determination of hesperidin in extract; lower part of standard curve.*—Six blank solutions (99% isopropanol) were prepared, containing 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 ppm hesperidin, respectively. The extract is read against these hesperidin standards to obtain spectrophotometric subtraction of the absorbance curve of hesperidin that has been extracted from the dry matter (in Fig. 1, compare curve 0 for extract versus the blank with curves 1–6 for extract versus hesperidin standards). Every increase of 2.5 ppm of hesperidin reduces $A_{290} - A_{300}$ by $39.3 \pm 0.8 \times 10^3$ and causes a leveling of the absorbance curve for the hesperidin in the extract.

When the same extract is read versus the blank, a typical peak is found at 286 mμ (Fig. 1, curve 0), for which $A_{290} - A_{300} = 181 \times 10^3$. In Fig. 1, curve 4 is the nearest to the horizontal line, but still has a small peak. This curve represents extract versus the 10.0 ppm hesperidin standard; $A_{290} - A_{300} = 24 \times 10^3$. Curve 5 in Fig. 1 shows a negative trend ($A_{290} - A_{300} = -15 \times 10^3$); in this case the standard contains more hesperidin than the extract. By interpolation, the amount of the natural hesperidin in the standard equals 11.0 ppm (or 11% hesperidin in the dry matter), and $A_{290} - A_{300} = 181 \times 10^3$.

The values for $A_{290} - A_{300}$ from curves 1–4 (Fig. 1) are used to build the lower part of the standard curve for hesperidin (Fig. 2).

*Upper part of the standard curve.*—Hesperidin was added to 2.5 ml of the alkaline extract before diluting with 99% isopropanol in a 50 ml volumetric flask. Hesperidin was diluted as above (2.5–15.0 ppm), giving six extract solutions, which were tested against a blank.

Curves 7–12 in Fig. 1 show the increase in absorbance peaks and the corresponding increasing values for $A_{290} - A_{300}$. As before, every increase in 2.5 ppm of hesperidin in the extract causes an increase in $A_{290} - A_{300}$ of $38.5 \pm 1.1 \times 10^3$. These values obtained from curves 7–12 are used to build the upper part of the standard curve for hesperidin (Fig. 2).

### *Calculations*

Since the concentration of dry matter in the cuvette is 100 ppm, 1.0 ppm of hesperidin equals 1.0% hesperidin. Using Hendrickson and Kesterson's procedure for hesperidin in orange juice (9), the same standard curve or regression equation below can be used, and results in ppm multiplied by 20. Hesperidin is then expressed as mg/100 ml of orange juice.

The regression equation for the standard curve is:

$$Y = -0.75993 + 0.64972\,X$$
$$r = +0.99$$

where Y = hesperidin content in ppm (or %) in dry matter, and X = difference in absorbances between 290 and 300 mμ, $\times 10^3$. From the equation given above a table can be prepared for routine use. If the standard procedure for dry matter or for orange juice (9) is followed, the above regression equation or standard curve (Fig. 2) of hesperidin may be used.

### Acknowledgments

We wish to thank Mr. R. Hendrickson for helpful discussions of problems in the development of the method.

The skilled technical assistance of Mr. J. Costo is gratefully acknowledged.

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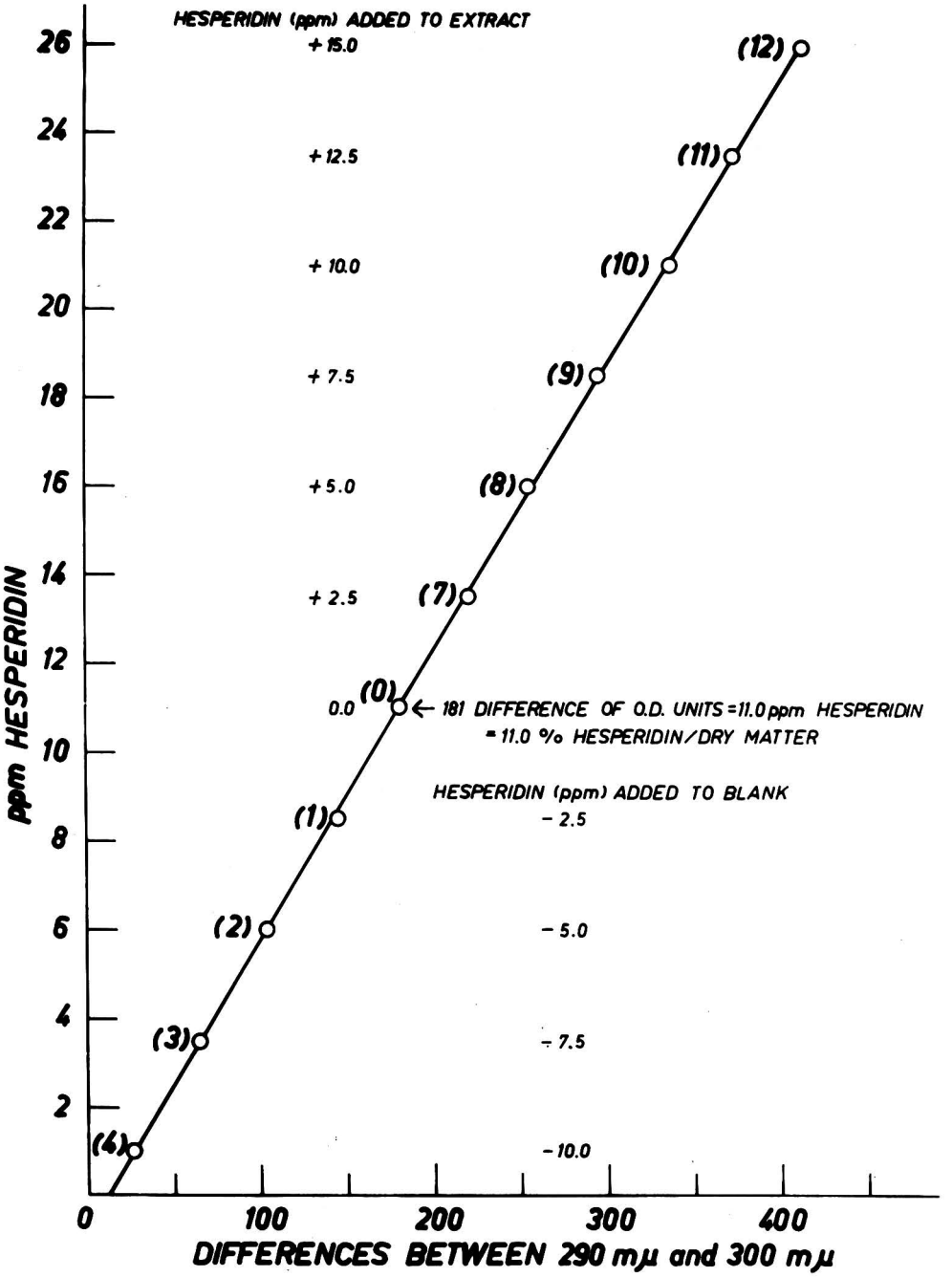


*Fig.* 2—Standard curve of hesperidin (numbers in parentheses refer to curves in Fig. 1).

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# RADIOACTIVITY

## Iodine-131 in Foods

By RICHARD J. VELTEN (Division of Radiological Health, Robert A. Taft Sanitary Engineering Center, 4676 Columbia Parkway, Cincinnati, Ohio)

**Two collaborative studies have been conducted on the determination of I-131 in milk. In the first study, eight collaborators analyzed four samples ranging from 58 to 227 pc/L I-131, but results were inconclusive. In the second study, 15 collaborators reported results for two samples. Results were much better. A third study is planned, on the basis of which a method will be proposed for adoption.**

At the October 1962 meeting of the AOAC a report (1) on methodology for the determination of iodine-131 in foods was presented. This report was quite general and discussed the problems inherent in both the physical and chemical methods of analysis. It also described some of the available methods of analysis, their sensitivities, and their accuracies. The data on the first set of referee samples, which were not available for the first report, are presented here.

The first set of samples submitted for iodine-131 determination was prepared according to the design described by Youden (2) in which, for the ideal case, a plot of the observed values versus the control values on log-log paper would delineate a straight line of slope unity, the points of which would be equidistant from each other. This set consisted of four milk samples containing iodine-131 concentrations of 58, 91, 144, and 227 pc/L as well as the radionuclides barium-140,

**Table 1. I-131 results from first collaborative study, pc/L**

| Coll. | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
|---|---|---|---|---|
| A | 90(90,90) | 165(170,160) | 45(40,50) | 135(140,130) |
| B | 60 ± 4 | 190 ± 7 | 50 ± 4 | 100 ± 7 |
| C | 96 | 231 | 57 | 143 |
| D | 105(99,111) | 245(235,255) | 70(70,71) | 147(173,121) |
| E | 56 | 142 | 47 | 99 |
| F | 97 ± 6 | 215 ± 11 | 54 ± 5 | 145 ± 9 |
| Control value | 91 ± 5[a] | 227 ± 11[a] | 58 ± 3[a] | 144 ± 7[a] |

[a] Calculated error includes error in the I-131 standard plus dilution error.

cesium-137, and potassium-40. The samples were prepared by counting a measured aliquot of an iodine-131 standard by gamma spectroscopy in an efficient geometrical configuration. After verification of a legitimate transfer of activity, the aliquot was mixed thoroughly into 76 L of milk free of iodine-131. Eight 4 L samples were removed for referee analysis; then 25.5 L of milk free of iodine-131 was added to the remaining volume of milk and mixed so that the resulting concentration was lowered by a factor of 1.58. Again, eight 4 L aliquots were removed for referee analysis. The third and fourth samples were also prepared by diluting the remaining volume of milk by a factor of 1.58 and withdrawing eight 4 L aliquots for each referee analysis. The four resulting sets were so labeled that no systematic numbering identified the rank of the various concentrations or suggested that the concentrations were unequal.

Eight collaborators were chosen who were known to be doing work in this field and whose experience would tend to validate the accuracy of the method. Each collaborator was asked to count for a 100-minute period the largest possible volume of milk for which adequate calibration data were known. The results of six collaborators for the first collaborative study are given in Table 1. The other two collaborators reported difficulties and did not submit data. The equations of the line of best fit as determined by the method of least squares are given in Table 2 and are represented graphically in Fig. 1. Since radioactive decay is a random phenomenon, the dotted area represents the over-all errors at the various concentrations. The over-all error includes the errors associated with the subtraction of background and the interfering radiations of the nuclides barium-lanthanum-140, cesium-137, and potassium-40 in the predominant iodine-131 gamma photopeak, as well as all errors associated in counting. This error has been defined by multiple analyses of samples of various concentrations at the 2 $\sigma$ confidence level to be 10 pc/L for iodine-131 concentrations of 100 pc/L or less and 10% for iodine levels of 100 pc/L or greater for the volume and time specified for this collaboration. Two collaborators (C and F) reported all the values that fall in the shaded area. A third collaborator's line of best fit (DD′) parallels the ideal line but is biased high; review of the individual points indicates an error, the origin of which could be in the background and/or calibration determination. Another collaborator's line of best fit (BB′) generally parallels the ideal line but the individual points show no systematic correlation to the control values, which indicates that something more than just calibration is amiss. No obvious conclusion as to the inconsistent results can be drawn from the other two

**Table 2. Equations of line of best fit for collaborative results**

| Coll. | Line of Best Fit | Equation |
|---|---|---|
| | OO′ (ideal line) | $y = x$ |
| A | AA′ | $y = 19.6 + 0.68x$ |
| B | BB′ | $y = -10.7 + 0.85x$ |
| C | CC′ | $y = 0.25 + 1.02x$ |
| D | DD′ | $y = 8.7 + 1.02x$ |
| E | EE′ | $y = 9.5 + 0.59x$ |
| F | FF′ | $y = 6.5 + 0.93x$ |

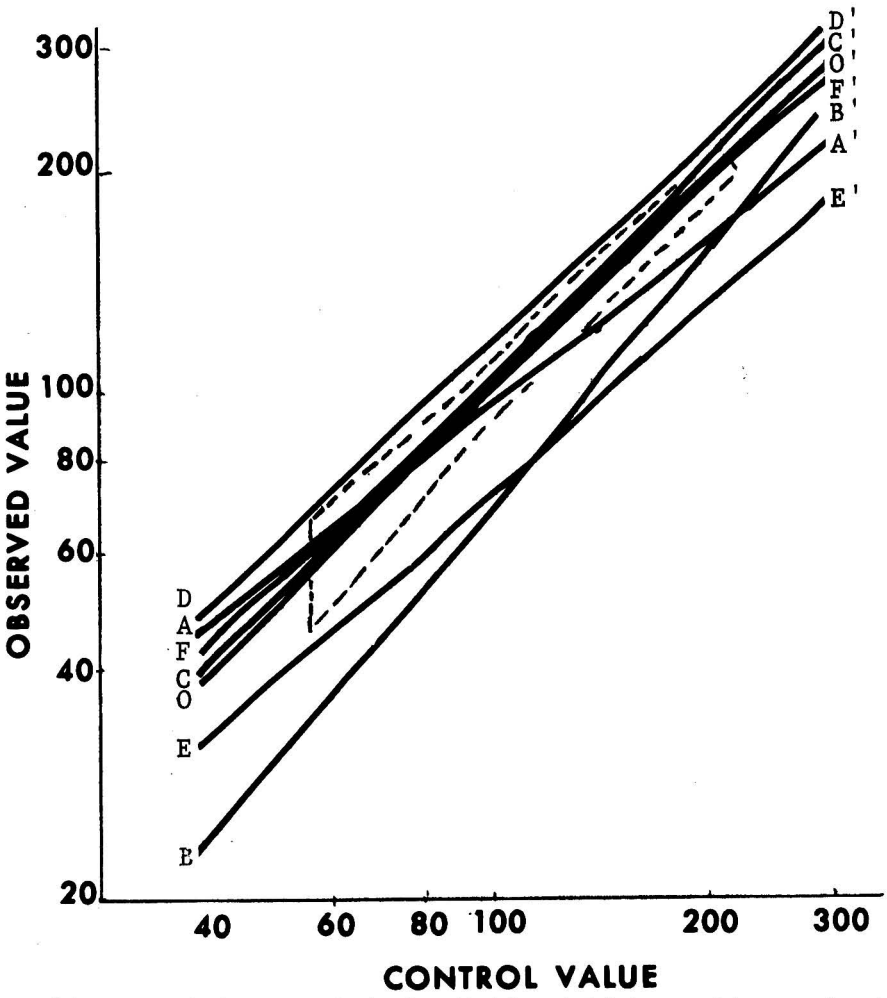


*Fig. 1*—Collaborative results for I-131 in foods. O = ideal line. A–E designate collaborators. Dotted areas = calculated over-all errors at various concentrations.

collaborators' data. Each participant was asked to review his analytical and data reduction methods for possible improvement.

Since the data obtained from the first collaborative study were not too conclusive, an attempt was made to increase the number of collaborators. As a result, 17 collaborators agreed to take part, 5 of whom had participated in the first venture.

Only two samples were used in the second collaborative study. These samples were prepared independently by counting a measured aliquot of an iodine-131 standard and transferring this aliquot to a measured 74 L of milk. After thorough mixing, nineteen 1-gallon aliquots were removed; the first and last aliquot were gamma-counted to test for homogeneity. The two samples agreed within 6%, the average being within 7% of the calculated control value. The collaborators were asked to analyze each sample three times for 50 minutes each so that counting and data reduction errors could be estimated. The results from 15 laboratories reporting triplicate values are given in Table 3. The sixteenth collaborator reported instrument malfunction, so that the validity of his data was voided. The seventeenth collaborator reported an average with an error term instead of individual values. The data from these two collaborators were therefore omitted.

In general, results are gratifying. The 2 $\sigma$

**Table 3. I-131 results of second collaborative study, pc/L**

| Coll. | Sample 1 | Sample 2 |
|---|---|---|
| A | 141(150,135,139) | 415(414,410,420) |
| B | 138(140,139,136) | 427(426,430,426) |
| C | 144(143,144,146) | 408(404,403,416) |
| D | 137(135,137,139) | 399(404,400,392) |
| E | 144(138,144,149) | 413(415,406,418) |
| F | 134(141,133,127) | 398(392,401,400) |
| G | 126(127,134,118) | 397(401,392,397) |
| H | 128(133,127,123) | 392(393,389,394) |
| I | 136(134,139,134) | 394(396,385,400) |
| J | 146(151,145,143) | 434(427,421,453) |
| K | 114(112,120,109) | 327(330,307,344) |
| L | Lost | 444(420,432,479) |
| M | 174(180,192,150) | 448(450,457,437) |
| N | 141(124,150,150) | 391(399,381,392) |
| O | 141(141,141,140) | 415(430,414,401) |
| Control value | 141 ± 7[a] | 402 ± 20[a] |

[a] Calculated error term includes error in the I-131 standard and dilution error.

"over-all" error, as previously defined, is used as a criterion. The 1 $\sigma$ error at the 141 pc/L level is ± 7 pc/L and at the 402 pc/L, ± 20 pc/L. Results of analysis of the data for accuracy are shown in Table 4. One collaborator is consistently out of the 3 $\sigma$ limit for both samples; this indicates an improper calibration. No assignable cause can be attributed to the other collaborator's values that are out of this limit. Only 8 collaborators report both results within ± 1 $\sigma$ and 11 with ± 2 $\sigma$.

A third collaborative study is planned, for which 10 of these laboratories will be chosen for a vigorous test of the method. The range of the extreme triplicate values will be used as a criterion. It is hoped that this third test will be sufficient to document the method. Contingent on the results of this test a method will be proposed for the measurement of iodine-131 by gamma spectroscopy.

It is recommended that study be continued.

**Table 4. Number of collaborators within the specified error terms**

| | Sample 1 | Sample 2 |
|---|---|---|
| Calculated control level, pc/L | 141 | 402 |
| Observed mean of all collaborators | 139 | 407 |
| No. of collaborators | 14 | 15 |
| Error term | | |
| ±1 $\sigma$ | 10 | 10 |
| ±2 $\sigma$ | 1 | 2 |
| ±3 $\sigma$ | 1 | 2 |
| >3 $\sigma$ | 2 | 1 |

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## FOOD ADDITIVES

# Determination of Polysorbate 80 in Bakery Products and Frozen Desserts

By MARTHA J. HALL (Food and Drug Administration, 3032 Bryan Street, Dallas, Texas 75204)

**A gravimetric method has been developed for determining polysorbate 80 at levels of 0.05 and 0.1% in bread, ice cream, and cake mix. The sample is hydrolyzed and saponified with hydrochloric acid and potassium hydroxide, respectively, and the liberated glycol is extracted with chloroform. The residue from the chloroform extracts is dissolved in water, and the fatty acids are extracted with petroleum ether. The glycol is then re-extracted from the aqueous solution into chloroform. The chloroform extract is evaporated to dryness, and the residue is dissolved in water and precipitated with phosphomolybdic acid. Recoveries at the 0.05% level ranged between 75 and 105%, and at the 0.1% level, between 85 and 107%.**

Food additive regulations (1) prescribe tolerances for certain polyoxyethylene esters as emulsifiers in ice cream, frozen desserts, cakes, and cake mixes. These emulsifying agents are sometimes added to shortening used in the production of bread (2).

Various methods have been described for the determination of polyoxyethylene esters (2, 4) and polyoxyethylene glycol (3). The former method is not quantitative but, under carefully controlled conditions, it is reproducible. The latter method determines only the polyoxyethylene glycol content present in polyoxyethylene(8)monostearate, a mixture of the glycol, monostearate, and distearate.

The procedure described below is a modification of the methods of Munsey (4) and Garrison, Harwood, and Chapman (2); nearly twenty times the amount of sample is used for analysis and changes have been made in the relative amounts of reagents. A sample is hydrolyzed with hydrochloric acid and saponified with potassium hydroxide. After this treatment, the glycol is extracted into chloroform, the chloroform evaporated to dryness, and the residue dissolved in water. Fatty acids are removed with petroleum ether extractions from the aqueous glycol solution, and the glycol is then extracted back into chloroform. The chloroform is evaporated to dryness, and the residue is dissolved in water and precipitated with phosphomolybdic acid in the presence of barium ions. Recovery studies were made by adding polysorbate 80 (polyoxyethylene-(20)sorbitan mono-oleate) to 10 g each of dried bread crumbs, finished ice cream, and packaged cake mix at levels of 0.05 and 0.1%. Final measurements were made gravimetrically.

## METHOD

### *Reagents*

(a) *Hydrochloric acid.*—Concentrated and 3*N*.

(b) *Potassium hydroxide.*—Analytical reagent grade.

(c) *Potassium carbonate.*—Anhydrous, analytical reagent grade.

(d) *Barium chloride dihydrate.*—10% solution.

(e) *Phosphomolybdic acid.*—10% solution (may be filtered if necessary).

(f) *Polysorbate 80 standard solution.*—1 mg/ml in water.

### *Procedure*

*Bread.*—Weigh 10 g dried, finely ground bread into a 500 ml Erlenmeyer flask. Add 10 ml water and 20 ml HCl and heat *in* steam bath for 10 min. with occasional swirling. Cool slightly and, while swirling and cooling under running tap water, add 20 g KOH at such a rate that the solution boils but does not spatter. Heat *in* steam bath for 30 min., add 120 g $K_2CO_3$, and heat for 30 min. more with

occasional stirring. Remove flask from steam bath and cool to room temperature.

Extract the nearly solid mass in the flask with five 50 ml portions of $CHCl_3$, and decant each $CHCl_3$ extract through a cotton plug wet with $CHCl_3$ into a 400 ml beaker.

Evaporate the $CHCl_3$ extract to dryness on a steam bath with the aid of a current of air. Dissolve the residue in water, warming if necessary, make acidic with HCl, and transfer to 125 ml separatory funnel with water (total water solution should be approximately 50 ml) and 30 ml petroleum ether. Shake vigorously, transfer the acidic solution to a second 125 ml separatory funnel containing 20 ml petroleum ether, and shake vigorously. Transfer the aqueous solution to a 250 ml separatory funnel and wash the petroleum ether extracts in the two separators, in succession, with one 50 ml portion of water. Combine the aqueous solutions in the 250 ml separatory funnel. (A drop of HCl may be added to the wash water to aid in the separation of the petroleum ether-water phases.) Extract the combined water solutions with six 25–30 ml portions $CHCl_3$ and filter each $CHCl_3$ extract through a cotton plug wet with $CHCl_3$ into a 400 ml beaker. Evaporate $CHCl_3$ extract to dryness on steam bath with the aid of a current of air, and add 250 ml water to the residue.

Heat the water solution to boiling; add, in order, 2 ml 3*N* HCl, 4 ml 10% barium chloride solution, and 4 ml 10% phosphomolybdic acid solution. Let stand overnight covered with a watch glass. Filter through a tared Gooch crucible, and wash precipitate with about 75 ml water. Rinse crucible and precipitate with a few ml of petroleum ether, dry in oven at 105° for 1 hour, cool, and weigh.

*Ice Cream and Cake Mix.*—Weigh a 10 g sample into a 500 ml Erlenmeyer flask. To ice cream, add 10 ml HCl; to cake mix, add 20 ml $H_2O$ and 20 ml HCl. Heat *in* steam bath for 10 min., swirling occasionally. Cool slightly and, while swirling and cooling under running tap water, add 10 g KOH to ice cream and 20 g KOH to cake mix. (Add the KOH cautiously so that the solution boils but does not spatter. Spattering may occur if there is not enough water in the flask; a little water may be added with a wash bottle before neutralization.) Heat *in* steam bath for 30 min.

Remove flask from steam bath and let it come to room temperature. Transfer to a 250 ml separatory funnel with water (total water solution should be about 50 ml) and 50 ml $CHCl_3$. Extract with a total of six 50 ml portions $CHCl_3$ and filter each $CHCl_3$ extract through a cotton plug wet with $CHCl_3$ into a 400 ml beaker.

Continue as in procedure for bread, beginning with the third paragraph: "Evaporate the $CHCl_3$ extract to dryness . . . . . ."

### *Standard Curve*

To prepare a standard curve, carry 0, 5, and 10 ml polysorbate 80 standard solution through the entire method. Plot mg ppt against mg polysorbate 80. The amount of polysorbate 80 in the sample is then read from the standard curve.

## Results and Discussion

Tables 1 and 2 give recovery data of polysorbate 80 standard solutions carried through the entire method and of polysorbate 80 standard solutions added to 10 g each of dried bread crumbs, finished ice cream, and packaged cake mix at levels of 0.05 and 0.1%.

The standard solutions of polysorbate 80

**Table 1. Recoveries of polysorbate 80 standard solutions and of polysorbate 80 standard solutions added to dried bread crumbs**

| Polysorbate 80 Std Solns | | Polysorbate 80 Std Solns Added to Bread | | % Recovery[a] |
|---|---|---|---|---|
| mg Added | mg Ppt | mg Added | mg Ppt | |
| 0 | 0 | 0 | 0.3 | |
| | | | 0.2 | |
| | | | 0.4 | |
| Av. | | | 0.3 | |
| 5.0 | 8.9 | 5 | 10.1 | |
| | 9.6 | | 10.8 | |
| | 9.4 | | 10.8 | |
| | 9.9 | | 9.3 | |
| Av. | 9.5 | | 10.3 | |
| Av.—Blank | | | 10.0 | 105.3 |
| 10.0 | 20.1 | 10 | 24.0 | |
| | 19.3 | | 24.0 | |
| | 19.2 | | 19.5 | |
| | 22.4 | | 20.7 | |
| Av. | 20.3 | | 22.1 | |
| Av.—Blank | | | 21.8 | 107.4 |

[a] Based on av. ppt wt from bread divided by av. ppt wt from standards alone.

**Table 2. Recoveries of polysorbate 80 standard solutions and of polysorbate 80 standard solutions added to finished ice cream and to packaged cake mix**

| Polysorbate 80 Std Solns | | Polysorbate 80 Std Solns Added to Packaged Cake Mix | | % Recovery[a] | Polysorbate 80 Std Solns Added to Finished Ice Cream[b] | | % Recovery[a] |
|---|---|---|---|---|---|---|---|
| mg Added | mg Ppt | mg Added | mg Ppt | | mg Added | mg Ppt | |
| 0 | 0 | 0 | 0 | | 0 | 7.3 | |
| | | | 0 | | | 5.5 | |
| | | | 0 | | | 6.5 | |
| | | | | | Av. | 6.4 | |
| 5.0 | 13.1 | 5.0 | 10.0 | | 5.0 | 16.3 | |
| | 13.7 | | 10.4 | | | 16.2 | |
| | 13.5 | | 10.0 | | | 16.8 | |
| | | | 9.5 | | | 17.6 | |
| Av. | 13.4 | | | | | | |
| | | Av. | 10.0 | 74.6 | Av. | 16.7 | |
| | | | | | Av.−Blank | 10.3 | 76.9 |
| 10.0 | 24.8 | 10.0 | 20.5 | | 10.0 | 30.7 | |
| | 25.7 | | 21.8 | | | 32.7 | |
| | 25.7 | | 21.8 | | | 30.8 | |
| | | | 22.4 | | | 29.2 | |
| Av. | 25.4 | | | | | | |
| | | Av. | 21.6 | 85.0 | Av. | 30.9 | |
| | | | | | Av.−Blank | 24.5 | 96.5 |

[a] Based on av. ppt wt from cake mix and from ice cream divided by av. ppt wt from standards alone.
[b] The initial $CHCl_3$ extractions were made from an acidic solution rather than from a basic solution because of the difficulty with emulsions and the low recoveries from the cake mix determination.

were carried through the entire procedure for bread with the following exceptions:

(1) The $K_2CO_3$ added varied from 20 to 120 g.

(2) The $CHCl_3$ extractions were made in separatory funnels and varied from four to six extractions.

It was observed that the larger the amount of $K_2CO_3$ used and the greater the number of $CHCl_3$ extractions made, the higher the recoveries.

The standard solution used for the bread recoveries was prepared in ethanol while the standard solution used for ice cream and cake mix recoveries was prepared in water. The weight of the precipitate from the standards in water was greater than that from the standards in ethanol. No explanation for this was found. However, this does not affect the results, since the recoveries from samples and standards are compared with the same standard solution.

Saturating the reaction mixture with $K_2CO_3$ in the bread determination gave a more favorable partition of the polysorbate 80 into the $CHCl_3$. However, in the ice cream and cake mix determinations, a nearly solid mass could not be achieved with the $K_2CO_3$ and its presence caused emulsions which made $CHCl_3$ extractions practically impossible. Therefore, no $K_2CO_3$ was added, the volume of water was kept to a minimum, and more $CHCl_3$ extractions were made. Recoveries were greater when the initial $CHCl_3$ extractions were made from an acidic solution as in the determination of the ice cream (Table 2).

It was found that the complete cleanup, i.e., extraction of fatty acids with petroleum ether and re-extraction of the glycol into

$CHCl_3$, was necessary to eliminate other substances which formed a precipitate with phosphomolybdic acid.

Although only polysorbate 80 was used in this study, this method should be applicable to all the polyoxyethylene esters, since their chemical properties are similar.

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## DRUGS

# Colorimetric Determination of Oxyphenisatin and Oxyphenisatin Diacetate in Pharmaceutical Preparations

By ANTOINE MAJOR, JR. (Food and Drug Administration, 3032 Bryan Street, Dallas, Texas 75204)

**A method is described which will quantitatively determine 0.1 mg oxyphenisatin or the diacetate in various pharmaceutical preparations. After removal of interferences by organic solvent extractions from aqueous solution and partition column chromatography, the reaction of oxyphenisatin (diacetate) with silver nitrate in alcoholic sodium hydroxide solution produces a violet solution, which follows Beer's law (1–15 μg per ml). The method was satisfactorily applied to the assay of commercial tablets, liquids, and powders with recoveries, as per cent found of declared, in the range 95–101%.**

Oxyphenisatin, 3,3-bis(*p*-hydroxyphenyl) 2-indolinone, and the diacetate; a white, odorless, tasteless, crystalline solid, are therapeutically used as laxatives (1–5). Previous colorimetric methods have used the addition of potassium ferricyanide to oxyphenisatin diacetate in basic solution to form a red solution which follows Beer's law (1–5 μg per ml) (6–8).

This paper describes a method for the separation and assay of oxyphenisatin and the diacetate by the addition of silver nitrate in basic solution to form a violet solution which follows Beer's law. The method is applicable to pharmaceutical liquids, tablets, and powders and can be performed rapidly and simply on small amounts of sample suitable for pharmaceutical control work. The sensitivity is about the same as for the potassium ferricyanide reaction.

In the proposed method, tablet samples are dissolved in water and alcohol. Powder samples, which form a mucilaginous mass with water because of the presence of sodium carboxymethylcellulose or Plantago seeds, are percolated with alcohol for several hours to remove the oxyphenisatin (diacetate) from the bulk of the excipients.

The amount of oxyphenisatin (diacetate) taken for the remainder of the analysis should be kept at a minimum because of limited solubilities in chloroform and ethyl ether.

The rest of the proposed method involves extractions with alcoholic sodium hydroxide from ethyl ether to remove neutral and basic interferences, ethyl ether extractions from an acidic solution to remove the oxyphenisatin (diacetate), partition chromatography on a sodium carbonate-diatomaceous earth column to remove strongly acidic interferences, and reaction with silver nitrate to form a colored soln.

Oxyphenisatin diacetate can be separated from oxyphenisatin by extracting the oxyphenisatin diacetate with chloroform from an acidic solution and percolating the chloroform extracts through a sodium carbonate-diatomaceous earth column. Chloroform will extract all of the oxyphenisatin diacetate and allow it to pass through the column while any oxyphenisatin that is extracted will be retained on the column. Ethyl ether will extract the remaining oxyphenisatin from the acidic solution and carry it through the column together with oxyphenisatin retained on the column from the chloroform wash.

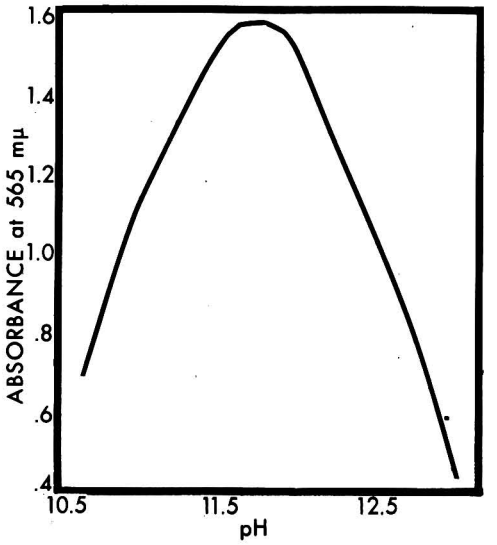


*Fig. 1*—**Effect of pH on the absorption spectrum at 565 mμ of the violet solution. (0.2 mg oxyphenisatin, 10 ml alcohol, 4 drops silver nitrate solution, made to 25 ml in volumetric flask with various normalities of NaOH.)**

A violet solution is produced when oxyphenisatin or the diacetate reacts with silver nitrate in alcoholic sodium hydroxide solution. The color reaction proceeds slowly; maximum color development is obtained in about 1 hour. The reaction solution is usually cloudy because of the presence of silver hydroxide but can be easily clarified by the addition of ammonium hydroxide. Ammonium hydroxide will not only clear the reaction solution but will also stop the reaction at any time during color development. The amount of ammonium hydroxide added to clarify the solution is not critical and an excess does not tend to destroy the color.

The color reaction was found to be stable and reproducible. Critical factors in color development were found by investigation to be pH, concentration of alcohol, and time (Figs. 1–3).

The method was satisfactorily applied to the assay of commercial tablets, liquids, and powders with recoveries, in terms of per cent found of declared, ranging between 95 and 101% (Table 1).

## METHOD

### *Reagents and Apparatus*

(a) *Alcohol.*—USP grade.

(b) *Ethyl ether.*—Analytical reagent grade.

(c) *Sodium carbonate solution.*—1*M*. Dissolve 10.6 g $Na_2CO_3$ in 100 ml water.

(d) *Sodium hydroxide solutions.*—0.1*N* and 0.010*N*.

(e) *Silver nitrate solution.*—0.1*N*. Dissolve 1.8 g $AgNO_3$ in 100 ml water.

(f) *Oxyphenisatin (diacetate) standard solution.*—1 mg oxyphenisatin or the diacetate in 100 mg alcohol.

(g) *Diatomaceous silica support.*—Celite 545 (Johns-Manville, New York 16, N.Y.).

(h) *Chromatographic columns.*—See 32.133 (b); with tamping rod consisting of disk of stainless steel, aluminum, etc., of diameter 1 mm less than i.d. of column, attached to 12–18" rod.

### *Preparation of Chromatographic Column*

Place a small wad of glass wool and about 0.5 g dry Celite 545 in the bottom of the chromatographic column. Add 2 ml sodium carbonate solution, (c), to 2 g Celite 545 in a beaker. Mix thoroughly with a spatula until the mixture appears fluffy and uniform, and transfer to the chromatographic column. Tap the sides of the column gently to let the mixture settle and press down firmly with a packing rod. Cover with a pad of glass wool to prevent disturbance of the surface.

### *Preparation of Assay Solutions*

*Tablets.*—Weigh and finely powder not less than 20 tablets. Weigh accurately a portion of the powder, equivalent to about 5 mg oxyphenisatin (diacetate), and transfer to a 100 ml volumetric flask with 30 ml water. Warm on a steam bath for 30 min. with frequent agitation. Cool, add alcohol to volume, and mix.

*Liquids.*—Use as is.

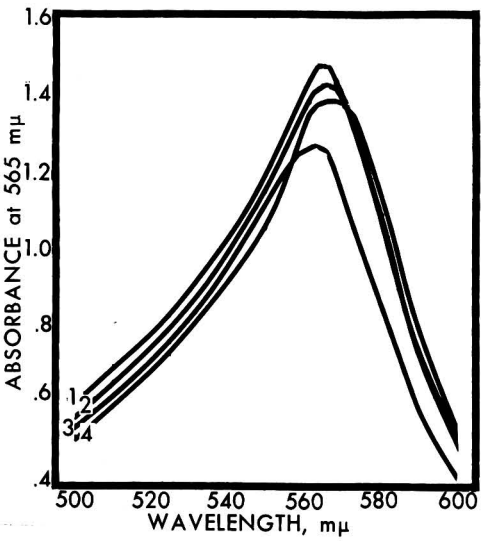


*Fig.* 2—Effect of concentration of alcohol on the absorption spectrum from 500 to 600 mμ of the violet solution. (0.2 mg oxyphenisatin, 4 drops silver nitrate solution, 1.5 ml 0.10N NaOH, various concentrations of alcohol, made to 25 ml in volumetric flask with water.) *1,* 10 ml; *2,* 15 ml; *3,* 5 ml, and *4,* 20 ml..

*Powders.*—Weigh accurately a portion of the powder, equivalent to about 5 mg oxyphenisatin (diacetate), transfer to the thimble of a Soxhlet or similar extractor, and percolate slowly with alcohol for at least 5 hr. Evaporate the alcoholic extract to about 50 ml on a steam bath, cool, and quantitatively transfer the extract to a 100 ml volumetric flask with alcohol.

***Removal of Interferences***

Pipet 10.0 ml of the assay solution into a 125 ml separatory funnel; for liquids, use a volume equivalent to 0.5 mg oxyphenisatin (diacetate). Add enough 0.1*N* NaOH and alcohol so that the volume of water will be 15 ml and alcohol 10 ml. Add 20 ml ethyl ether and shake vigorously. Let the layers separate, and transfer the lower aqueous alcoholic layer to another 125 ml separatory funnel. Extract the ethyl ether again with a solution consisting of 15 ml 0.1*N* NaOH and 10 ml alcohol. Combine the aqueous alcoholic layers; discard the ethyl ether. Add 50 ml water to the combined aqueous alcoholic extractions, and make the solution acidic with HCl. Extract the oxyphenisatin (diacetate) with five 20 ml portions of ethyl ether, letting each extract percolate through the sodium carbonate-Celite 545 column before the next ethyl ether extraction is added to the column. Evaporate the combined ethyl ether extracts to dryness, quantitatively transfer the residue to a 50 ml volumetric flask with alcohol, and make to volume (final assay solution).

***Separation of Oxyphenisatin and Oxyphenisatin Diacetate***

If quantitative separation of oxyphenisatin and the diacetate is desired, extract the oxy-

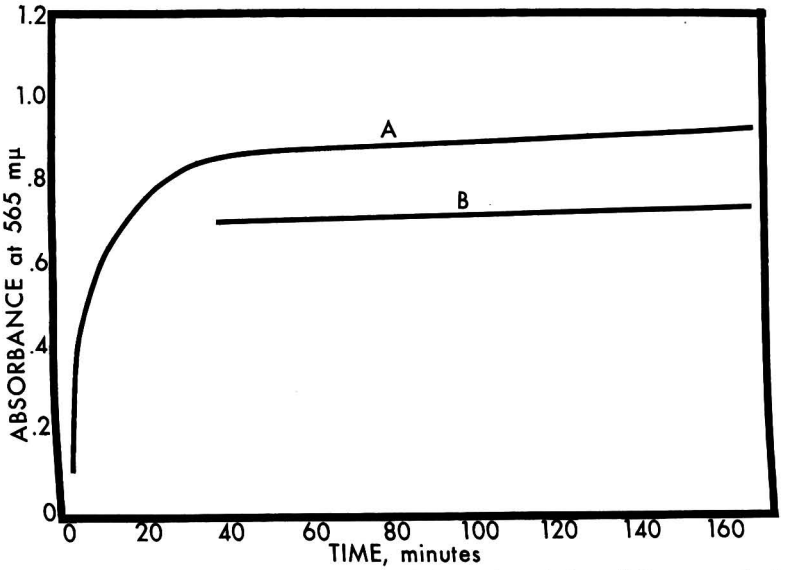


Fig. 3—Effect of time on the absorbance at 565 mμ of the violet solution. (0.2 mg oxyphenisatin, 10 ml alcohol, 4 drops silver nitrate solution, made to 25 ml in volumetric flask with 0.010N NaOH). A = no $NH_4OH$ added; B = $NH_4OH$ added.

**Table 1. Per cent found of declared oxyphenisatin (diacetate) in commercial pharmaceutical preparations**

| Type of Prepn | Declared | Found, mg | % Found of Declared |
|---|---|---|---|
| Tablet | 5 mg/tab (250 mg) | 4.96, 4.98 | 99.2, 99.6 |
| Tablet | 5 mg/tab (200 mg) | 5.03, 4.97 | 100.6, 99.4 |
| Powder | 3 mg/7 g | 2.87, 2.90 | 95.7, 96.7 |
| Powder | 5 mg/7 g | 4.91, 4.87 | 98.2, 97.4 |
| Liquid | 20 mg/180 ml | 19.0 , 19.2 | 95.0, 96.0 |

phenisatin diacetate from the acidic solution with ten 20 ml portions of chloroform, and let each extract percolate through the column. Evaporate the combined chloroform extracts to dryness and make to an appropriate volume with alcohol. This fraction contains only oxyphenisatin diacetate. Extract the oxyphenisatin from the same acidic solution with five 20 ml portions of ethyl ether and let each extract percolate through the column. Evaporate the combined ethyl ether extracts to dryness and make to an appropriate volume with alcohol. This fraction contains only the oxyphenisatin.

***Colorimetric Development***

(Standard and sample colorimetric developments should be performed at the same time.)

To 10.0 ml of the final assay solution in a 50 ml glass-stoppered Erlenmeyer flask, add 4 drops silver nitrate solution, (e), and 15.0 ml 0.010*N* NaOH. Stopper and shake the flask; then let the color develop for 90 min. The reaction solution first appears straw-colored, then turns violet. At the end of 90 min., add 1 drop ammonium hydroxide to clarify the solution. Measure the maximum peak absorption (565 mμ) of the solution against a blank prepared in the same manner in a 1 cm cell on the Beckman Model DU or by recording the visible spectral characteristics from 500 to 600 mμ, using a recording spectrophotometer.

Prepare a standard curve, using various amounts of the oxyphenisatin (diacetate) standard solution (from 0 to 10 ml) so that the final reaction solution contains a total of 10.0 ml alcohol, 4 drops silver nitrate solution, and 15.0 ml 0.010*N* NaOH. Let the color develop for 90 min.; then clarify with 1 drop ammonium hydroxide.

By comparing the absorbances of the standard and the sample, calculate the quantity of oxyphenisatin (diacetate) in the sample. The absorbance of the standard is proportional to the molecular weight of whichever standard is used.

**Table 2. Recoveries of oxyphenisatin from various excipients**

| Excipients[a] | % Recovered |
|---|---|
| 1 Stearic acid | 105.4 |
| 2 Calcium sulfate | 104.3 |
| 3 Talc | 103.3 |
| 4 Sucrose and cornstarch (2:1) | 100.8 |
| 5 Citric acid | 100.6 |
| 6 $KHCO_3$ | 97.6 |
| 7 $KHCO_3$, citric acid, and sucrose (1:1:1) | 95.3 |
| 8 Citric acid | 96.2 |
| 9 $KHCO_3$ | 99.2 |
| 10 $KHCO_3$, citric acid, and sucrose (1:1:1) | 98.4 |

[a] Water and alcohol preparation for all excipients, except excipients 8–10, which were Soxhlet prepared. Excipients 1–4 = 5 mg oxyphenisatin/200 mg excipient; excipients 5–10 = 5 mg oxyphenisatin/10 g excipient.

**Results and Discussion**

The method was satisfactorily applied to various commercial preparations. No difficulties were encountered once the tablet preparations were completely dissolved or the powder preparations percolated with alcohol. In the tablet and liquid preparations the basic alcoholic extractions from ethyl ether were not necessary. For powder preparations the basic alcoholic extractions were necessary to remove substances that interfered with the color reaction. Authentic sample preparations were made with sucrose, stearic acid, calcium sulfate, corn starch, potassium bicarbonate, citric acid, and talc as excipients. No interferences were obtained when these authentic samples were subjected to the method. Ten recoveries of oxyphenisatin, made with various excipients and mix-

tures of excipients, gave a range between 95 and 105% with a standard deviation of 3.42% (Table 2). The violet solution produced when oxyphenisatin is reacted with silver nitrate in basic solution is believed to be caused by complex formation: ammonium hydroxide, added to a basic solution of oxyphenisatin before the silver nitrate solution is added, will prevent the color from appearing; the violet reaction solution can be destroyed by acid, and an orange solid can be extracted with ethyl ether which gives an ultraviolet and infrared spectra similar to oxyphenisatin. Attempts at isolating the violet compound and obtaining the ultraviolet and infrared spectra have been unsuccessful.


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# ANTIBIOTICS

## Note on a Source of Unwanted Inhibition in Antibiotic Assays

By LESTER HANKIN (The Connecticut Agricultural Experiment Station, New Haven, Conn.)

In the assay for chlortetracycline by the AOAC method[1] the water of syneresis that forms during the incubation period is absorbed by tops made either of porcelain (glazed on one side) or of Brewer petri metal (aluminum), with filter paper inserts. Recently, new metal tops and inserts were purchased. The use of these tops and inserts brought about complete inhibition of the test organism, *Bacillus cereus.* When porcelain or glass tops were used, satisfactory growth was obtained. The use of a metal top with the insert removed also allowed good growth of the organism, indicating that the paper insert was to blame for the inhibitory effect. The use of a glass top with a paper insert confirmed this. When an inoculated plate covered with a metal top and paper insert was incubated for 24 hours, no growth occurred. If the insert was then removed (or a porcelain or glass top substituted) and the plate was incubated again, growth still did not take place, indicating that the agar had absorbed some of the toxic material.

When pieces of previously unused inserts were sterilized by autoclaving, or by dry heat while in contact with either a metal or a glass top, and were then placed directly on an inoculated agar surface, they were found to be inhibitory. Experience indicates that if the insert is repeatedly sterilized by dry heat in contact with the metal top, it becomes more inhibitory, as determined by the size of the zones of inhibition.

To see if the inhibitory effect is inherent only in the inserts, duplicate samples of several grades of common laboratory filter papers were sterilized by dry heat; one sample of each was in contact either with a glass or a metal top. None of the papers in contact with the glass were inhibitory, but some of the papers in contact with the metal showed zones of inhibition when placed on an inoculated agar surface. It would appear that the inhibitory material is predominantly inherent in the insert, but that a like response may be obtained during sterilization by action of the metal on certain filter papers. No attempt has been made to identify the toxic material.

This note is to call attention to an unusual phenomenon so that other investigators will be judicious in the selection of paper inserts used to absorb water of syneresis.

The technical assistance of Kirsti Grevskott is acknowledged.

[1] *Official Methods of Analysis,* 9th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1960, sections **33.057–33.062** and **33.071–33.074.**

## PESTICIDES

# Chromatographic Separation of Malathion Prior to Infrared Spectrophotometric Estimation

By R. C. DOUBLE (Government Chemical Laboratories, Mines Department, Western Australia)

**Column chromatography was used to separate malathion from other pesticide formulations prior to spectrophotometric measurement. Malathion is separated by a petroleum ether-acetone system on a Florisil column. The chromatographic procedure is applicable to technical preparations, emulsifiable concentrates, dusts, and water-based fruit fly bait.**

Colorimetric methods of malathion estimation were found to be unsatisfactory for commercial dusts containing malathion, DDT, and lindane; reproducible results could not be obtained. Column chromatography has therefore been used to separate malathion from pesticide formulations prior to infrared spectrophotometric estimation. With Florisil (magnesium trisilicate) as adsorbent, and a petroleum ether-acetone solvent system, malathion has been separated quantitatively from several types of formulations containing malathion alone, or malathion with DDT and/or lindane as other actives.

The chromatographic procedure was applied to technical preparations, emulsifiable concentrates, dusts, and water-based fruit fly baits containing protein hydrolysate. Malathion was estimated by infrared spectrophotometry, based upon absorption at 12.2 and 11.45 $\mu$ in acetonitrile solution and comparison with a standard graph prepared by subjecting weighed amounts of 95% technical malathion to the chromatographic procedure.

Sample solutions were prepared, and their absorbances measured, over a 5 day period. A linear relationship was found between malathion concentration and absorbance over the range 50–200 mg (Fig. 1).

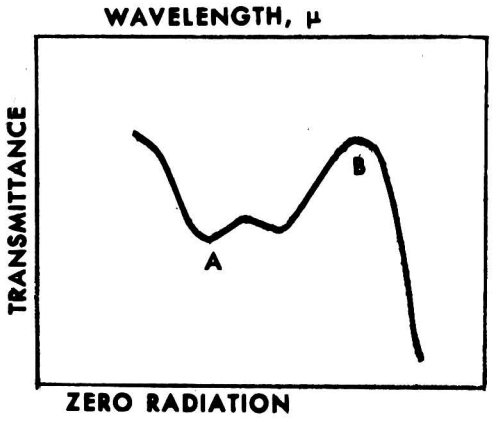


*Fig. 1*—Standard curve for malathion. (A = 12.2 $\mu$; B = 11.45 $\mu$.)

## METHOD

### *Apparatus and Reagents*

(a) *Spectrophotometer.* — A Perkin-Elmer Model 112 was used in this work.

(b) *n-Hexane* (or petroleum ether redistilled, b.p. → 70°C).

(c) *Acetone.*—Redistilled.

(d) *Acetonitrile.* — A nonalkaline solvent transparent in the 11–13 $\mu$ region. (May and Baker reagent was found to be satisfactory.)

(e) *Florisil.* — 60–100 mesh; activated at 1200°F as supplied by the Floridin Co., Tallahassee, Fla.

### *Preparation of Standard Curve*

Slurry 35 g Florisil in a beaker with about 75 ml petroleum ether. Transfer with a stream of petroleum ether to a chromatographic tube, leaving a head (about 10 cm) of petroleum ether above the adsorbent. Tap the tube to remove air bubbles and settle the adsorbent. Cover the adsorbent with a layer (about 2 mm) of anhydrous sodium sulfate.

Weigh 50, 100, 150, and 200 mg portions of 95% technical malathion into small beakers, and dissolve each in 5 ml petroleum ether. Pass each solution through a prepared column as follows: Adjust the flow rate of petroleum ether through column to about 2–5 ml/min.

Transfer sample solution to column with three 5 ml washings of petroleum ether. Add second and third washings just before level of previous washing drains below the surface of adsorbent. Then add 250 ml petroleum ether. Discard filtrate.

Add three 5 ml portions of acetone followed by 150 ml acetone, and collect filtrate in a wide-mouth flask. Let column drain. Evaporate to low bulk on a water bath, using a current of air. Transfer residue to a small evaporating dish, and evaporate to dryness.

### *Infrared Procedures (1, 2)*

Transfer residue to a 10 ml volumetric flask with acetonitrile, and make to volume with acetonitrile. Add a few crystals of anhydrous sodium sulfate, and mix thoroughly. Transfer to a 0.5 mm NaCl cell and scan over the 11–13 $\mu$ range, starting with the most concentrated solutions. Adjust the gain in each case to 95% at 11.45 $\mu$. Plot mg malathion against the ratio of the transmittance at 11.45 $\mu$ to the transmittance at 12.2 $\mu$, i.e., log B/A in Fig. 2. (This technique was found satisfactory with the Perkin-Elmer Model 112; other instruments would require a modified technique.)

### *Preparation of Sample*

*Dusts.*—Weigh a suitable amount of sample (10–20 g for dusts containing up to 10% malathion) into a stoppered graduated cylinder, and add 100 ml acetonitrile. Shake vigorously for 2 minutes, and let settle. Transfer as quantitatively as possible to a centrifuge tube and centrifuge until clear. Pipet a suitable aliquot (to contain 150–200 mg malathion) into an evaporating dish, and evaporate to dryness on a water bath, using a current of air.

Transfer the residue with three 5 ml portions of petroleum ether to a prepared column, and proceed as for standard samples.

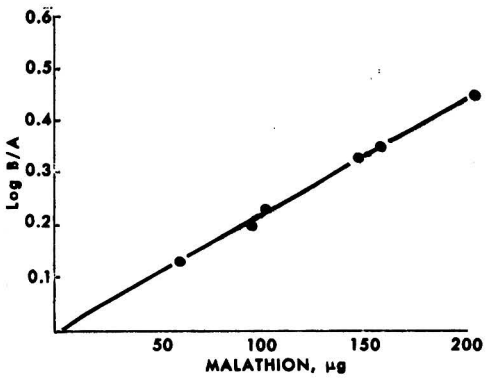


*Fig. 2*—Malathion absorption curve.

*Emulsifiable concentrates and technical preparation.*—Weigh enough sample to yield 150–200 mg malathion into a small beaker, and dissolve in 5 ml petroleum ether. (Concentrates not completely soluble in petroleum ether can be quantitatively transferred to the column by washing with benzene-petroleum ether (1 + 1).) Transfer solution to prepared column with three 5 ml portions of petroleum ether, and proceed as for standard samples.

*Fruit fly baits.*—(Prepared from malathion concentrates, water, and protein hydrolysate.) Extract suitable volume of sample with at least three 50 ml portions of benzene. Combine benzene extracts, discarding interfacial cuffs, and wash with water. Evaporate to dryness on a water bath, using a current of air, and dissolve residue in 5 ml petroleum ether.

Transfer solution to a prepared column with three 5 ml portions of petroleum ether, and proceed as for standard samples.

**Table 1. Typical analytical results for various malathion formulations**

| Sample | % Malathion Claimed | % Malathion Found | Other Actives, % |
|---|---|---|---|
| Dust[a] | 1.0 | 1.01 | DDT 1 |
| | | 0.94 | Lindane 0.13 |
| Emulsifiable concentrate | 20 | 19.7 | |
| | | 20.0 | DDT 20 |
| | | 19.7 | |
| Emulsifiable concentrate | 50 | 49.0 | |
| | | 48.5 | None |
| | | 49.7 | |
| | | 49.4 | |
| Emulsifiable concentrate | 50 | 46.6 | |
| | | 46.1 | None |
| | | 47.3 | |
| Technical malathion | 95 | 99 | |
| | | 100 | |
| Fruit fly bait | 0.32 | 0.25 | None |

[a] Synthetic dust sample prepared to contain 1% malathion.

## Results and Discussion

Since pure malathion was not available, the determination was made with a 95% technical malathion sample, manufactured

by Cyanamid Co., and supplied by a reputable formulator. (Whenever pure malathion is available, absorbance standards can be made directly in acetonitrile solution without prior chromatography.)

In preparing the standard curve, care should be taken during the evaporation from acetone to avoid loss of malathion. Temperature should be kept low during evaporation, and final drying can be accomplished under vacuum.

If DDT and/or lindane are present in the sample, they will be extracted with acetonitrile and will pass through the column in the petroleum ether, from which either can be recovered.

The chromatographic procedure separates malathion from DDT, lindane, and emulsifying agents. Pesticides containing other active materials should be tested for interference.

Recovery of the method was checked by preparing a synthetic dust containing 1% malathion, 0.13% lindane, and 1% DDT in a talc base. Results of the malathion estimation are given in Table 1.

REFERENCES

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## MEAT AND MEAT PRODUCTS

# Use of Enzymes in the Determination of Added Lactose in Meat Products Containing Corn Sirup Solids

By LESTER HANKIN and ALPHONSE F. WICKROSKI (The Connecticut Agricultural Experiment Station, New Haven, Conn.)

**An enzymatic method is proposed to overcome the difficulties encountered with the AOAC yeast method for the determination of lactose in prepared meat products which also contain corn sirup solids in addition to nonfat dry milk. The AOAC method does not allow for complete hydrolysis of the corn sirup solids, and, when reducing sugars are calculated as lactose, inflated values are obtained since some of the unhydrolyzed reducing material from the corn sirup solids is measured. In the enzyme method, a combination of crude maltase and glucose oxidase are used to remove all reducing sugars except lactose. Preliminary yeast treatment is included either routinely or to remove sucrose, if present. Comparative values obtained on prepared frankfort samples showed that the enzyme method was superior to the AOAC method.**

Until recently, skim milk powder (technically, nonfat dry milk) was the common additive to prepared meat products; dextrose and cereal or starch were sometimes added also, either as fillers or for flavoring. Federal regulations, as well as those of Connecticut, forbid the addition of more than a combined total of 3.5% of fillers for all types of sausage. For this reason, manufacturers and control laboratories require reliable methods to determine skim milk powder and other fillers.

The analysis of meat products for added skim milk powder is generally a straightforward procedure based on the determination of lactose (1, 2). In this method, which has proved to be satisfactory over the years, the yeast *Saccharomyces cerevisiae* removes all reducing sugar except lactose by selective adsorptive action; the lactose is then determined by copper reduction.

Satisfactory results were obtained by this method until some manufacturers of prepared meat products such as frankforts began to add corn sirup solids as well as skim milk powder to the product. Corn sirup solids generally contain dextrose, maltose,

and dextrins (3). The *Saccharomyces cerevisiae* method for the analysis of lactose in such products fails to give satisfactory results, since the values obtained—and calculated as skim milk powder—are in excess of the actual or declared amounts. The yeast does not ferment or adsorb all of the corn sirup solids, and when the reducing sugars are calculated as lactose, the result is obviously in error since some of the reducing material from the corn sirup solids is included in the determination. To compensate for this development in manufacturing technology, a method using yeast acclimated to maltose was adopted as first action by the AOAC (2). The acclimated yeast is assumed to be capable of fermenting or adsorbing all of the corn sirup solids. Several AOAC referee reports discuss the background of this procedure (4–6) and note that further work is needed to improve the method.

In our laboratory (and other laboratories) the AOAC method has continually given inflated values for skim milk powder in frankforts and similar products when they also contain corn sirup solids. Furthermore, if the sample is aerated during the incubation period, we have obtained results with the regular or unacclimated yeast that are comparable to those with the yeast acclimated to maltose or even to corn sirup solids.

Therefore, we have investigated the possibility of using commercial enzyme preparations to hydrolyze all fermentable carbohydrates other than lactose.

## METHOD

### *Reagents*

(a) *Yeast suspension.*—Suspend 3 packages of commercial dried yeast (or equivalent in moist yeast cake) in about 100 ml water. Collect cells by centrifugation and wash twice with water. After final centrifugation, suspend the cells in 150 ml water. Freeze in 20 ml portions. Cells may be kept frozen for long periods of time.

(b) *Acetate buffer, pH 5.6.*—Prepare solutions containing 2.4 parts 1*M* acetic acid (57.75 ml glacial acetic acid/L) to 22.6 parts 1*M* sodium acetate (82 g anhydrous sodium acetate/L).

(c) *Antifoam.*—Any water-soluble antifoam known not to interfere with the test may be used. (Dow-Corning Silicone AF antifoam has been satisfactory.)

(d) *Enzymes.*—Glucose oxidase: 15,000 oxidase units/g; and maltase: standardized 600 *p*-nitrophenyl glucoside units. (Both may be obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.)

(e) *Enzyme suspension.*—Triturate together in water enough glucose oxidase and maltase for samples to be run on the same day. Use 25 mg glucose oxidase and 50 mg maltase for not more than 50 mg expected corn sirup solids in 50 ml aliquot of meat solution.

A convenient set-up for 5 samples is to triturate 125 mg glucose oxidase and 250 mg maltase in water, transfer to 25 ml volumetric flask, add trace of antifoam, dilute to volume, and shake well. Use 5 ml of this suspension for each 50 ml meat solution.

### *Procedure*

*Preparation of meat sample.*—Place 20 g sample in 200 ml volumetric sugar flask, add small amount of water, and break up by agitation. Add about 100 ml water and warm on steam bath 30 min., shaking frequently. Cool to room temperature. Add 4 ml HCl and dilute to volume, using bottom of fat layer as meniscus. Add 10 ml 20% phosphotungstic acid solution, mix well, and let stand few min. Filter through moist filter paper. (Centrifugation before filtration is recommended but not indispensable.) Pipet 160 ml filtrate into 200 ml volumetric flask and neutralize to pH 4.8–5.0 (chlorophenol red indicator). Dilute to volume and mix.

*Preliminary yeast treatment.*—Centrifuge 20 ml thawed yeast suspension in 100 ml centrifuge tube, and decant water. To the packed cells, add about 60 ml meat solution, and mix thoroughly. Incubate at 30°C for 30 min., with air bubbling through the mixture. Centrifuge to remove cells. Use 50 ml supernatant solution for enzyme treatment. (This preliminary yeast treatment may be omitted if sucrose is known to be absent from the sample.)

*Enzyme treatment.*—Add the following to a 200 ml volumetric flask: 4 ml pH 5.6 acetate buffer, 50 ml yeast-treated meat solution (or 50 ml original meat solution), 1–2 ml 1% water suspension of antifoam, and 5 ml enzyme suspension containing 25 mg glucose oxidase and

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50 mg maltase. (Total volume should not be more than 70 ml nor less than 50 ml in order to keep buffer concentration within optimum range for enzymes.)

Place flask in water bath so controlled that contents are kept at 39 ± 2°C. Bubble air rapidly through mixture for entire 3-hour incubation. If foaming is excessive, add additional antifoam.

After incubation, heat contents of flask just to boiling, remove from heat, and add 95% alcohol *slowly and carefully* with mixing, bringing nearly to volume. Cool to room temperature and adjust volume with water. Shake flask well, and filter contents through Whatman No. 2 filter paper. Transfer 100 ml filtrate (or any desired aliquot) to a beaker and evaporate on steam bath to about 20 ml or until alcohol aroma disappears. Do not permit to go to dryness. After evaporation, adjust volume to 50 ml.

*Reducing sugar determination.*—Determine reducing sugars on evaporated material (diluted to 50 ml) by Munson-Walker method, **29.038–29.039** (or any other applicable method).

*AOAC yeast method.*—Proceed as in **23.030–23.032**, but use acclimated yeast, and bubble air through the incubation mixture instead of shaking it.

## Results and Discussion

In experimental studies the following sequence or combination of enzymes was used to eliminate all reducing carbohydrates (except lactose) which might normally be found in prepared meat products: first, commercial maltase to hydrolyze the maltose to glucose; next, crude diastase, containing both $\alpha$ and $\beta$-amylases, to hydrolyze the dextrins to maltose and glucose; next, glucose oxidase to oxidize all of the glucose to gluconic acid, which is nonreducing. This treatment should leave lactose as the only remaining reducing sugar. In practice, a simpler system is satisfactory: commercial crude glucose oxidase contains enough contaminating enzymes to hydrolyze corn sirup solids to nonreducing material; and commercial crude maltase contains enough amylases as contaminants to hydrolyze the dextrins. Consequently, when amylase is added to a system containing crude glucose oxidase and maltase, no further benefit is derived.

In the present investigation a combination of maltase and glucose oxidase was used to determine lactose in prepared meat products containing both skim milk powder and corn sirup solids, and this enzyme combination coupled with a preliminary yeast treatment was used for products that also contain sucrose.

The amount of glucose oxidase needed was determined by testing increasing quantities on 100 mg of glucose and measuring the sugar remaining after 3 hours (Table 1). The level selected (50 mg of glucose oxidase per 100 mg of glucose) provides safety factors with regard to both concentration and time.

**Table 1. Oxidation of glucose by glucose oxidase[a]**

| mg Glucose Oxidase/ 100 mg Glucose | % Glucose Remaining After 3 hours Incubation |
|---|---|
| 0 | 100 |
| 1 | 58 |
| 5 | Trace |
| 10 | 0 |
| 20 | 0 |
| 40 | 0 |
| 100 | 0 |

[a] NBC; 15,000 oxidase units/g.

**Table 2. Increase in $Cu_2O$ due to hydrolysis of maltose by maltase[a]**

| mg Maltase/100 mg Maltose | mg $Cu_2O$ Found After 3 hours Incubation |
|---|---|
| 1 | 120.8 |
| 5 | 117.6 |
| 10 | 117.6 |
| 25 | 126.4 |
| 50 | 149.6 |
| 100 | 200.4 |
| 150 | 198.4 |
| 200 | 214.0 |

[a] NBC; Standardized 600 *p*-nitrophenyl oxidase units.

The amount of maltase was estimated similarly, with maltose as the substrate, by determining the increase of reducing material formed (Table 2). Maltose was converted to glucose satisfactorily with 100 mg of maltase. Since it had already been found that the crude glucose oxidase possesses maltase activity, this level was selected for use rather than a higher one to avoid overloading the system.

An incubation temperature of 39°C and a pH 5.6 acetate buffer were considered to be within the optimum range for the enzymes (7, 8). Enough antifoam must be used to prevent excessive foaming or frothing during the incubation period; such foaming may cause surface denaturation of the enzyme proteins. Alcohol is added after the incubation to precipitate the enzyme proteins; otherwise filtration is almost impossible with Gooch crucibles. A blank determination should be made on each batch of alcohol to check the presence of reducing substances.

To test the entire system, samples were prepared by adding combinations of skim milk powder, corn sirup solids, and dextrose to commercial frankforts known to include none of the additives, and these samples were analyzed for lactose. Several combinations were tested—even some with larger amounts than might normally be expected in commercially available products. Enzyme levels were kept constant to determine if the safety factor was adequate. The results of these tests are shown in Table 3. Enough enzyme was present to hydrolyze all of the corn sirup solids in the samples containing 2% of this material. When 4% of corn sirup solids was added, the percentage recovery of skim milk powder was somewhat high but still acceptable. This in no way invalidates the test, since a rerun would be made either with higher concentrations of enzyme or a smaller aliquot of meat solution in routine work where excessive skim milk powder is found. In any case, a level of 4% of corn sirup solids is more than twice that generally used in good manufacturing practice. Starch did not appear to affect the test.

Table 4 shows a comparison of the AOAC yeast method and the present enzyme method on similarly prepared samples. A preliminary yeast treatment was not used, since none of the samples contained sucrose. The yeast used for the AOAC method had been acclimated, and air was bubbled through the incubation mixture instead of shaking it. Data in Table 4 conclusively show that unhydrolyzed material is carried over and calculated as lactose when the AOAC method is used. The enzyme method in all cases yields results that are closer to theoretical recoveries. Where 4% of corn sirup solids is used, the percentage recoveries of skim milk powder are higher, and in practice a rerun should be made.

In both Tables 3 and 4 the standard deviation has been computed without deleting those samples with high recoveries. These should be omitted to give a more accurate picture of the reliability of the method under more usual circumstances. However, they were purposely retained to demonstrate any limitations which might be present.

In some prepared meat products sucrose is added either alone or combined with skim milk powder, corn sirup solids, or other fillers. The enzyme method for determining lactose in such products is limited, since the crude enzymes used cause partial inversion of the sucrose. When reducing sugars are calculated, some of the inversion products are thus included. If the enzymes were extremely pure, the sucrose would presumably not be altered and would remain nonreducing. Inclusion of invertase in the system causes complete inversion of the sucrose; this was ascertained on several samples by comparison with the AOAC method for inversion of sucrose. The glucose formed during the inversion is oxidized by the glucose oxidase present, but fructose is not altered. As yet, there is no simple *in vitro* enzyme system which will convert fructose to a nonreducing form. Therefore, to obtain valid results for lactose in products containing sucrose, the sucrose must be removed. This is effectively accomplished by including a 30 minute yeast treatment of the meat solution prior to the enzyme step. Since the yeast treatment is short, the amount of yeast used is greater than normally employed in the AOAC method. The amount of yeast needed was estimated by testing various amounts of yeast on selected samples containing a known amount of sucrose.

Table 5 shows a comparison of data obtained on prepared frankfort samples by the AOAC yeast method and the enzyme method with the preliminary yeast treatment. With samples containing no corn sirup solids, satisfactory values are obtained by both methods. However, the AOAC method gives

**Table 3. Recovery of skim milk powder added to commercial frankforts with enzyme hydrolysis of corn sirup solids**

| Sample Contains: | | | SMP[a] Found, % | Recovery, % |
|---|---|---|---|---|
| % SMP[a] | % CSS[b] | % Dext.[c] | | |
| 3.5 | 2.0 | 0 | 3.74 | 106.9 |
| 3.5 | 2.0 | 0 | 3.30 | 94.3 |
| 3.5 | 2.0 | 0 | 3.45 | 98.6 |
| 3.5 | 2.0 | 0 | 3.47 | 99.1 |
| 3.5 | 2.0 | 0 | 3.55 | 101.4 |
| 3.5 | 2.0 | 0 | 3.40 | 97.1 |
| 3.5 | 2.0 | 1.0 | 3.38 | 96.6 |
| 3.5 | 3.0 | 3.0 | 3.78 | 108.0 |
| 3.5 | 4.0 | 0 | 3.96 | 113.1 |
| 4.45 | 4.0 | 0 | 4.97 | 111.7 |
| 5.0 | 2.0 | 0 | 5.17 | 103.4 |
| 5.0 | 2.0 | 0 | 4.62 | 92.4 |
| 5.0 | 2.0 | 0 | 5.04 | 100.8 |
| 5.0 | 2.0 | 0 | 4.91 | 98.2 |
| 5.0 | 2.0 | 0 | 5.06 | 101.2 |
| 5.0 | 2.0 | 0 | 4.81 | 96.2 |
| 5.0 | 2.0 | 1.0 | 5.04 | 100.8 |
| 5.0 | 2.0 | 2.0 | 5.28 | 105.6 |
| 5.95 | 2.0 | 0 | 5.98 | 100.5 |
| 8.95 | 2.0 | 0 | 8.79 | 98.2 |
| | | | | Av. 101.2 ± 1.2% |

[a] SMP = skim milk powder.
[b] CSS = corn sirup solids.
[c] Dext. = dextrose.

**Table 4. Comparison of yeast and enzyme method on prepared frankfort samples**

| Sample Contains: | | | Found | | | |
|---|---|---|---|---|---|---|
| | | | Enzyme Method | | Yeast Method | |
| % SMP[a] | % CSS[b] | % Dext.[c] | % SMP[a] | % Recovery | % SMP[a] | % Recovery |
| 3.5 | 2.0 | 1.0 | 3.74 | 106.8 | 4.19 | 119.7 |
| 3.5 | 3.0 | 0 | 3.78 | 108.0 | 4.44 | 126.9 |
| 3.5 | 4.0 | 0 | 3.96 | 113.1 | 4.58 | 130.8 |
| 3.5 | 4.0 | 0 | 4.15 | 118.6 | 4.93 | 140.9 |
| 4.45 | 4.0 | 0 | 4.97 | 111.7 | 6.83 | 153.5 |
| 5.0 | 2.0 | 0 | 5.17 | 103.4 | 5.53 | 110.6 |
| 5.0 | 2.0 | 2.0 | 5.28 | 105.6 | 5.72 | 114.4 |
| 5.0 | 2.0 | 2.0 | 5.51 | 110.2 | 5.82 | 116.4 |
| 5.95 | 2.0 | 0 | 5.98 | 100.5 | 7.17 | 120.5 |
| 8.0 | 2.0 | 0 | 8.44 | 105.5 | 8.67 | 108.4 |
| 8.95 | 2.0 | 0 | 8.79 | 98.2 | 9.82 | 109.7 |
| | | | Av. 107.1 ± 1.8% | | | 122.9 ± 4.3% |

[a] SMP = skim milk powder,
[b] CSS = corn sirup solids,
[c] Dext. = dextrose,

**Table 5. Comparison of AOAC yeast method and enzyme method with preliminary yeast treatment on prepared frankfort samples**

| Sample Contains: | | | | | Found | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | AOAC Yeast Method | | Comb'n Yeast-Enzyme | |
| % SMP[a] | % CSS[b] | % Suc.[c] | % Malt.[d] | % Dext.[e] | % SMP[a] | % Recovery | % SMP[a] | % Recovery |
| 3.36 | – | 1.86 | 1.86 | – | 3.15 | 93.8 | 3.08 | 91.7 |
| 3.41 | – | 0.93 | 1.86 | 0.93 | 3.13 | 91.8 | 3.17 | 93.3 |
| 3.53 | – | – | – | – | 3.20 | 90.7 | 3.24 | 91.8 |
| 3.29 | 1.88 | 0.94 | – | – | 3.66 | 111.2 | 3.35 | 101.8 |
| 3.26 | 1.86 | 0.93 | – | 0.93 | 3.84 | 117.8 | 3.15 | 96.6 |
| 3.32 | – | 1.9 | – | – | 3.19 | 96.1 | 3.03 | 91.3 |
| 3.26 | 1.86 | 1.86 | – | – | 3.67 | 112.6 | 3.33 | 102.1 |
| 3.32 | – | 1.9 | – | – | 3.26 | 98.2 | 3.22 | 97.0 |
| 3.32 | 1.9 | – | – | – | 3.81 | 114.8 | 3.49 | 105.1 |
| 3.5 | 2.0 | 2.0 | – | – | 4.17 | 119.1 | 3.45 | 98.6 |
| 3.5 | 2.0 | – | – | – | 4.22 | 120.6 | 3.31 | 94.6 |
| 5.0 | 2.0 | 2.0 | – | – | 5.32 | 106.4 | 4.83 | 96.6 |
| | | | | | | Av. 96.7 ± 1.3 | | |

[a] Skim milk powder. [b] Corn sirup solids. [c] Sucrose. [d] Maltose. [e] Dextrose.

inflated values for skim milk powder when corn sirup solids are added (see Table 4). The mean and standard deviation are not shown for the AOAC yeast method results, since the figure is not meaningful. Acclimated yeast was not used in this test, but air was bubbled through the mixture during the 3 hour incubation period. As previously noted, the method with unacclimated yeast and aeration, and the AOAC method are equally satisfactory. The preliminary yeast treatment has no adverse effect if samples do not contain sucrose. If samples are known to contain no sucrose, the yeast treatment step may be eliminated. Obviously, this is more practical for the manufacturer than for a regulatory laboratory, since sausage-type samples are highly variable as to declared carbohydrate content.

As additional advantages, the enzymatic method eliminated the somewhat arduous task of acclimating the yeast to maltose or corn sirup solids, and the dried enzymes, purchased commercially, can be kept for long periods of time at refrigeration or freezer temperatures.

Although the incubation period could be shortened, we believe that the longer incubation time affords a wide margin of safety if a sample should be unusually high in carbohydrates.

Crude enzyme preparations are used because they are always available. If highly purified enzymes were used, a differential assay for all sugars in the sample might be possible.

The method may be applicable to the determination of skim milk powder or lactose in other types of products. We have tried this procedure for the determination of skim milk powder in bread but, although the method looks promising, insufficient data are available for presentation.

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## FISH AND OTHER MARINE PRODUCTS

# Determination of Sodium and Potassium in Fish and Other Marine Products

By MARY H. THOMPSON (U.S. Bureau of Commercial Fisheries Technological Laboratory, Pascagoula, Miss.)

**Wet-ash and dry-ash extraction methods for the flame photometric determination of sodium and potassium in fish and other marine products were studied collaboratively. The wet-ash method was more accurate primarily because potassium was lost during the dry-ashing procedure at 550°C. Statistical analyses of precision indicate that wet-ashing is equal to or superior to dry-ashing. The Associate Referee, using the wet-ash procedure, obtained 98% recoveries for sodium and 102% for potassium, and relative standard deviations of 1.3–2.2 for sodium, and 1.5–3.6 for potassium. Over-all average recoveries obtained by the collaborators were 112% for sodium and 96% for potassium. Collaborative study will be continued.**

Flame photometry is generally conceded to be the method of choice for the determination of sodium and potassium, since it can easily be applied to a wide variety of materials with an accuracy of 1–2% (1). In applying flame photometry to the determination of sodium and potassium in fish and other marine products, we had to choose the most accurate method for solubilizing the biological material to be assayed. The methods listed in *Official Methods of Analysis* (2) for fertilizer (**2.067–2.075**) and plant materials (**6.016–6.019**) involve the use of ion exchange columns and water extraction, respectively, and are not suitable for animal materials. The methods most commonly employed in the past have been dry-ashing followed by extraction of the sodium and potassium with dilute nitric acid (3) and wet-ashing in which the material was extracted directly by concentrated nitric acid (4). Recently attention has been called to the loss of sodium and potassium during the dry-ashing, particularly at the high temperatures required to obtain a white ash from biological materials (5).

This report describes (a) a method for determining sodium and potassium in fish and other marine products by flame photometry, including both wet-ash and dry-ash methods of extraction; and (b) a study, both by the Associate Referee and by collaborating laboratories, to determine the possible loss of sodium and potassium during ashing procedures.

It is recommended that collaborative study of the wet-ash flame photometric method for the determination of sodium and potassium in fish and other marine products be continued.

### Procedure

#### *Apparatus*

(a) *Flame photometer.*—Preferably Beckman Model DU or equivalent.

(b) Borosilicate glassware and intact porcelain, Vycor, platinum, or silica crucibles precleansed with dilute $HNO_3$ and rinsed in distilled water, immediately before use.

#### *Reagents*

(a) *Distilled water.*—Water freed from sodium and potassium; either double-distilled or de-ionized.

(b) *Nitric acid.*—Reagent grade concd $HNO_3$ meeting ACS specifications.

(c) *Stock sodium standard.*—1 mg/ml. Dry reagent grade NaCl 2 hr at 110°C. Cool in desiccator. Weigh 2.5422 g into a 1 L volumetric flask and make to volume with distilled water.

(d) *Stock potassium standard.*—1 mg/ml. Dry and cool reagent grade KCl as above. Weigh 1.9068 g into a 1 L volumetric flask and make to volume with distilled water.

(e) *Dilute sodium standards.*—Prepare working sodium standards, 0.01, 0.03, and 0.05 mg per ml, as follows: pipet into 100 ml volumetric flasks 1, 3, and 5 ml stock Na standard; add 7 ml stock K standard and 2 ml concd $HNO_3$ to each flask; make to volume with distilled water.

(f) *Dilute potassium standards.*—Prepare working potassium standards, 0.04, 0.07, and 0.10 mg per ml, as above, using 4, 7, and 10 ml stock K standard, and adding 3 ml stock Na standard.

### *Wet-ash Method*

Prepare sample as in **18.001** (*Official Methods of Analysis* (2)).

Weigh 1 g sample into a 50 ml Pyrex beaker. Dry for 2½ hr at 110°C. Cool and weigh if % solids is to be determined.

*Samples with an unknown or a known high oil content.*—Add about 10 ml petroleum ether to each sample, and warm on a steam bath or a low temperature hot plate until oil is extracted. Decant and repeat until sample is defatted. Proceed as for low oil samples.

*Samples with low oil content.*—Add 2 ml concd $HNO_3$ to each beaker (if total chloride is desired, add enough 0.1*N* $AgNO_3$ as in **18.008**). Digest on steam bath or low-temperature hot plate until sample dissolves, adding more $HNO_3$ if necessary. Adjust final volume of digest to about 1 ml by heating or adding concd $HNO_3$.

Transfer digest to 25 ml volumetric flasks with hot distilled water. Wash down sides of beaker with distilled water, and add washing to flask; repeat twice. Cool and make to volume. Mix thoroughly, and let stand until particles settle (if necessary, centrifuge aliquot at 1000 × g to clear the solution). For total chlorides take aliquot and proceed as in **18.008**, beginning "Cool, add 50 ml $H_2O$. . . . "

### *Dry-ash Method*

Prepare sample as in **18.001**.

Weigh 4 g sample into a porcelain crucible. Char on electric hot plate or over low flame. Ash for at least 4 hr—longer if necessary—to white ash at 550°C in a muffle furnace. Cool, and weigh if total ash content is desired.

Add 15 ml (1 + 9) $HNO_3$ to each crucible, breaking up ash with stirring rod if necessary. Filter contents of crucible through Whatman No. 42 filter paper (or other suitable acid-washed paper) into 100 ml volumetric flasks. Wash crucible, residue, and filter paper three times with distilled water. Make to volume.

### *Flame Photometry*

Prepare blank by diluting 2 ml $HNO_3$ to 100 ml with distilled water.

Follow established procedure for type of flame photometer available. Dilute samples if necessary to bring transmittance readings within range of working standards. Read blank, standards, and samples at 589 mμ for sodium until results are reproducible, and record % T. Read blank, standards, and samples at 767 mμ for potassium until results are reproducible, and record % T.

### *Calculations*

For photometers not equipped with direct readout:

$$\text{mg Na or K/100 g} = 100 \times F \times \left(\left[\frac{(E_x - E_1)}{(E_2 - E_1)} \times (C_2 - C_1)\right] + C_1\right) / \text{g sample}$$

where $E_x$ = (% T of unknown) − (% T of blank); $E_1$ = (% T of standard of lower concentration than sample) − (% T of blank); $E_2$ = (% T of standard of higher concentration than sample) − (% T of blank); $C_1$ = mg Na or K per ml in standard of lower concentration than sample; $C_2$ = mg Na or K per ml in standard of higher concentration than sample; F = dilution factor.

## Experimental

The Associate Referee performed two experiments to test the precision and accuracy of the proposed method. Brown shrimp (*Penaeus aztecus*) meats were homogenized in a food grinder three times as in **18.001**, and a series of 12 replicates was removed for analysis by both wet-ash and dry-ash methods (Series I). A second sample of brown shrimp was prepared in the same manner, and a series of six replicates was removed for analysis by wet-ash and dry-ash methods (Series II A); concurrently, a second series of five samples was removed and a solution containing the equivalent of 100 mg of sodium per 100 g sample and 150 mg of potassium per 100 g sample was added prior to analysis by the wet-ash and dry-ash methods (Series II B).

Two additional samples, one of brown shrimp (*Penaeus aztecus*) and one of red snapper (*Lutianus blackfordi*), were prepared in this laboratory by **18.001** for the collaborative study. These samples were wrapped in aluminum foil, sealed in a tin

sardine-flat, and quick-frozen. One sample of each type was shipped frozen in Dry Ice to each of seven collaborating laboratories; three samples of each were retained by the Associate Referee. The collaborators were instructed to report the sodium and potassium values in triplicate by the wet-ash and the dry-ash methods, and to add an unknown solution equivalent to 50 mg sodium per 100 g sample and 150 mg potassium per 100 g sample to a second series of triplicates to be determined by both ashing methods.

Since the samples were of biological origin and particularly subject to loss of mineral constituents through exuded liquor, three samples of each type were analyzed by this laboratory, in accordance with the instructions given the collaborators, to determine the range of acceptable results and to provide comparable data for statistical measurement of accuracy.

## Results and Discussion

### *Comparison of Wet-ash and Dry-ash Methods*

In analysis of Series I, the mean value for sodium was 91 mg/100 g by wet-ashing compared with 86 mg/100 g by dry-ashing; the mean value for potassium was 150 mg/100 g by wet-ashing compared with 129 mg/100 g by dry-ashing. The difference between these means is significant at the 99.7% level.

Series II A and II B were analyzed by both methods to determine whether the error noted above was positive or negative. The mean potassium value by wet-ashing (126 mg/100 g) was significantly different from that by dry-ashing (96 mg/100 g) at the 99.7% level, as is the mean value for wet-ashing with added potassium (272 mg/100 g) compared to dry-ashing with added potassium (227 mg/100 g). The average recoveries were 102% for wet-ashing and 93% for dry-ashing. Thus, the difference between the means appears to be due to loss during dry-ashing—24% and 17%, respectively—as reported previously (5).

The results for sodium recovery, however, are not as clear-cut. The difference between the mean for sodium by wet-ashing (91 mg/100 g) and by dry-ashing (84 mg/100 g) is significant at the 99.7% level and indicates some loss (8%). At the same time, the difference between the mean for sodium content plus added sodium by wet-ashing (182 mg/100 g) is not significantly different from that by dry-ashing (183 mg/100 g). The recoveries averaged 98% and 101%, respectively. Apparently, any loss during dry-ashing is more than canceled through gain of sodium from other sources. Recent studies by Nesbett and Ames (6) show that sodium may be eluted by de-ionized water or KCl solutions from glassware that has been washed with nitric acid but allowed to remain unused for varying periods. The sodium appeared to be an original constituent of the glass, and the amount eluted depended on type of glass, temperature at which the glass was stored, and length of time between nitric acid wash and use.

Recoveries of sodium and potassium by wet-ashing were accurate enough for the method to be of value. Since flame photometry is accurate to 1–2%, most of the error involved probably originates in the determination in the flame.

In determining sodium, wet-ashing appears to yield more precise data. The differences between wet-ashing and dry-ashing may be governed by incidental outside contamination. The standard deviations for Series II A by dry-ashing do not differ as greatly from those by wet-ashing as the standard deviations for Series I; nevertheless, they show that in general, dry-ashing is not as precise as wet-ashing. The 95% confidence limits of the mean confirm this.

Recoveries of known amounts of material indicate that wet-ashing is the more accurate procedure. Comparison of the mean error and the relative mean error for potassium determination by the two methods confirm this (+4.4 vs. − 16.0; 3.1 vs. 10.9). With sodium, however, incidental outside contamination or other error obscures any preference between the methods based on accuracy alone, as the mean error and the relative mean error for wet-ashing is greater than that for dry-ashing (−3.6 vs +0.8; 3.8 vs. 0.8). These are the only values in favor of dry-ashing and run counter to other statistical indications.

As stated above, the wet-ash method of

determining sodium and potassium by flame photometry is precise and accurate enough to be of value. With a relative standard deviation of 1.3–2.2 for sodium and 1.5–3.6 for potassium, all sodium and potassium values will lie within 4–11% of the true mean 99.7% of the time and within 3–7% of the true mean 95% of the time.

In view of the above evidence, and to investigate further the apparent loss of potassium during dry-ashing, both methods were submitted to collaborating laboratories.

***Collaborative Study***

Samples were sent to each of seven collaborating laboratories. Three collaborators reported complete results and a fourth returned partial data. Results by both methods are reported in Tables 1 and 2. As mentioned above, frozen samples of this type often give considerable difficulty because fluid exuded from the sample as the material is thawed for analysis contains large quantities of soluble trace minerals such as sodium and potassium. The washed aluminum foil in which samples were wrapped to prevent contamination probably made it difficult for collaborators to properly remix the flesh and exuded liquor. Therefore, mean sample values, even from the same lot of material, might not agree closely from laboratory to laboratory. This collaborative study was useful, however, to test the accuracy of the two types of extraction procedures on the material to be worked with.

Two values reported for sodium content of red snapper were omitted in computing the average deviation and per cent error and in further computations, since they were outside the limits of 3 standard deviations from the mean in the expected population range. The ranges of the average deviation and the per cent error compare favorably with data obtained by the Associate Referee for three samples from the same lot.

With the exception of 4 sodium values and 1 potassium value (Collaborator D), the individual values submitted by the collaborators (total of 84) did not vary from the individual means by an amount greater than indicated by the 95% confidence interval estimated by the Associate Referee. Thus the precision of the methods remained essentially as determined by the Associate Referee.

The degree of accuracy to be expected from both extraction procedures is indicated by the average per cent recoveries and the over-all averages obtained by the collaborating laboratories, shown in Tables 1 and 2. Collaborators C and D typically obtained high sodium values with red snapper.

Collaborator C reported extremely high blank values for sodium (14 mg), which are probably a function of the instrument used for flame work. Collaborator A obtained low potassium recoveries for red snapper (wet-ash) and Collaborator D for shrimp. In general, however, the participating laboratories obtained acceptable recoveries with the wet-ash method. A decrease in per cent recovery of potassium and a slight trend toward decreased sodium recovery are discerned when the wet-ash and dry-ash methods are compared. The loss of potassium during dry-ashing indicated the unsuitability of this method of extraction.

Average per cent recoveries and other statistical measures are compared between the collaborating laboratories and the Associate Referee (on similar samples) in Table 3. The over-all average per cent recoveries of the collaborators show a general decrease in recovery of sodium from wet-ashing to dry-ashing, as well as a loss in potassium. The most obvious difficulty apparent in both methods is the addition of sodium through contamination, either through improperly cleaned glassware, leaching, or air-borne contamination. Great caution must be exercised in this regard.

The standard deviation, relative standard deviation, standard error, average deviation, and per cent error, also shown in Table 3, were computed on the basis of a series of grouped data. The Associate Referee's values are based on the three samples retained from the lot distributed among the collaborators. A comparison of the standard deviation, relative standard deviation, and standard error values for wet-ashing obtained by all laboratories with those for dry-ashing indicates either that there is no difference between the methods or else that wet-ashing is the more precise procedure.

**Table 1. Collaborative results for sodium in shrimp and red snapper by wet-ash and dry-ash methods**

| Species | Wet-Ash Method | | | | | Dry-Ash Method | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Av. Content, mg/100 g | % Error | Calc. Added, mg/100 g | Found, mg/100 g | Recovery, % | Av. Content, mg/100 g | % Error | Calc. Added, mg/100 g | Found, mg/100 g | Recovery, % |
| **Collaborator A** | | | | | | | | | | |
| Shrimp | 211[a] | – | 47[a] | 269[a] | 104[a] | 230 | 0.9 | 50 | 254 | 91 |
| | | | | | | | | 49 | 278 | 100 |
| | | | | | | | | 49 | 283 | 101 |
| Red snapper | 48[a] | – | 47[a] | 96[a] | 101[a] | 47 | 0.0 | 50 | 102 | 105 |
| | | | | | | | | 49 | 100 | 104 |
| | | | | | | | | 48 | 101 | 106 |
| Av. | | | | | 103 | | | | | 101 |
| Range | | | | | 101–104 | | | | | 91–106 |
| **Collaborator B** | | | | | | | | | | |
| Shrimp | 222 | 0.9 | 49 | 271 | 100 | 249 | 1.6 | 48 | 295 | 99 |
| | | | 45 | 267 | 100 | | | 47 | 283 | 96 |
| | | | 42 | 269 | 102 | | | 50 | 285 | 95 |
| Red snapper | 40[b] | 2.5 | 45 | 87 | 102 | 91 | 2.2 | 49 | 112 | 80 |
| | | | 51 | 99 | 109 | | | 49 | 136 | 97 |
| | | | 47 | 87 | 100 | | | 50 | 145 | 103 |
| Av. | | | | | 102 | | | | | 95 |
| Range | | | | | 100–109 | | | | | 80–103 |
| **Collaborator C** | | | | | | | | | | |
| Shrimp | 192 | 2.1 | 50 | 261 | 108 | 189 | 1.6 | 50 | 273 | 114 |
| | | | 50 | 272 | 112 | | | 50 | 277 | 116 |
| | | | 49 | 270 | 112 | | | 50 | 274 | 115 |
| Red snapper | 46 | 0.0 | 50 | 136 | 142 | 62 | 3.2 | 50 | 125 | 112 |
| | | | 49 | 134 | 141 | | | 50 | 136 | 121 |
| | | | 51 | 136 | 140 | | | 50 | 131 | 117 |
| Av. | | | | | 126 | | | | | 116 |
| Range | | | | | 108–142 | | | | | 112–121 |
| **Collaborator D** | | | | | | | | | | |
| Shrimp | 214 | 3.3 | 44 | 274 | 106 | 252 | 1.2 | 49 | 310 | 103 |
| | | | 41 | 265 | 104 | | | 50 | 304 | 101 |
| | | | 40 | 258 | 102 | | | 50 | 330 | 109 |
| Red snapper | 87 | 5.7 | 37 | 146 | 118 | 85[b] | 1.2 | 47 | 151 | 114 |
| | | | 40 | 147 | 116 | | | 47 | 145 | 110 |
| | | | 44 | 162 | 124 | | | 49 | 147 | 110 |
| Av. | | | | | 112 | | | | | 108 |
| Range | | | | | 102–124 | | | | | 101–114 |
| Over-all av. | | | | | 112 | | | | | 105 |

[a] Incomplete series reported.
[b] Average of two values.

**Table 2. Collaborative results for potassium in shrimp and red snapper by wet-ash and dry-ash methods**

| Species | Wet-Ash Method | | | | | Dry-Ash Method | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Av. Content, mg/100 g | % Error | Calc. Added, mg/100 g | Found, mg/100 g | Recovery, % | Av. Content, mg/100 g | % Error | Calc. Added, mg/100 g | Found, mg/100 g | Recovery, % |
| **Collaborator A** | | | | | | | | | | |
| Shrimp | 228[a] | — | 142[a] | 368[a] | 100[a] | 207 | 1.4 | 150 | 310 | 87 |
| | | | | | | | | 148 | 335 | 94 |
| | | | | | | | | 146 | 367 | 104 |
| Red snapper | 435[a] | — | 140[a] | 512[a] | 89[a] | 429 | 0.9 | 149 | 577 | 100 |
| | | | | | | | | 146 | 547 | 95 |
| | | | | | | | | 144 | 524 | 91 |
| Av. | | | | | 95 | | | | | 95 |
| Range | | | | | 89–100 | | | | | 87–104 |
| **Collaborator B** | | | | | | | | | | |
| Shrimp | 225 | 1.3 | 130 | 355 | 100 | 202 | 3.5 | 140 | 346 | 101 |
| | | | 163 | 372 | 96 | | | 150 | 322 | 92 |
| | | | 134 | 359 | 100 | | | 149 | 301 | 86 |
| Red snapper | 442 | 0.9 | 150 | 607 | 103 | 401 | 2.0 | 140 | 537 | 99 |
| | | | 154 | 613 | 103 | | | 148 | 542 | 99 |
| | | | 151 | 602 | 102 | | | 160 | 491 | 88 |
| Av. | | | | | 101 | | | | | 94 |
| Range | | | | | 96–103 | | | | | 86–101 |
| **Collaborator C** | | | | | | | | | | |
| Shrimp | 215 | 2.8 | 149 | 352 | 97 | 211 | 1.9 | 150 | 348 | 96 |
| | | | 149 | 341 | 94 | | | 151 | 346 | 96 |
| | | | 148 | 344 | 95 | | | 150 | 338 | 94 |
| Red snapper | 385 | 2.6 | 150 | 515 | 96 | 374 | 2.1 | 150 | 469 | 90 |
| | | | 148 | 513 | 96 | | | 149 | 469 | 90 |
| | | | 153 | 526 | 98 | | | 150 | 452 | 86 |
| Av. | | | | | 96 | | | | | 92 |
| Range | | | | | 94–98 | | | | | 86–96 |
| **Collaborator D** | | | | | | | | | | |
| Shrimp | 265 | 4.2 | 133 | 351 | 88 | 209 | 4.8 | 147 | 282 | 79 |
| | | | 124 | 336 | 86 | | | 150 | 297 | 83 |
| | | | 120 | 335 | 87 | | | 149 | 292 | 82 |
| Red snapper | 408 | 1.7 | 110 | 501 | 97 | 369 | 1.4 | 142 | 430 | 84 |
| | | | 120 | 498 | 94 | | | 141 | 431 | 85 |
| | | | 132 | 516 | 96 | | | 148 | 460 | 89 |
| Av. | | | | | 91 | | | | | 84 |
| Range | | | | | 86–97 | | | | | 79–89 |
| Over-all av. | | | | | 96 | | | | | 91 |

[a] Incomplete series reported.

**Table 3. Comparison of statistical measurements of precision and accuracy between data submitted by collaborators and the Associate Referee on similar samples of shrimp and red snapper**

| | Shrimp | | | | | | | | Red Snapper | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sodium | | | | Potassium | | | | Sodium | | | | Potassium | | | |
| | Wet-ash Method | | Dry-ash Method | | Wet-ash Method | | Dry-ash Method | | Wet-ash Method | | Dry-ash Method | | Wet-ash Method | | Dry-ash Method | |
| Measure | C[a] | A.R.[b] | C | A.R. | C | A.R. | C | A.R. | C | A.R. | C | A.R. | C | A.R. | C | A.R. |
| Std dev. | 5.9 | 2.8 | 3.4 | 3.7 | 9.2 | 5.5 | 7.6 | 7.5 | 3.5 | 1.1 | 1.9 | 5.1 | 8.4 | 11.3 | 7.7 | 14.6 |
| Relative std dev. | 2.8 | 1.2 | 1.5 | 1.6 | 3.9 | 2.3 | 3.7 | 3.7 | 6.0 | 2.2 | 2.7 | 9.6 | 2.0 | 2.5 | 2.0 | 3.6 |
| Std error of mean | 2.0 | 1.1 | 1.0 | 1.5 | 3.1 | 2.3 | 2.2 | 3.0 | 1.2 | 0.5 | 0.6 | 2.1 | 2.8 | 4.6 | 2.2 | 6.0 |
| Av. dev. | 4 | 2 | 3 | 3 | 7 | 4 | 6 | 7 | 2 | 1 | 1 | 4 | 7 | 7 | 6 | 12 |
| % Error | 2.1 | 0.9 | 1.3 | 1.3 | 2.8 | 1.7 | 2.9 | 3.5 | 2.7 | 2.0 | 1.7 | 7.5 | 1.7 | 1.6 | 1.6 | 3.0 |
| % Recovery | 105 | 100 | 103 | 94 | 94 | 104 | 91 | 98 | 119 | 107 | 107 | 108 | 97 | 101 | 91 | 96 |

[a] Statistical measures computed from all data submitted by collaborators.
[b] Statistical measures computed from data recorded by originating laboratory for same samples.

The relative standard deviation indicates good agreement between the collaborating laboratories and the Associate Referee, with the exception of the sodium values for red snapper. Apparently, this divergence can be attributed to a small amount of contamination when dealing with very low sodium values. The necessity for meticulous care of glassware and avoidance of air-borne contamination is effectively shown. Comparison of the values of the other precision measurements obtained by the collaborators and the Associate Referee indicates that the flame photometric method is reliable enough for determining sodium and potassium.

Since precision measurements indicate that precision of the wet-ash method is equal to or better than that of the dry-ash method, the choice between methods is dictated by the superior accuracy, particularly with regard to potassium, of the wet-ash method.


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This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, 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 *This Journal*, **47**, 127 (1964).

# Total Solids and Ether Extract in Fish and Other Marine Products

By H. M. RISLEY (Food and Drug Administration, 501 Federal Office Bldg., Seattle, Wash. 98104)

**The first action rapid modified Babcock method for crude fat in canned fish (18.013) was again studied collaboratively. Two samples of fresh (unfrozen) fish were examined by 6 analysts in 3 different laboratories. The same fish (frozen) together with samples of canned tuna, canned salmon, and a mixture of raw sablefish and canned tuna were examined by a total of 12 analysts in 6 different laboratories. Each analyst also determined fat by the official acid hydrolysis method (18.012). The results show that the rapid modified Babcock method (18.013) has the same degree of reliability as the acid hydrolysis method; it has been rewritten for greater clarity following the suggestions of the collaborators, and the revised form is recommended for adoption as official, final action.**

The first action rapid modified Babcock method for crude fat in canned fish (18.013) was studied collaboratively again this year. At the suggestion of the Association, the study included raw and frozen fish as well as canned fish. All samples were examined by the official acid hydrolysis method (18.012) as well as by the method under study. The rapid method (18.013) has been rewritten for greater clarity and changed slightly in accordance with suggestions received from collaborators. The results have been summarized in two tables.

### Experimental

Raw, unfrozen sole and sablefish were submitted to three laboratories. Two of the participating analysts in one laboratory obtained results both before and after freezing. Frozen sole and sablefish together with samples of canned salmon, canned tuna, and a mixture of canned tuna and raw sablefish were submitted to a total of 6 laboratories and 12 different analysts. All of these samples were frozen before shipment. In every case the lot of fish was ground three times with a conventional food grinder, with thorough mixing between grindings. After the last grinding it was vigorously mixed mechanically for 10 minutes before it was placed in pint jars. Collaborators were instructed to thaw and thoroughly mix the contents of each jar before analysis. Each sample was analyzed by method (18.013) and also by the official acid hydrolysis method (18.012).

## METHOD

(Applicable to raw, canned, and frozen fish)

### *18.013 Determination*

Weigh 9.0 g ground and mixed sample into Paley-type Babcock cheese bottle (Kimble Glass No. 508, 20% size), stopper, and add ca 30 ml of reagent prepd by mixing equal vols HOAc and 70–72% $HClO_4$. Place in $H_2O$ bath (2 L stainless steel beaker is satisfactory) maintained at 92 ± 2°, swirling occasionally until no lumps remain (usually ca 20 min.). Remove from bath, add reagent until fat is well up in calibrated neck of bottle, centrifuge 2 min. at ca 600 rpm, and read % fat with dividers, using bottom of top meniscus. If fat falls below calibration, add more reagent, centrifuge 1 min., and read again.

With very fat fish, it may be necessary to use less than 9 g sample. In this case, correct reading by multiplying % fat by factor 9/g sample.

### Results and Recommendation

Table 1 summarizes the results obtained on raw, unfrozen fish compared to results obtained on the same fish after freezing and thawing.

This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C, and was adopted by the Association. See *This Journal*, 47, 126 (1964).

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

**Table 1. Comparison of rapid modified Babcock method (18.013) with official acid hydrolysis method (18.012) on raw sole and sablefish, before and after freezing (figures represent per cent fat)**

| | Before Freezing | | | | After Freezing | | | |
|---|---|---|---|---|---|---|---|---|
| | Sole | | Sablefish | | Sole | | Sablefish | |
| Analyst | Rapid | Official | Rapid | Official | Rapid | Official | Rapid | Official |
| A | 2.5 | 3.18 | 21.2 | 23.55 | 2.1 | 3.29 | 22.0 | 22.82 |
| | 2.5 | 3.20 | 21.6 | 23.79 | 2.2 | 3.20 | 22.0 | 22.72 |
| | | | | 23.52 | | | | |
| B | 2.8 | 4.38 | 23.6 | 23.73 | 3.0 | 3.54 | 23.0 | 23.65 |
| | 2.8 | 4.40 | 24.0 | 24.74 | 3.0 | 3.58 | 22.8 | 23.02 |
| C | 2.3 | 3.00 | 23.6 | 23.60 | — | — | — | — |
| | 2.3 | 3.06 | 24.0 | 23.70 | | | | |
| D | 2.3 | 3.03 | 24.0 | 23.59 | — | — | — | — |
| | 2.4 | 3.05 | 24.0 | 23.49 | | | | |
| E | 3.0 | 3.15 | 23.9 | 23.28 | — | — | — | — |
| | 3.0 | 3.09 | 23.3 | 23.04 | | | | |
| F | 3.1 | 3.24 | 23.8 | 23.37 | — | — | — | — |
| | 3.0 | 3.22 | 23.6 | 23.31 | | | | |
| Av. | 2.67 | 3.33 | 23.38 | 23.59 | 2.58 | 3.40 | 22.45 | 23.05 |

**Table 2. Comparison of rapid modified Babcock method (18.013) with official acid hydrolysis method (18.012) (all samples frozen prior to analysis; figures represent per cent fat)**

| | Raw Sole | | Raw Sablefish | | Canned Tuna | | Canned Salmon | | 3 + 5 Mixture[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| Analyst | Rapid | Official | Rapid | Official | Rapid | Official | Rapid | Official | Rapid | Official |
| A | 2.1 | 3.29 | 22.0 | 22.82 | 8.2 | 8.79 | 15.4 | 15.66 | 13.5 | 14.07 |
| | 2.2 | 3.20 | 22.0 | 22.72 | 8.4 | 8.77 | 15.6 | 15.60 | 13.3 | 14.18 |
| | | | | | 8.4 | | | | | |
| B | 3.0 | 3.54 | 23.0 | 23.65 | 8.8 | 9.27 | 15.8 | 16.06 | 14.4 | 15.11 |
| | 3.0 | 3.58 | 22.8 | 23.02 | 8.8 | 9.32 | 15.7 | 16.24 | 14.5 | 15.16 |
| C | — | — | — | — | 8.0 | 8.84 | 15.0 | 15.90 | 13.8 | 14.70 |
| | | | | | 8.2 | 8.90 | 15.0 | 15.90 | 13.6 | 14.50 |
| D | — | — | — | — | 8.2 | 8.70 | 15.2 | 16.25 | 13.2 | 14.35 |
| | | | | | 8.2 | 8.80 | 15.4 | 16.25 | 13.2 | 14.39 |
| E | — | — | — | — | 8.4 | 8.98 | 14.0 | 16.06 | 15.6 | 14.55 |
| | | | | | 8.4 | 8.96 | 13.8 | 16.02 | 15.5 | 14.47 |

(*Continued*)

**Table 2.** (*Continued*)

| Analyst | Raw Sole | | Raw Sablefish | | Canned Tuna | | Canned Salmon | | 3 + 5 Mixture[a] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Rapid | Official | Rapid | Official | Rapid | Official | Rapid | Official | Rapid | Official |
| F | — | — | — | — | 8.3 | 8.83 | 15.6 | 15.69 | 13.9 | 14.60 |
| | | | | | 8.3 | 8.79 | 15.4 | 15.69 | 13.9 | 14.50 |
| G | 2.2 | 3.00 | — | — | 8.4 | 9.53 | 15.6 | 16.66 | 13.2 | 14.55 |
| | 2.2 | 2.95 | | | 8.4 | 9.40 | 15.7 | 16.87 | 13.25 | 14.38 |
| H | 2.2 | 3.15 | — | — | 8.6 | 9.35 | 15.0 | 16.25 | 12.8 | 14.68 |
| | 2.2 | 3.11 | | | 8.6 | 9.11 | 15.0 | 16.05 | 12.8 | 14.40 |
| I | 2.4 | 2.87 | 22.3 | 21.81 | 8.7 | 8.98 | 15.6 | 15.54 | 14.0 | 14.30 |
| J | 2.0 | 3.43 | 21.1 | 21.43 | 7.8 | 9.18 | 15.9 | 15.96 | 12.8 | 14.63 |
| K | 2.3 | 3.15 | 22.0 | 23.1 | 8.1 | 8.61 | 15.3 | 15.8 | 13.6 | 14.2 |
| | 2.2 | 3.09 | 21.8 | 23.3 | 8.2 | 8.65 | 15.4 | 15.6 | 13.9 | 14.3 |
| L | 2.2 | 2.29 | 22.9 | 23.3 | 8.3 | 9.15 | 16.6 | 15.9 | 14.0 | 14.7 |
| | 2.4 | 2.43 | 23.3 | 24.0 | 8.3 | 8.93 | 16.4 | 15.9 | 14.0 | 14.7 |
| Av. | 2.33 | 3.08 | 22.32 | 22.92 | 8.35 | 8.99 | 15.38 | 15.99 | 13.76 | 14.52 |

[a] Approximately 3 parts raw sablefish and 5 parts canned tuna.

Table 2 summarizes all results obtained on samples that had been frozen for preservation, and repeats the results on frozen fish in Table 1.

The results obtained are as consistent as those obtained last year. Table 1 shows that the rapid modified Babcock method (**18.013**) can be applied to raw, unfrozen fish as well as to frozen and canned fish. Both tables show that replicate results of individual analysts are as good by the rapid method as by the official method. The differences between analysts and laboratories may be due in part to difficulties in preparing uniform samples of ground fish.

It is recommended that the first action rapid modified Babcock method (**18.013**) for crude fat in canned fish, as rewritten in this report, be adopted as official, first action and that it be applicable to raw and frozen fish as well as canned fish.

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## ALCOHOLIC BEVERAGES

# Enzymatic Determination of Carbon Dioxide in Lightly Carbonated Wine—1963 Collaborative Study

By ROBERT L. MORRISON (E. & J. Gallo Winery, Modesto, Calif.)

**The titrimetric method for determining carbon dioxide in wine, using carbonic anhydrase, was studied collaboratively for the second year. Sample preparation was improved, and two modifications were made in the apparatus: a 5 ml instead of a 10 ml buret and a 25–30 ml automatic pipet instead of a 50 ml buret. Results were good, and it is recommended that the method be adopted as official, first action.**

With the advent of lightly carbonated wines it became necessary to determine the carbon dioxide in these products. The present first action method (1) was developed as a result of this necessity, but while it is an improvement over previous methods, collaborative studies show that a more straight-forward method would be desirable. Therefore the enzymatic method (2) was developed in 1961 and, subsequently, collaboratively studied in 1962 (3).

The results of the 1962 collaborative study were not as satisfactory as anticipated, but since most of the trouble was explained, it was decided that further collaboration was warranted.

### Collaborative Study

The samples used for this study were lightly carbonated wines procured as follows: an iron tank was partially filled with carbonated wine and 7 oz. bottles were submerged into the wine. When all of the bottles were in the tank, the liquid level of wine was approximately 3 inches over the open tops of the submerged bottles. This entire operation was carried out in a 0°C cold room. The tank was sealed and allowed to stand quiet in the cold room for 6 weeks. The samples were then removed and capped with crown caps.

The method, as originally published, remains unchanged with two exceptions: a 5 ml buret graduated in 0.01 ml is used for dispensing the acid solution rather than a 10 ml buret; and a 25 ml or 30 ml automatic pipet (Corning #96125 with Teflon stopcock) is used for dispensing the aliquot of sodium hydroxide rather than a 50 ml buret.

All apparatus and solutions were furnished to each collaborator. The pH meters used were the Radiometer model PM-22 and the Beckman Zeromatic. Beckman glass and reference electrodes were used on both pH meters.

### Results and Discussion

Table 1 shows the results obtained by each collaborator. Collaborator 4 reported that he went past the end point on Sample 3; his result was omitted from all calculations even though it did not vary far enough from the mean to justify the action. The last three results of Collaborator 2 were omitted from

**Table 1. Collaborative results for $CO_2$ content of lightly carbonated wines (mg/100 ml)**

| | Lab. 1 | Lab. 2 | Lab. 3 | Lab. 4 | Lab. 5 |
|---|---|---|---|---|---|
| | 215 | 220 | 220 | 224 | 221 |
| | 216 | 223 | 219 | 224 | 219 |
| | 212 | 221 | 220 | 220[b] | 220 |
| | 216 | 218 | 220 | 224 | 220 |
| | 212 | 220 | 219 | 225 | 221 |
| | 213 | 220 | 219 | 224 | 222 |
| | 220 | 223 | 219 | 223 | 220 |
| | 215 | 204[a] | 220 | 224 | 221 |
| | 214 | 206[a] | 219 | 223 | 222 |
| | 214 | 208[a] | 220 | 224 | 221 |
| Mean | 215 | 221 | 220 | 224 | 221 |
| Std Dev. | 2.4 | 1.8 | 0.7 | 0.6 | 1.0 |

[a] Not used in calculations.
[b] Collaborator reported he over-titrated; not used in calculations.

the calculation because they did vary far enough from the mean to justify their exclusion. A total mean difference of 9 mg $CO_2$/100 ml sample is not very large when the inexperience of all but the initiating laboratory is considered. Collaborator 3 had no previous experience with the method, and Collaborators 1, 2, and 4 had only the experience gained during last year's collaborative study.

### Recommendation

It is recommended that the enzymatic determination of carbon dioxide in lightly carbonated wine, as described in *This Journal*, **45**, 627 (1962), with the modifications mentioned in this report, be adopted as official, first action.

### Acknowledgments

The Associate Referee wishes to express thanks and appreciation to the following collaborators, without whose assistance this study would not have been possible:

C. S. Ough, Department of Viticulture and Enology, University of California, Davis, Calif.; Hershel Frye, Department of Chemistry, University of the Pacific, Stockton, Calif.; and the following members of the Alcohol and Tobacco Tax Laboratories: C. E. Hubach and Herman Meuron, San Francisco, and William Gowans, Seattle.

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This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted by the Association. See *This Journal*, **47**, 129–130 (1964).

# Determination of Esters in Whisky by Gas-Liquid Chromatography

By GLENN E. MARTIN, ROBERT L. SCHOENEMAN, and HOWARD L. SCHLESINGER (Alcohol and Tobacco Tax Division, Internal Revenue Service, Washington, D.C. 20224)

**A rapid and direct method has been developed for the determination of ethyl acetate in whisky by gas-liquid chromatography. Volatile esters are comprised mainly of ethyl acetate, but in addition, traces of methyl acetate are present.**

The official AOAC method for the determination of esters in distilled spirits (1) requires distillation of the esters, neutralization of the distillate, saponification, and titration. This method is not direct and errors may result from incomplete recovery of the distillate (2) or incomplete saponification (3).

Schneyder (4) found by gas-liquid chromatography that the main esters present in wine yeast oil were the ethyl esters of capric, lauric, and caprylic acid. Zientara, *et al.* (5) found that ethyl and amyl acetate were the predominant esters in American and foreign beers.

This paper presents a gas-liquid chromatographic method for the quantitative determination of ethyl acetate in whisky, and determination of the presence of additional esters.

### Procedure

An F & M Model 609 gas chromatograph with flame ionization detector was used for the ester determination. Components were separated on a column 27′ long × ¼″ o.d. containing Diatoport S (60–80 mesh) as the solid

**Table 1. Comparison of methods for the determination of ethyl acetate in whisky**

| Sample | Ethyl Acetate, g/100 L | |
|---|---|---|
| | Gas-Liquid Chromatography | AOAC Hydrolysis Method |
| Std | 22.5 | 23.8 |
| A | 41.4 | 42.2 |
| B | 52.3 | 53.6 |
| C | 16.8 | 18.0 |
| D | 15.4 | 17.5 |
| E | 33.2 | 33.0 |
| F | 33.5 | 34.9 |
| G | 36.2 | 37.6 |
| H | 36.9 | 37.8 |

support and 20% (w/w) Tide as liquid phase. Block and injection port temperatures were 100°C; the column was operated isothermally at 50°C. Helium was used as the carrier gas at a rate of 90 ml/min. Approximately 1 μl samples of standard and unknown solution were injected for the respective determinations.

The standard consisted of 22.5 g ethyl acetate/100 L (25 μl ethyl acetate/100 ml) in 40% ethyl alcohol solution. Samples were checked to insure absence of isopropyl acetate before isopropyl acetate was added to each at a concentration of 21.9 g/100 L sample (25 μl isopropyl acetate/100 ml sample). Thus, isopropyl acetate served as an internal standard for both standard and unknown solutions.

Determinations were made in the following manner: The individual ratio of ethyl acetate to isopropyl acetate was determined for both standard and unknown solutions as the quotient of the areas (triangulation method) beneath the curves of the respective acetates. Since the concentration of isopropyl acetate is the same for both standard and unknown solutions, the concentration of ethyl acetate/100 L sample can be determined as:

[(ratio of ethyl acetate to isopropyl acetate in unknown sample)/(ratio of ethyl acetate to isopropyl acetate in standard sample)] × (g/100 L ethyl acetate in standard solution) = g ethyl acetate/100 L.

The standard solution was run four times. Each sample was run in triplicate. Qualitative determinations were made by retention time and addition of known ester.

## Results

The ethyl acetate content of the samples (Table 1) was determined by both the AOAC and the GLC methods. The values by the AOAC method for Samples A–D are averages of the results obtained by 12 collaborators. The average standard deviation was 3.0 for these four samples. The values obtained by the GLC method checked within 5% of those obtained by the AOAC method, except for Samples C and D, which had low ester concentrations. These samples gave results 7 and 12% lower, respectively, by the GLC method.

Samples E–H are whiskies that had been routinely analyzed by the AOAC method by only one analyst. The values obtained by the GLC method checked within 2.3% of those obtained by the AOAC method.

Traces of methyl acetate were found in 6 samples. The relative retention times of methyl acetate and ethyl acetate to isopropyl acetate are 0.58 and 0.87, respectively.

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This paper was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

# A Simple Screening Procedure for the Detection of Traces of EDTA-Type Compounds in Beer

By IRWIN STONE (Wallerstein Company, Wallerstein Square, Staten Island, N.Y. 10303)

**A rapid and simple screening procedure is presented for detecting the presence of traces of EDTA-type compounds in beer. The procedure is based upon the color change of the red zinc dithizonate to the bluish-green of dithizone in the presence of strongly chelating compounds. The presence of a water-miscible organic solvent provides sufficient solubility to keep the colored aqueous-insoluble dithizonates dispersed and discernible.**

The introduction of traces of EDTA into beer as a sequestrant improves its resistance to oxidation and increases its shelf life. It also inhibits the formation of wild beer nuclei (1). The presence of an excess of these nuclei causes the beer to gush violently from the package when it is opened by the consumer. It is permissible to use up to 25 ppm of EDTA as the anhydrous calcium disodium salt in fermented malt beverages (2).

The presently available methods for the detection or determination of trace levels of EDTA in a material such as beer are rather lengthy and involved. In a recent paper by the author, it was found that traces of EDTA and related compounds interfered in the direct method for the determination of traces of zinc in beer (3). While this interference can easily be circumvented in the determination of zinc, the reactions involved can serve as the basis for a rapid and simple test for the presence of EDTA and related compounds in beer.

In the method presented in this paper, the red zinc dithizone complex dissolved in a water-miscible organic solvent is used as the reagent. This type of solvent avoids the many operations in the immiscible solvent extractions usually associated with the two-phase dithizone methods. The solvent provides sufficient solubility on dilution with beer to prevent precipitation, separation, and loss of visibility of the colored insoluble dithizonates. When this reagent is added to beer (10 ml reagent to 10 ml beer) the beer turns pinkish in the absence of EDTA compounds. When traces of EDTA are present, the zinc forms the more stable EDTA chelate and the beer assumes the greenish hue of the uncomplexed dithizone.

## EDTA-type Compounds in Beer—Qualitative Screening Test

### *Reagents*

(a) *Ethylene glycol monomethyl ether.*—(Available as "Methylcellosolve" from Carbide & Carbon Chemical Co.) Distill from an all-glass distillation equipment (b.p. about 125°C), discarding the first and last 5% distillate. Add 1 ml glacial acetic acid to each 100 ml distillate.

(b) *Dithizone solution.*—Dissolve 50 mg diphenylthiocarbazone (dithizone) in (a). When completely dissolved, dilute to 100 ml with (a).

(c) *Zinc solution.* — (*1*) Dissolve 4.398 g $ZnSO_4.7H_2O$ in water and dilute to 1 L (1 ml = 1 mg Zn). (*2*) Transfer 1 ml (*1*) to a 100 ml volumetric flask and dilute to volume with (a). 1 ml = 0.01 mg zinc.

(d) *Color reagent.*—Transfer about 40 ml (a) to a 100 ml volumetric flask, add 2 ml (b) and 5 ml (c)(*2*), mix, and dilute to volume with (a). Prepare fresh before use.

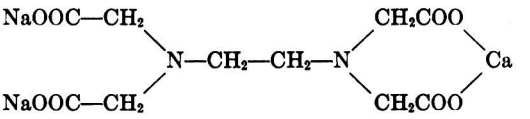
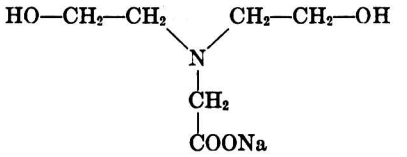
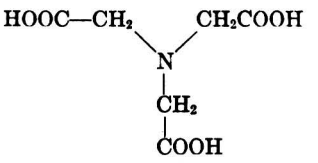
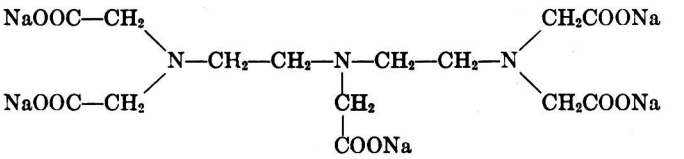
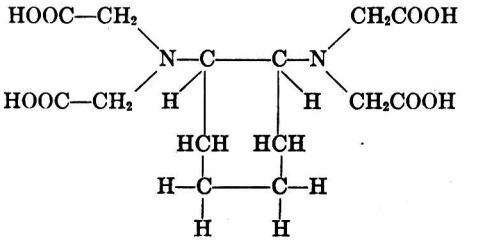
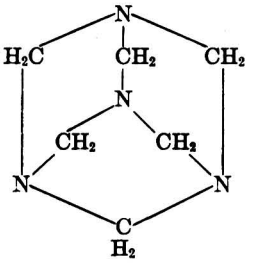
### *Determination*

Place 10 ml beer in large (25 × 200 mm) test tube. Add 10 ml *color reagent,* (d), and mix by swirling. Observe color (pink in the absence of EDTA compounds; various shades of

**Table 1. Color changes due to presence of EDTA in a typical beer**

| $Na_2CaEDTA$ Added, ppm | Visual Color |
|---|---|
| None | Grayish orange-pink |
| 1 | Darker grayish orange-pink |
| 2 | Dark bluish-pink |
| 3 | Grayish green |
| 4 | Green |
| >4 | Bluish green |

**Table 2. Response in screening test of structurally related amino carboxylic acid compounds and other nonrelated "chelating" agents**

| Material | Structure | Amount Added, ppm | Response in Screening Test |
|---|---|---|---|
| EDTA ($Na_2Ca$ salt) |  | 10 | Positive |
| Diethanol glycine (Na salt) |  | 25 | Negative |
| Nitrilotriacetic acid |  | 25<br>10 | Positive<br>Positive |
| Diethylene triamine pentaacetic acid pentasodium salt |  | 25<br>10 | Positive<br>Positive |
| Diaminocyclohexane tetraacetic acid |  | 25<br>10 | Positive<br>Positive |
| Hexamethylene Tetramine |  | 25 | Negative |
| Citric acid | | 25<br>500 | Negative<br>Negative |
| Gluconic acid (50%) | | 25<br>500 | Negative<br>Negative |
| Potassium acid saccharate | | 25<br>500 | Negative<br>Negative |

green, depending on amount of chelating agent, in the presence of EDTA-type compounds).

### Results and Discussion

*Sensitivity.*—The test has a visual color sensitivity of about 3 to 5 ppm, depending upon the beer. Typical color changes are listed in Table 1. With 3 ppm of $Na_2Ca$-EDTA the reagent changes from pink to a definite green; amounts below this level are discernible but the color change is not so striking.

The presence of abnormal amounts of trace metals in the beer will affect the sensitivity and response of the test. Iron or copper in amounts in excess of about 0.5 ppm will give off-shades of color but these amounts are not common in present American bottled beers. No interference is encountered at their normal levels (about 0.25 ppm or less). No interference from zinc would be expected, since zinc is present in beer only at very low levels (3). Rapid methods for determination of trace metals in beer are now available, so any suspect sample can easily be checked for metal interference.

*Specificity.*—This method is based solely on the strong chelating ability of EDTA to displace zinc from its colored chelate compound with dithizone. Thus, complete specificity could not be expected for a complex molecule like EDTA. The method appears to be fairly specific and responds positively only to amino carboxylic acid compounds closely related to the EDTA molecule (Table 2). Replacement of two of the carboxylic acid groups in a molecule such as nitrilotriacetic acid is a sufficient structural modification to cause a change in response of the compound in this test. Other so-called chelating agents like citrates, saccharates, or gluconates do not give a positive reaction, and no interference will be encountered from these materials.

If a positive reaction is obtained and exact identification of the chelating agent is required, other more complicated separation procedures would have to be employed for the complete identification.

The method is useful as a rapid qualitative screening test. It can be adapted as a quantitative procedure by reading the developed color in a photometer in the red region. The quantitative method, however, has certain drawbacks and objections, i.e., a single calibration curve covers only a rather narrow range of EDTA concentrations, a spread of no more than about 10 ppm. To calibrate a photometer for the range 0 to 50 ppm would require the preparation of five or six color reagents containing increasing increments of the dilute zinc reagent (c) (*2*). The calibration graph for this range would be a series of five or six S-shaped curves, each covering only about a 10 ppm segment with some overlap.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# Malt Beverages, Sirups, Extracts, and Brewing Materials

By IRWIN STONE (Wallerstein Company, Wallerstein Square, Staten Island 3, N.Y.)

**A review is given of the collaborative work conducted by the committees of the American Society of Brewing Chemists for the two methods (sulfur dioxide in beer and hop acids by spectrophotometry) previously presented for adoption by the AOAC. Eight new methods have been accepted by the ASBC for publication and are in various stages of editorial completion.**

In the previous report by the liaison officer for the American Society of Brewing Chemists and the Associate Referee on Malt Beverages (1), two methods published by the ASBC—the determination of total sulfur dioxide in beer and the determination of alpha and beta acids in hops by spectrophotometry—were recommended for adoption by the AOAC.

Before acceptance by the ASBC, collaborative work was conducted for several years on both methods.

*Total sulfur dioxide in beer.*—The work of the ASBC's sulfur dioxide committee for its first year, 1957, comprised a survey of available methods used for this determination by the industry (2). In 1959 the committee presented a comprehensive report (3) covering the collaborative analysis of beers by 11 collaborators using four different methods. The report of this committee for 1960 (4) gave further extensive collaborative results on a comparison of two colorimetric procedures with the rather cumbersome and insensitive Monier-Williams distillation procedure (5). It is difficult to briefly summarize the data of several years' work by these 11 collaborators on many samples of beer. Data from the 1960 report, containing the results of tests immediately preceding the Committee's final recommendation, are typical and appear in this paper as Tables 1 and 2. The Committee recommended adoption of the Stone-Laschiver colorimetric procedure (6) and publication of the method.

*Alpha and beta acids in hops.*—The ASBC Subcommittee on Hop Analysis, in its first report in 1956 (7), gave preliminary collaborative results on several methods and cited the need for clarification of the nomenclature of hop terminology. The 1957 report (8) gave the Subcommittee's tentative recommendations for hop nomenclature arrived at in conjunction with the corresponding committees of the European Brewery Convention. Comprehensive results were reported for the analysis of hops by 16 collaborators using gravimetric, spectrophoto-

**Table 1. Results of collaborative test (Spring 1960) on sulfur dioxide by various analytical methods, ppm**

| Coll. | Bottle No. | Sample A | | | Sample B | | |
|---|---|---|---|---|---|---|---|
| | | Method[a] | | | Method[a] | | |
| | | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | 1 | 6.9 | | 7.2 | 13.8 | | 13.5 |
| | | 6.4 | | 7.2 | 13.3 | | 13.3 |
| | 2 | 6.4 | | 6.3 | 12.9 | | 13.3 |
| | | 6.1 | | 6.1 | 13.1 | | 13.3 |
| 2 | 1 | 8.8 | 4.9 | 6.0 | 14.3 | 6.0 | 12.1 |
| | | 8.8 | 4.8 | 6.2 | 13.7 | 6.6 | 12.1 |
| | 2 | 7.9 | 3.7 | 5.6 | 14.3 | 7.2 | 11.9 |
| | | 8.8 | 3.2 | 5.8 | 14.2 | 6.4 | 12.2 |
| 3 | | 8.5 | 7.0 | 5.8 | 10.7 | 11.6 | 12.2 |
| | | 9.7 | | | 11.8 | | |
| 4 | 1 | 6.8 | 4.9 | 5.1 | 13.7 | 10.5 | 13.0 |
| | | 6.7 | 5.0 | 5.1 | 13.6 | 10.5 | 13.0 |
| | 2 | 6.7 | 5.1 | 5.1 | 13.1 | 9.9 | 13.0 |
| | | 6.0 | 4.9 | 5.2 | 13.5 | 9.9 | 13.2 |
| 5 | | | 7.2 | 7.8 | | 12.8 | 14.7 |
| | | | 6.6 | 7.0 | | 12.8 | 14.7 |

(*Continued*)

**Table 1.** (*Continued*)

| Coll. | Bottle No. | Sample A, Method[a] 1 | Sample A, Method[a] 2 | Sample A, Method[a] 3 | Sample B, Method[a] 1 | Sample B, Method[a] 2 | Sample B, Method[a] 3 |
|---|---|---|---|---|---|---|---|
| 6 | | 5.1 | 5.5 | 4.9 | 10.5 | 9.8 | 10.6 |
| 7 | | 6.1 | 5.3 | 6.6 | 12.2 | 7.9 | 13.4 |
| | | 6.1 | 3.8 | 6.5 | 12.2 | 7.1 | 13.8 |
| 8 | | 7.8 | 4.7 | 4.0 | 12.0 | 8.4 | 11.0 |
| | | 7.0 | 4.8 | 4.3 | 13.0 | 8.3 | 11.0 |
| 9 | 1 | 5.3 | 4.3 | 5.3 | 10.8 | 9.8 | 11.4 |
| | | 4.8 | 4.1 | 5.2 | 10.6 | 8.7 | 11.9 |
| | 2 | 6.3 | 3.4 | 6.2 | 10.7 | 10.0 | 11.5 |
| | | 5.8 | 4.2 | 5.7 | 11.1 | 9.5 | 11.7 |
| 10 | 1 | 5.5 | 9.9 | 6.6 | 10.6 | 9.7 | 14.2 |
| | | 3.5 | 6.0 | 7.3 | 10.5 | 9.7 | 14.2 |
| | 2 | 5.9 | 3.7 | 7.3 | | | |
| | | 5.9 | 7.8 | 7.3 | | | |
| Range: | | | | | | | |
| High | | 9.7 | 9.9 | 7.8 | 14.3 | 12.8 | 14.7 |
| Low | | 3.5 | 3.2 | 4.0 | 10.5 | 6.0 | 10.6 |
| Av. | | 6.6 | 5.2 | 6.0 | 12.4 | 9.2 | 12.7 |

[a] Method: *1*, Monier-Williams; *2*, Brenner-Owades-Fazio; *3*, Stone-Laschiver.

metric, polarimetric, and other procedures. The reports of 1958 (9) and 1959 (10) gave additional collaborative analytical data on the basis of which the method for determining alpha and beta acids in hops by spectrophotometry was published (1). The analytical data on three samples of hops by 18 collaborators using the spectrophotometric method, as published in the Committee's 1959 report, are given in this paper as Table 3. Benzene was the solvent of choice.

*Further studies.*—Eight more new methods have been adopted by the ASBC and are presently in various stages of editorial completion: (*1*) wort color by spectrophotometry; (*2*) enzyme method for corn grits; (*3*) assortment of corn grits; (*4*) measurement of total haze in beer after chilling; (*5*) and (*6*) beer foam evaluation by collapse rate of foam by the foam flashing method and the modified Carlsberg method; (*7*) and (*8*) microbiological examination of

**Table 2. Standard deviation of samples stored in quart containers**

| Sample | Method 1 | Method 2 | Method 3 |
|---|---|---|---|
| A (Av. $SO_2$, 6.6 ppm) | | | |
| Within bottles[a] | 0.54 | 1.27 | 0.25 |
| Among bottles[b] | 1.37 | 1.24 | 0.94 |
| B (Av. $SO_2$, 12.4 ppm) | | | |
| Within bottles[a] | 0.37 | 0.39 | 0.16 |
| Among bottles[b] | 1.35 | 1.81 | 1.16 |

[a] Std Dev. $= \sqrt{\left(\frac{1}{n}\right)\Sigma d^2}$

[b] Std Dev. $= \sqrt{\left(\frac{1}{n}\right)\Sigma\bar{X}^2 - \left(\frac{1}{n^2}\right)(\Sigma\bar{X})^2}$.

$\bar{X}$ is mean of results on same bottle (in one case by each method one result was reported, and this was used as mean).

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

**Table 3. % Total alpha acids by spectrophotometric methods[a]**

| Coll. | California 1958 | | | Hallertau 1957 | | | Bullion 1957 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Petroleum Ether | Benzene | Toluene | Petroleum Ether | Benzene | Toluene | Petroleum Ether | Benzene | Toluene |
| A | 6.5 | 6.1 | 3.8 | 0.9 | 1.9 | ... | 0.7 | 0.7 | 1.1 |
| B | 6.1 | 6.4 | 6.2 | 1.8 | 2.7 | 2.5 | 2.7 | 2.5 | 3.4 |
| C | 5.8 | 6.2 | 5.9 | 2.1 | 2.5 | 2.5 | 3.4 | 3.5 | 3.4 |
| D | 6.1 | 6.2 | 6.1 | 2.2 | 2.3 | 2.4 | 3.2 | 3.1 | 3.1 |
| E | 6.1 | 6.3 | 6.2 | 2.5 | 2.8 | 2.7 | 3.7 | 3.6 | 3.6 |
| F | 5.8 | 6.2 | 6.4 | 2.4 | 2.4 | 2.6 | 3.7 | 3.4 | 3.7 |
| G | 6.1 | 6.5 | 6.3 | 2.5 | 2.7 | 2.8 | 3.5 | 3.6 | 3.6 |
| H | 5.2 | 6.0 | 6.3 | 1.1 | 2.0 | 2.2 | 1.6 | 2.7 | 2.3 |
| I | 6.4 | 6.8 | 6.8 | 2.5 | 2.6 | 2.8 | 3.8 | 3.2 | 3.7 |
| J | 5.3 | 5.7 | 5.8 | 4.8 | 2.3 | 2.5 | 2.5 | 3.4 | 3.3 |
| K | 6.0 | 5.8 | 6.0 | 2.1 | 2.3 | 2.4 | 3.5 | 3.4 | 3.4 |
| L | 5.5 | 5.6 | 5.9 | 2.1 | 2.1 | 2.2 | 2.9 | 3.2 | 3.5 |
| M | 6.1 | 6.3 | 6.0 | 1.4 | 2.6 | 2.6 | 2.0 | 2.9 | 2.6 |
| N | 5.6 | 6.3 | 6.1 | 2.0 | 2.3 | 2.4 | 2.5 | 3.2 | 3.4 |
| Q | 5.9 | 6.5 | 6.0 | 1.9 | 2.8 | 2.6 | 2.3 | 2.8 | 2.6 |
| R | | 6.1 | | | 1.8 | | | 2.1 | |
| S | 5.6 | 6.0 | 6.3 | 2.3 | 2.2 | 2.2 | 2.8 | 3.0 | 2.9 |
| T | 6.2 | 6.5 | 5.7 | 2.2 | 2.1 | 2.6 | 2.8 | 3.1 | 2.8 |
| Mean % | 5.9 | 6.2 | 6.0 | 2.2 | 2.4 | 2.5 | 2.8 | 3.0 | 3.1 |
| AD | 0.33 | 0.24 | 0.36 | 0.50 | 0.27 | 0.48 | 1.10 | 0.46 | 0.49 |
| % AD | 5.6 | 3.9 | 6.0 | 22.7 | 11.2 | 19.2 | 39.2 | 15.3 | 15.8 |
| ADD | 0.13 | 0.18 | 0.13 | 0.06 | 0.10 | 0.13 | 0.07 | 0.11 | 0.15 |

[a] AD = average deviation among collaborators. % AD = average deviation among collaborators expressed as a percentage of the mean per cent alpha acids. ADD = average deviation between duplicate analyses obtained by each collaborator; duplicate values obtained by each collaborator are not tabulated.

yeast, wort, and beer by the gram staining procedure and the actidione plating technique. As soon as they are released by the ASBC, they will be forwarded to the AOAC.

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# Sodium and Potassium in Wines and Distilled Spirits

By HERMAN J. MEURON (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, 450 Golden Gate Ave., San Francisco, Calif.)

**Collaborative study of the official, first action flame photometric method of Pro and Mathers for sodium and potassium in wines and distilled spirits was continued and showed that this method is applicable also to sherry wine, fruit wines, and apple brandy. The sodium determination, using essentially simple dilution of wines and little or no dilution of distilled spirits, varied considerably among collaborators for concentrations below 100 ppm, but this is not considered objectionable for routine determinations of these products. Studies will continue to determine the applicability of the method to beer and related malt beverages.**

The flame photometric method for the determination of sodium and potassium in wines and distilled spirits, studied collaboratively in 1962, was adopted as official, first action by the Association (1, 2).

Collaborative study of the method was continued to make the sodium determination more accurate at the lower concentrations found in these products without resort to elaborate treatment or equipment. Fruit wines, an apple brandy, and a sherry were tested to see if the method could be extended to include these products.

Samples were furnished to collaborators in flint glass bottles with plastic screw caps and plastic liners. The samples were: *W*, apple brandy; *H*, sherry wine (composite of about two dozen sherries); *E*, loganberry wine; *M*, apple wine; *G*, blackberry wine. With the samples, collaborators were sent small portions of ACS grade NaCl and KCl in plastic bags to be used in preparing standards.

Collaborators were asked to follow the methods referred to above. In addition they were cautioned to be especially careful with the sodium determinations and "to wash all flasks, beakers, cylinders, pipets etc., with warm nitric acid and rinse well with distilled water; to pipet solutions with vacuum or suction bulb, not by mouth; and to adjust the dilutions of the samples so that % T falls on the straightest portion of the standard curve . . ." (The standard curve for sodium is not a straight line. The straight portion is usually between 2 and 8 ppm when the standards range from 1 to 10 ppm.)

### Results and Discussion

A preliminary recovery experiment by the Associate Referee indicated that the method should be applicable to sherry and apple wine. Experiments in which 100 ppm Na and 1000 ppm K were added to samples of these two wines showed a recovery of at least 90% in all samples and almost 100% in most.

Results reported by collaborators (Table 1) indicate that the method can be used for sherry and fruit wines and fruit brandy, since there are no substances in these products that interfere with the flame determination. Results also indicate that the sodium determination varies considerably when the concentration is below 100 ppm, despite the precautions used. This is not objectionable when the method is used for control purposes by wine and distilled spirits manufacturers or by government regulatory and enforcement agencies for routine checks.

Again, as previously reported, the "Unit Rise" concept of calculation gave no significant values for $T_a$ and $T_b$ which could be considered as zero, making "Unit Rise" equal to $T_{max}$. It should be noted that in many flame photometers this concept cannot be utilized; it is of value only for reference to the method in other chapters of *Official Methods of Analysis* for products which might have $T_a$ and $T_b$ values.

Some wineries are known to prepare the standards with higher concentrations of Na or K, adding alcohol and possibly other constituents in the range found in their products. While there is no objection to this practice, which is even desirable under cer-

**Table 1. 1963 Collaborative results on sodium and potassium in wine and distilled spirits, ppm[a]**

| | Sample W Apple Brandy | | Sample H Sherry | | Sample E Loganberry | | Sample M Apple | | Sample G Blackberry | |
|---|---|---|---|---|---|---|---|---|---|---|
| Coll.[b] | Na | K | Na | K | Na | K | Na | K | Na | K |
| 1 | 19 | 24 | 49 | 800 | 83 | 700 | 19 | 1114 | 157 | 788 |
| 2 | 18 | 24 | 43 | 880 | 76 | 700 | 19 | 1120 | 145 | 880 |
| 3 | 20 | 26 | 47 | 1032 | 83 | 869 | 20 | 1212 | 165 | 1015 |
| 4 | 20 | 23 | 43 | 910 | 75 | 720 | 18 | 1180 | 148 | 900 |
| 5 | 21 | 24 | 62 | 905 | 87 | 707 | 25 | 1130 | 159 | 892 |
| 6 | 21 | 24 | 57 | 880 | 91 | 725 | 25 | 1100 | 164 | 880 |
| 9 | 18 | (50) | 39 | 905 | 97 | 718 | 12 | 1092 | 170 | 866 |
| 10 | 25 | 26 | 50 | 910 | 93 | 730 | 21 | 1160 | 180 | 900 |
| 11 | 18 | 23 | 47 | 925 | 74 | 730 | 19 | 1150 | 160 | 910 |
| 12 | — | — | 49 | 921 | 87 | 736 | 32 | 1148 | 167 | 914 |
| Av. Less ( ) | 20 | 24 | 49 | 907 | 85 | 734 | 21 | 1141 | 162 | 895 |

[a] Collaborators' results averaged. Results in parentheses are not included in average. (Collaborator number corresponds with those in Table 1 of 1962 report (*This Journal*, **46**, 299 (1963)) for those participating for the second time.)
[b] Collaborators 1–5 and 12 used the Beckman DU; Collaborator 6, the Beckman B; Collaborator 9, the Coleman 21; Collaborators 10 and 11, the EEL Model A flame photometers.

tain conditions, it should be noted that when no dilutions are made, the effects of possible interferences are also heightened. For critical determinations, the dilutions, as described in the method, are to be preferred.

### Recommendations

It is recommended—

(1) That the flame photometric method for sodium and potassium, adopted last year as official, first action, be adopted as official, final action.

(2) That continued study be directed to determine the applicability of the method to beer and related malt beverages.

### Acknowledgments

The work of the following collaborators is greatly appreciated:

S. N. Nasledov, Berkeley Yeast Laboratory, Berkeley, Calif.; E. J. Lowe, Paul Masson Vineyards, Saratoga, Calif.; Duane Strunk, Joseph E. Seagram and Sons, Inc., Louisville, Ky.; Donna Karasek, Italian Swiss Colony, Asti, Calif.; Robert L. Morrison, E. and J. Gallo Winery, Modesto, Calif.; and, the following members of the Alcohol and Tobacco Tax Laboratories: C. E. Hubach, San Francisco; John E. Beck, Washington, D.C.; E. H. Richards, Cincinnati; F. P. Scaringelli, New York; and Paul A. Reeves, Philadelphia.

### References

(1) Meuron, H. J., *This Journal*, **46**, 299 (1963).
(2) "Changes in Methods," *ibid.*, **46**, 127 (1963).

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D, and were adopted by the Association. See *This Journal*, **47**, 129–130 (1964).

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

# Total Volatile Acidity in Wines

By W. J. GOWANS (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, 210 Federal Office Bldg., Seattle, Wash.)

**The determination of the total volatile acidity of wines has been studied collaboratively, using modern equipment now available. Procedures studied employed the Cash Electric Still and conventional apparatus, an all-glass unit. Results were good on both types of apparatus and between collaborators. It is recommended that the method, using either type of apparatus, be adopted as official, first action.**

In reviewing the present AOAC method for the determination of total volatile acidity in wines, **11.030**, a survey of government laboratories and commercial practices showed that this method has been modified in several respects. In order to bring the AOAC method into conformity with present practices, a collaborative study was undertaken. Considerable interest was shown in the use of the Cash Electric Still, so this was compared with a self-contained glass still with ground glass joints throughout.

Collaborators were provided with six samples containing acetic acid; three were aqueous acetic acid solutions, and three were wine samples containing acetic acid. Collaborators were asked to analyze each of the solutions in duplicate for total volatile acid by one or both methods.

## METHOD

### *Apparatus*

(a) *Cash electric still.*—Consists of outer chamber, inner chamber, trap, 2-way stopcock, elec. coil heater, and glass "T" inlet-outlet for $H_2O$. All parts are of Pyrex. Residue in inner chamber after distn has been completed is flushed out automatically by vac. action when current is shut off. Addn of $H_2O$ thru funnel above stopcock gives automatic spray bath to inner chamber, and waste drains thru outlet in glass "T." Two-way stopcock permits introduction of sample, serves as escape vent for $CO_2$, and allows introduction of wash $H_2O$. (Available from Braun-Knecht Heimann Co., 1400 16th Street, San Francisco 19, Calif. as Cash still.)

(b) *Steam distillation apparatus.*—*J. Assoc. Offic. Agr. Chemists* **44**, 138(1961); Fig. 3.

### *Preparation of Sample*

Remove dissolved $CO_2$ from ca 50 ml sample by either: placing under low vac. ($H_2O$ aspirator) 2 min. with continuous stirring; or bringing to incipient boiling under air condenser and cooling immediately.

### *Steam Distillation Apparatus*

Add ca 600 ml $H_2O$ to outer chamber of still. Pipet 25 ml freshly prepd sample into inner chamber and stopper. Boil $H_2O$ 3 min. with sidearm open. Close and distill ca 300 ml into erlenmeyer. Add 0.5 ml phthln to distillate and titr. rapidly with 0.1*N* NaOH until pink persists 15 sec. Express results as g HOAc/100 ml = ml 0.1*N* NaOH × 0.006 × 4.

### *Cash Electric Still*

Add $H_2O$ and pipet sample as above. Rinse funnel with ca 5 ml $H_2O$. Distill ca 300 ml into erlenmeyer. Titr. and express results as above. (Disconnect heating coil immediately and empty still by opening drain tube and stopcock to inner tube. Rinse still with two 10–15 ml portions $H_2O$ by adding thru funnel; evacuate each portion by closing drain tube.)

### Comments of Collaborators

Most collaborators commented on the Cash still, and generally agreed that they preferred it to the steam distillation apparatus. Speed of analysis, convenience, and ease of cleaning were the comments most cited.

Several collaborators preferred eliminating $CO_2$ by opening the outer water boiler of the Cash apparatus for a few minutes during boiling. The need for this is avoided by using freshly boiled distilled water.

Two of the collaborators preferred using smaller sample aliquots and collecting less distillate to save time. However, the Associate Referee has found that this somewhat diminished the accuracy of the method.

Some collaborators preferred to boil the distillate just before titration to eliminate $CO_2$. It has been found, however, that all

**Table 1. Collaborative results on total volatile acidity in wine[a] (g HOAc/100 ml)**

| Coll. | Soln A | Soln B | Soln C | Soln D | Soln E | Soln F |
|---|---|---|---|---|---|---|
| | | | Cash Still Method | | | |
| 1 | 0.292 | 0.150 | 0.077 | 0.052 | 0.137 | 0.207 |
| 2 | 0.282 | 0.140 | 0.076 | 0.048 | 0.134 | 0.204 |
| 3 | 0.282 | 0.142 | 0.073 | 0.051 | 0.132 | 0.201 |
| 4 | 0.285 | 0.143 | 0.074 | 0.050 | 0.131 | 0.201 |
| 5 | 0.289 | 0.147 | 0.087 | 0.061 | 0.140 | 0.209 |
| Av. | 0.286 | 0.144 | 0.077 | 0.0524 | 0.135 | 0.204 |
| Known HOAc[b] | 0.291 | 0.144 | 0.081 | 0.0045 | 0.0033 | 0.0032 |
| Std Dev. | 0.004 | 0.004 | 0.0045 | 0.0045 | 0.0033 | 0.0032 |
| | | | Conventional Method (*This Journal*, 44, 138 (1961)), similar apparatus | | | |
| 6 | 0.292 | 0.152 | 0.085 | 0.056 | 0.142 | 0.217 |
| 3 | 0.287 | 0.144 | 0.073 | 0.052 | 0.136 | 0.206 |
| 2 | 0.291 | 0.146 | 0.079 | 0.050 | 0.138 | 0.208 |
| 7 | 0.286 | 0.140 | 0.078 | 0.053 | 0.133 | 0.207 |
| 8 | 0.295 | 0.150 | 0.081 | 0.056 | 0.141 | 0.215 |
| 5 | 0.294 | 0.150 | 0.087 | 0.056 | 0.140 | 0.210 |
| Av. | 0.291 | 0.147 | 0.081 | 0.054 | 0.138 | 0.211 |
| Known HOAc[b] | 0.291 | 0.144 | 0.081 | | | |
| Std Dev. | 0.00333 | 0.0041 | 0.0046 | 0.0023 | 0.0031 | 0.0041 |

[a] Solutions A, B, and C are acetic acid solutions; Solutions D, E, and F are wine solutions.
[b] By direct titration.

but trace amounts of $CO_2$ are eliminated by following the procedure exactly.

## Results

Eight laboratories participated in the collaborative study; five used the Cash still, and six used the all-glass still. The results (averages of duplicate or triplicate determinations) are shown in Table 1.

Evaluation of these results shows that both types of apparatus give comparable values. The amount of acetic acid present within the limits surveyed does not appreciably affect the accuracy. The precisions of both sets of analyses are about the same as shown by the standard deviation.

## Recommendations

It is recommended—

(1) That the method for total volatile acidity, **11.030**, be deleted.

(2) That the method for total volatile acidity in wine, using either type of still described in this report, be adopted as official, first action.

(3) That this method be subjected to further collaborative study.

## Acknowledgments

The Associate Referee wishes to express his thanks and appreciation to the following collaborators:

Edmund Rossi, United Vintners, Asti, Calif. and the following members of the Alcohol and Tobacco Tax Laboratories: C. E. Hubach, San Francisco; Arthur Shaffer, Chicago; James Kluckholn, Omaha; Richard Hall, Detroit; Alex P. Mathers, Washington, D.C.; and Charles Milos, New York City.

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee D, and were adopted by the Association. See *This Journal*, **47**, 129–130 (1964).

# Quantitative Methods for Trace Amounts of Polyvinylpyrrolidone

By D. A. SHIRAEFF (Central Research Laboratory, Analytical Research & Services, General Aniline & Film Corp., Easton, Pa.)

**Two approaches were used for a simple procedure for quantitative estimation of polyvinylpyrrolidone: I. PVP forms complexes with textile dyes, which can be identified by paper chromatography. When PVP is added to a chromatographing solvent, the spread of a spot of dye placed on a paper strip is a function of PVP concentration in the solvent. Pyramine Orange 2R and Congo Red were used in the test. II. At concentrations of perchloric acid greater than 1.6–1.7%, PVP falls out of solution and forms a finely dispersed precipitate like a cloud. The clouding is proportional to PVP concentration and can be measured by a nephelometer or spectrophotometer.**

Polyvinylpyrrolidone (PVP) has been shown to complex with and effectively remove certain undesired constituents of a variety of naturally derived beverages. In beer, for example, such treatment has been applied successfully on a large scale to remove chill-haze components as an alternative to the older and lengthier enzyme treatment (1–3). Also, clarification of wines (4) with PVP has been practiced commercially. The tendency of fruit vinegars to settle or haze with time is also improved by treatment with PVP. The increased interest in such applications has led to the need for assay methods for practical application and control of the treatment, and also to meet requirements for regulatory aspects when PVP is encountered as a food additive.

In the search for simple and rapid methods for quantitative estimation of traces of polyvinylpyrrolidone, two new procedures have demonstrated their utility in specific systems and show promise of adaptability to many others. One employs the technique of paper chromatography and the other depends on the determination of the degree of turbidity formed by the addition of perchloric acid to PVP solutions. The methods are applicable to the ranges of molecular weight grades (represented by K values) of PVP available.

### Paper Chromatographic Method

PVP is used in the textile industry in dyeing with direct cotton dyes and leuco compounds of vat dyes. Both these classes of dyes have an affinity for cellulose. Some members of these two classes have so great an affinity that "retardants" must be used to prevent uneven dyeing. The active ingredient of some of these retardants is PVP.

The affinity of both cellulose and PVP for direct cotton dyes has been adapted to analytical purposes. A spot of a selected dye is placed on paper in such concentration that it is completely adsorbed by a small area of the paper, and PVP is made a component of a chromatographing solvent; then the paper and PVP are in competition for the same amount of dye in the spot. The paper tends to retain the dye where it was originally placed; the PVP pulls it along with the chromatographing solvent. Any movement of the dye away from the original spot exposes it to an ever greater adsorptive force from the cellulose in the increased paper area occupied. The result is a dynamic equilibrium adaptable to measuring the concentration of PVP in the chromatographing solvent, if other variables (dye, paper, and other components of the chromatographic solvent) are kept constant.

The chromatographic method detects concentrations of PVP as low as about 5 ppm. The technique and apparatus are extremely simple, the test is rapid and requires only one reagent, and the amount of sample needed is only 5–10 ml.

The method requires reference standards comprising a set of chromatograms of solutions of known concentrations of PVP in each solvent of the same K range (molecular weight range). Chromatograms of aqueous solutions containing 0, 2, 5, 10, 15, 20, 25, and 30 ppm of PVP K-90 (mol. wt 200,000) are shown in Fig. 1. The chromatograms of the two beers

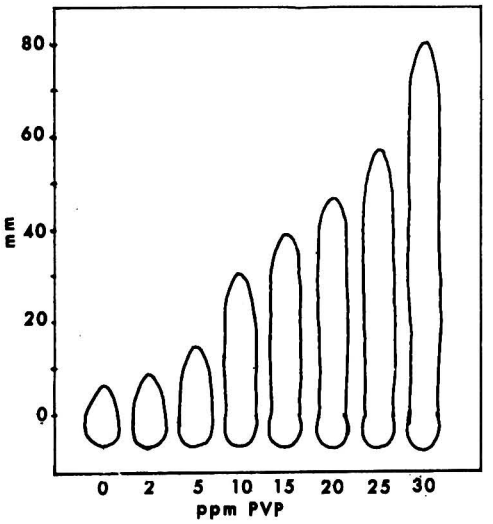


*Fig. 1*—Chromatogram of PVP K-90 in water.

in Figs. 2 and 3 demonstrate the ability of the method to determine the optimum amount of PVP to add to a beer in process for reducing chill-haze. As the figures show, the first additions of PVP are completely used up in precipitating certain tannins and protein constituents that lead to chill-haze. The first 27 ppm PVP added to Beer A (Fig. 2) are precipitated completely, and the first appearance of PVP registered by the chromatogram takes place after 32 ppm PVP is added. Beer B (Fig. 3) originally contained somewhat less precipitable components and dissolved PVP shows its appearance as soon as 27 ppm PVP is added.

However, the precipitation of PVP by beer is not stoichiometric but is the function of PVP concentration in solution. The perchloric acid method, described later, shows that the amounts of PVP removed from the solution as precipitate increase with the increased amounts of PVP added. Therefore, in the chromatographic method the differences in the distance of the spread of the dye between any two concentrations of PVP register the difference in concentrations of PVP remaining in the solution and not a numerical difference between the amounts originally added.

Chromatograms of such systems could be calibrated for closely estimating residual PVP present by applying the perchloric acid method or another method to the same set of samples. Acetic acid solutions of PVP have been shown to serve as proper chromatography standards for the less complicated vinegar system. The following method includes a general procedure (I) and a specific adaptation (II) for measuring PVP K-30 (Polyclar L[1]) in vinegar.

### *Apparatus*

(a) *Pipet.*—5 μl (0.005 ml).

(b) *Vessels.*—(1) A set of identical vessels for holding test samples (Fisher Cat. No. 11-505-155 was used in this study). (2) A shallow rectangular vessel in the form of a tray fitted with a frame to hold a lattice of thin glass plates for holding the paper strips horizontal during the chromatography (Fig. 4).

### *Reagents*

(a) *Pyramine Orange 2R.*—(C.I. 369, First Ed.), 0.3% water solution.[2]

(b) *Polyvinylpyrrolidone.* — 0.1% solution (1000 ppm).

(c) *Filter paper.*—Whatman No. 1 sheets.

### *Preparation of the Paper*

Cut strips of Whatman No. 1 paper about 19 × 23 cm. With a pencil, line them vertically into eighteen 1.0 cm strips and horizontally, starting 1.5 cm from the top, followed by 18 lines 1.0 cm apart and two final lines at the bottom, 1.5 cm apart.

Into each first 1.0 cm square from the bottom, place 5 μl of 0.3% Pyramine Orange 2R solution, using a 5 μl pipet. When the drops are dry, cut the paper longitudinally into eighteen strips each 1.0 cm wide. Crease each strip at right angles to its plane at the middle line between the two bottom 1.5 × 1.0 cm rectangles. Use the top 1.5 × 1.0 cm rectangle for marking.

### *Preparation of PVP Reference Solutions*

Prepare reference solutions from the 0.1% stock solution of PVP by dilution with the appropriate solvent (e.g., a 5% water solution of acetic acid for analysis of PVP in vinegar). Choose reference solutions according to the expected content of PVP in the unknown sample and the desired accuracy of the estimation. For example, in the special case of assaying for ± 40 ppm, the established tolerance for PVP in vinegar (4), a set of 20, 40, and 60 ppm standards ordinarily is sufficient. If standards and unknowns are being run simultane-

[1] Polyclar is the registered trademark of General Aniline & Film Corp. for the grades of PVP used in beverage clarification.

[2] Samples are available from Acetylene Chemicals Marketing Dept., Dyestuff & Chemical Division, General Aniline & Film Corp., 435 Hudson St., New York, 14, N.Y. Certain other dyes, such as Congo Red, have also been used.

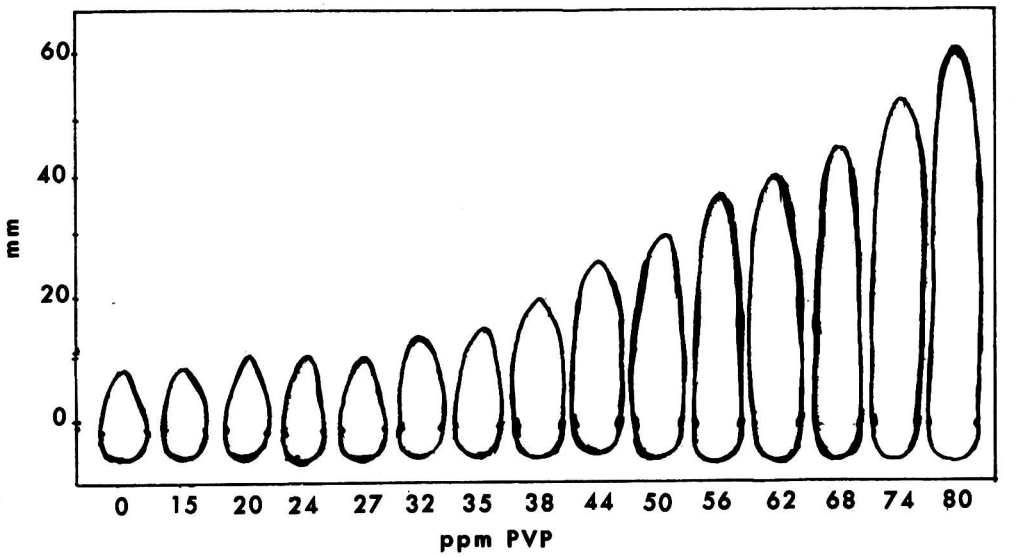


*Fig. 2—Chromatograms of PVP K-90 in Beer A.*

ously, a relatively short chromatographing period (1–2 hr) will show when unknowns are well below 40 ppm.

### *I. General Procedure*

Place samples in 5 ml beakers at one end of the chromatographing tray. Dip the short portion of the creased strip into each sample beaker in such a manner that the strip does not touch the rim of the beaker and the rest of the paper strip is laid horizontally across the glass lattice of the frame. To keep the moisture content within the tray constant and evenly distributed, put large-surfaced vessels containing water at the far end of the paper strips and run a similar strip without dye spot from a beaker of distilled water at each side of the chromatographing set of paper strips. Cover the tray with a heavy glass plate fitted with a foam rubber gasket to prevent drafts that might interfere with the uninterrupted flow of solution through the paper strips.

Chromatograph for a period from 15 minutes to overnight, depending on the purpose of the analysis and the concentrations of PVP.

### *II. Procedure for PVP K-30 in Vinegar*

Fill the cup-beakers almost to the brim with the samples of vinegar to be tested for Polyclar L (PVP K-30) content and develop the chromatograms as in Procedure I. For maximum accuracy, let the strips develop 18–20 hr; remove them, and lay them flat on a large sheet of filter paper until dry. Compare with a reference set of chromatograms prepared in the same way from solutions of known concentration of Polyclar L in 5% acetic acid, as above.

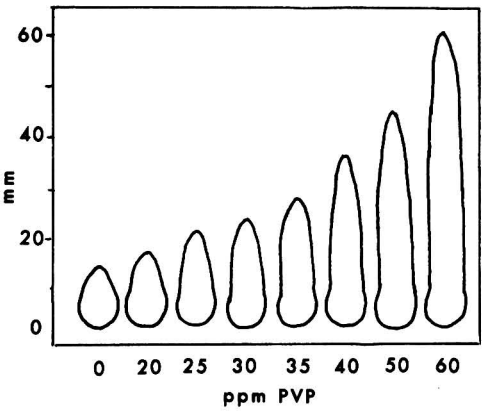


*Fig. 3—Chromatograms of PVP K-90 in Beer B.*

### Nephelometric Method

Solutions of polyvinylpyrrolidone do not give a turbidity with perchloric acid of concentrations below 1.6% and above 30%. Solutions of perchloric acid between these two concentrations are suitable for turbidimetric measurements, since they precipitate PVP as highly dispersed particles that do not settle for hours.

The turbidity of the mixtures is proportional to the concentration of PVP. At 2 ppm PVP, the turbidity is apparent to the naked eye; at 1 ppm the turbidity is clearly registered by a spectrophotometer or nephelometer.

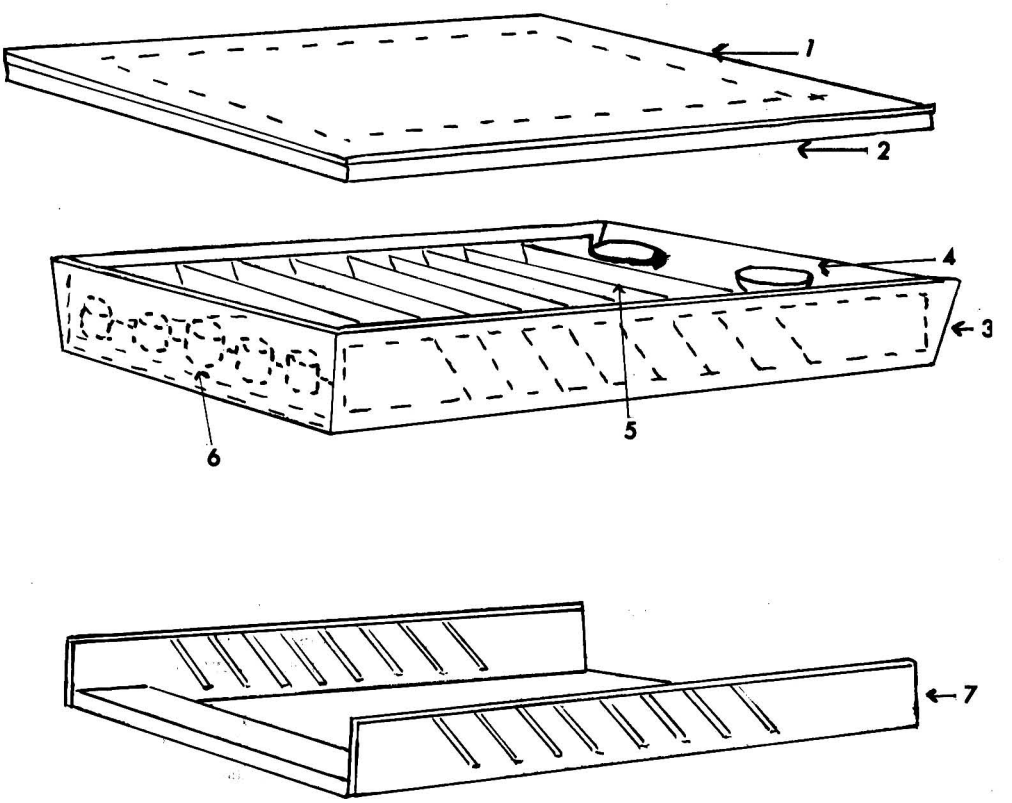


*Fig. 4*—Apparatus for chromatography. *1*, glass cover plate, 15 × 20″. *2*, foam rubber strip, 1.5″ wide, attached at edge of cover plate. *3*, pyrex glass tray, 18 × 12 × 2⅛″. *4*, shallow dishes for humidifying the apparatus. *5*, Kodak lantern slide medium plates, 2 × 10″ (cleaned of emulsion, or equivalent glass strips). *6*, 5 ml beakers. *7*, detail of wooden inset rack.

Turbidities of PVP solutions increase somewhat with time (Fig. 5) and depend on the concentrations of perchloric acid (Table 1) so that both these factors must be taken into account when devising an analytical procedure. In addition, the oxidizing power of perchloric acid increases considerably with an increase in temperature that can occur, for example, through dilution of the concentrated acid. In the analysis of PVP solutions containing substances easily susceptible to oxidation, the strength of perchloric acid should be chosen so that further dilution of this reagent produces no rise in temperature. Also the amount of perchloric acid should be chosen so that only a slight excess is present in the test solution.

**Table 1. Variations in opacity at different concentrations of perchloric acid at constant PVP concentration (PVP K-90 in 50 ml)**

| ml 18% $HClO_4$ Added | Absorbance at 500 mμ[a] |
|---|---|
| 30 | 0.78 |
| 25 | 0.83 |
| 20 | 0.83 |
| 15 | 0.81 |
| 12 | 0.78 |
| 9 | 0.73 |
| 7 | 0.53 |
| 5 | 0.25 |
| 4.5 | 0.11 |
| 4 | 0.03 |
| 0 | 0.03 |

[a] Cary Recording Spectrophotometer.

### *Reagents*

(a) *Polyvinylpyrrolidone.*—0.1% aqueous solution (1000 ppm).

(b) *Perchloric acid.*—18% aqueous solution. Dilute 250 ml 70–71% acid to 1 L with distilled water.

This paper was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

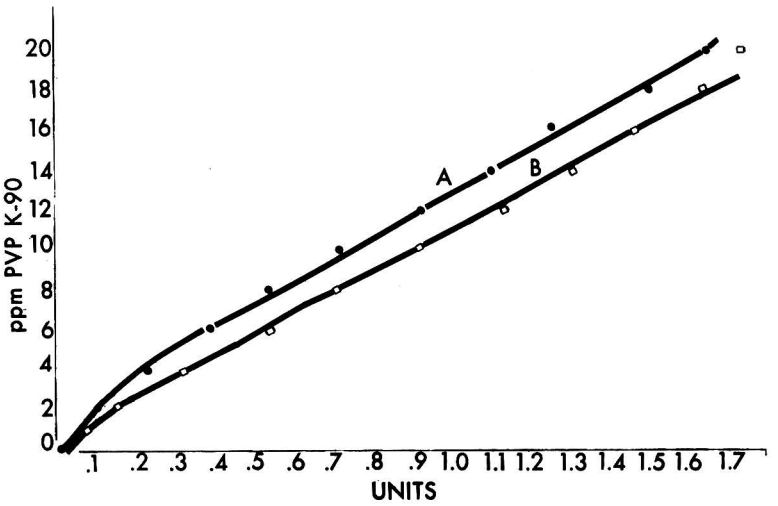


*Fig. 5*—**Influence of time on turbidity. Reference scale, PVP K-90 in water. *A*, read after 1 hr. *B*, read after 5 hr.**

***Preparation of Reference Standards***

To a number of 50 ml volumetric flasks add different amounts of 0.1% PVP (a) from a 10 ml microburet to produce the desired concentrations of PVP in ppm on dilution to 50 ml. Fill the flasks with water to about 70–75% of capacity and add 10 ml 18% perchloric acid to each flask from a 50 ml buret. Dilute to volume with water, shake, let stand 1 hr, and measure the absorbance against distilled water. To eliminate the influence of time, add perchloric acid to each succeeding flask at a definite interval of 5, 10, or 15 min., whichever is convenient. (See Fig. 5 for the standard reference scale for PVP K-90 obtained with a Cary Recording Spectrophotometer.)

***Preparation of PVP-treated Beer Solutions***

(For examination of the method, regular finished beers, enzyme-treated, were treated as described below. To assay finished beers that had been treated with PVP K-90, Polyclar H, only degassing is necessary before proceeding to the determination. For brewery practice in determining the amount of PVP required for the chill-haze treatment, the entire following procedure is used to choose a concentration below which PVP substantially appears in the beer. Adequate chill-haze treatment may usually be effected with essentially zero residues in the treated beer, as indicated by this or the preceding method.)

Place the beer for a short time under gradually increasing vacuum to liberate dissolved carbon dioxide gas and reduce foaming in the following measurements and manipulations. To several 100 ml volumetric flasks, add different amounts of 0.1% water solution of PVP to form the desired concentrations of PVP when the flasks are brought to volume with degassed beer. Let the flasks stand 3–4 days to insure complete interaction between the PVP and the precipitable matter in the beer. Refrigerate the samples to prevent possible mold growth. (Depending on the particular beer and the amount of PVP added, all of it will precipitate or part of it will precipitate and part will remain in solution.) Filter beer treated as above through Whatman No. 42 filter paper.

***Determination of PVP in Beer by Spectrophotometer***

(Beers differ greatly in color. The absorbance of dark beer is quite high, and it may be necessary to reduce the aliquot for such beers below the maximum of 40 ml. Absorbance due to color must be excluded in correlating the PVP content of beer with that of the reference standards. The addition of perchloric acid to beers containing no PVP produces no turbidity and has little effect on the absorbance which is solely due to beer color.)

To determine the PVP content of a given beer, place equal volumes of the beer in each of two 50 ml volumetric flasks. Dilute flask 1 to volume with water alone. To flask 2, add 10 ml 18% perchloric acid, and if the aliquot taken is less than 40 ml, dilute to the mark with water. Measure the absorbances of the two solutions after 1 hr. The absorbance of flask 2 is due to the beer color and the tur-

bidity caused by perchloric acid precipitation of PVP. The difference between the two readings is equal to the light absorption due only to the turbidity of the PVP precipitated by perchloric acid and is therefore proportional to the values for the standard reference scale (Fig. 5). To find the concentration of PVP in ppm in the original beer, read the values for PVP content from this scale, multiply by 50 (volume of the flask), and divide by ml aliquot taken.

## Results

Tables 2–4 illustrate the use of the spectrophotometric method for determining PVP concentrations remaining in solution after treatment of two commercial beers with various amounts of PVP. The numerical values for these residual concentrations are given in Table 4; they are derived from the data in Tables 2 and 3 by subtracting the absorbance of the original beer without added perchloric acid from the absorbance of individual concentrations, then locating the differences obtained on the absorbance curve for water solutions of PVP treated with perchloric acid against the concentrations of PVP (Fig. 5) and correcting for the dilution factor.

**Table 2. Absorbance after various known additions of PVP K-90 to Beer C**

| ppm PVP Added | Absorbance at 500 mμ[a] | |
|---|---|---|
| | After 1 hr | After 5 hr |
| 68 | 2.58 | 2.70 |
| 62 | 2.15 | 2.50 |
| 56 | 1.77 | 2.10 |
| 50 | 1.63 | 1.88 |
| 44 | 1.43 | 1.67 |
| 38 | 1.26 | 1.51 |
| 35 | 1.23 | 1.43 |
| 32 | 1.14 | 1.39 |
| 27 | 1.14 | 1.36 |
| 24 | 1.15 | 1.37 |
| 20 | 1.13 | 1.35 |
| 15 | 1.13 | 1.35 |
| 0 | 1.13 | 1.35 |
| 0[b] | 1.02 | 1.00 |

[a] Tables 2 and 3 show readings obtained by using 5.0 ml stronger perchloric acid and 45 ml beer. The concentration of PVP as found from the standard reference curve should be multiplied in this case by 50/45 to obtain the PVP content of the original beer.
[b] Control or blank; no acid.

**Table 3. Absorbance after various known additions of PVP K-90 to Beer D[a]**

| ppm PVP Added | Absorbance at 500 mμ | |
|---|---|---|
| | After 1 hr | After 5 hr |
| 60 | 2.69 | 3.00 |
| 50 | 2.01 | 2.38 |
| 40 | 1.49 | 1.72 |
| 35 | 1.29 | 1.48 |
| 30 | 1.14 | 1.32 |
| 25 | 1.05 | 1.20 |
| 20 | 1.00 | 1.14 |
| 0 | 0.96 | 1.04 |
| 0[b] | 0.96 | 0.96 |

[a] See footnote to Table 2.
[b] Control or blank; no acid.

The adaptation of the method to nephelometric measurement (scattered light) is independent of the color of the beer. Nephelometric measurements of the samples (multiplied by sample dilution factor, usually 5/4) are compared directly with a PVP-in-water nephelometric standard scale.

**Table 4. Determination of PVP content of Beers C and D by turbidimetric method**

| Beer Treated Originally with Following Amounts of PVP K-90, ppm | Polyvinylpyrrolidone Found in the Filtrate,[a] ppm | |
|---|---|---|
| | Beer C | Beer D |
| 15 | 2.3 | — |
| 20 | 2.3 | 0.9 |
| 24 | 2.3 | – |
| 25 | – | 2.0 |
| 27 | 2.7 | – |
| 30 | – | 3.5 |
| 32 | 2.6 | – |
| 35 | 4.0 | 5.7 |
| 38 | 4.5 | – |
| 40 | – | 8.6 |
| 44 | 4.6 | – |
| 50 | 6.9 | 15.7 |
| 56 | 11.3 | – |
| 60 | – | 23.1 |
| 62 | 16.3 | – |
| 68 | 20.8 | – |

[a] Using spectrophotometer readings after 1 hr.

In this method, as in the paper chromatographic method, this incremental PVP addition (i.e., to beer or vinegar before haze-

proofing) defines the amount of PVP needed (i.e., point at or just before where the dissolved PVP baseline breaks). It should be emphasized that beers already treated by the enzyme process were used in both these and the chromatography examples, so that they do not illustrate typical PVP quantities required for actual treatment of beer. In brewery practice, it has been found that 2–4 lb per 100 barrels or 80–150 ppm PVP K-90 (Polyclar H) is ordinarily needed to remove the components leading to chill-haze to an adequate level and to give a residue of PVP of essentially 0 ppm.

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# Determination of $CO_2$ in Wines by Gas Chromatography

By HOWARD L. ASHMEAD (F & M Scientific Corp., Route 41 and Starr Rd., Avondale, Pa.), GLENN E. MARTIN (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington, D.C.), and JOHN A. SCHMIT (F & M Scientific Corp., Route 41 and Starr Rd., Avondale, Pa.)

**A fast and direct method is presented for the determination of $CO_2$ in wines. Partial pressures from other ingredients of alcoholic beverages do not interfere. The method was compared to the manometric procedure under vacuum. An F & M Model 450 Gas Chromatograph with a filament detector was used for $CO_2$ determinations. Components were separated by a column 9″ long and ⅛″ o.d. using charcoal (60–80 mesh) as solid adsorbent. Injection port temperature was ambient; detector and column temperatures were 40°C. Helium was used as a carrier gas at the rate of 50 ml/min. Approximately 50 μl samples of standard and unknown solutions were used for the respective determinations.**

Congress has defined wines containing less than 0.256 g of carbon dioxide per 100 ml as still wines and those containing larger amounts as carbonated or effervescent wines (1). Since the tax rate on carbonated wines is 15–20 times greater than that on still wines, there is considerable interest in rapid and accurate methods for determining the $CO_2$ content of these products. Although gravimetric (2, 3), volumetric (4–6), and manometric (7) methods have been widely applied, they do not appear to possess the combination of high speed and accuracy offered by gas chromatography.

Descriptions of gas chromatographs equipped with gas "stripping" devices to extract gases from blood prior to injection have been reported in the literature (8, 9). Difficulties encountered with these systems include the inability to accurately measure a sample volume, nonreproducibility of sample gas injection time, and inadequate control of reaction time. These parameters must be closely controlled to attain acceptable precision. Since the design of the F & M Model 450 Blood Gas Analyzer effectively eliminates these difficulties, its application to analysis of wines for $CO_2$ content was investigated and compared to the manometric method.

## Manometric Procedure

The manometric apparatus, reagents, and procedure used in the comparison were those described by Pro, *et al.* (7).

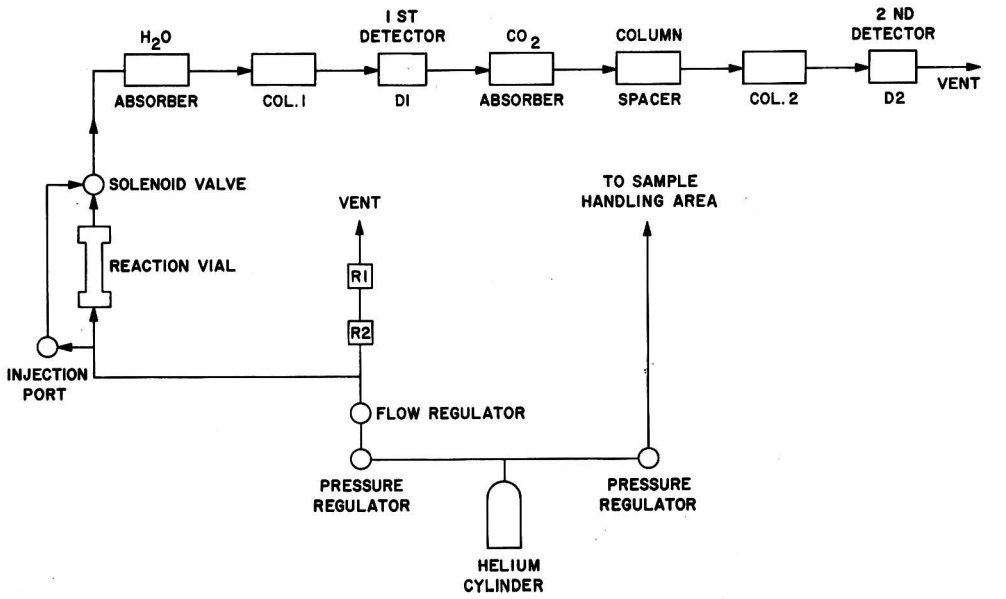


*Fig. 1*—Schematic diagram of chromatographic system.

## Gas Chromatographic Procedure

### *Reagents*

(a) *$CO_2$ releasing reagent.*—Dilute 80 µl concentrated $H_2SO_4$ and 0.5 ml F & M Antifoam 450-1A to 25 ml with distilled $H_2O$. Transfer mixture to the reagent reservoir of the instrument, and degas for 15 min. before use.

(b) *Sodium bicarbonate standard wine solution.*—Prepared in the following manner: Acidify wine with 1 ml/L concd HCl, and boil to remove all $CO_2$. Bring 5 L of the boiled wine to a pH above 10 by adding 16 ml 50% (w/w) NaOH. Dissolve $NaHCO_3$ (reagent grade; dried over $H_2SO_4$ for 24 hr) in the wine in the following quantities:

| | *g $NaHCO_3$/500 ml wine* | *mg $CO_2$/100 ml wine* |
|---|---|---|
| (1) | 2.1478 | 225 |
| (2) | 2.3864 | 250 |
| (3) | 2.6250 | 275 |
| (4) | 2.8636 | 300 |
| (5) | 3.3409 | 350 |
| (6) | 3.8182 | 400 |
| (7) | 4.2955 | 450 |
| (8) | 4.7727 | 500 |
| (9) | 5.2500 | 550 |

(c) *Aqueous bicarbonate standard.*—Dissolve 0.2100 g $NaHCO_3$ in recently boiled distilled $H_2O$ and dilute to 100 ml. Standard contains 110 mg $CO_2$/100 ml $H_2O$.

### *Apparatus*

(a) *Chromatograph.*—An F & M Scientific Corp. Model 450 Blood Gas Analyzer was used for the chromatographic determinations. Substitution of wine for blood samples required no instrument modifications.

(b) *Carrier gas flow system (Fig. 1).*—Two pressure regulators were used on the supply cylinder to eliminate any effects in the chromatograph caused by pressure surges in the sample handling system. A timer-operated solenoid valve diverted He flow from injection port through gas stripper and reaction vial during gas stripping step. First column, 9″ long × ⅛″ o.d. copper, is packed with 60–80 mesh charcoal to separate $CO_2$ from air. (This charcoal[1] is specially treated to produce a sharp $CO_2$ peak with minimal tailing.)

$CO_2$ is detected by a filament-type thermal conductivity detector and the signal displayed on a 1 millivolt full scale Minneapolis-Honeywell Model 18 recorder. (Both the $H_2O$ and $CO_2$ are absorbed on replaceable absorber tubes to eliminate column deactivation and to insure constant elution times of peaks.) Empty ¼″ o.d. polyethylene tubing, about 10′ long, is used to space the $O_2$ and $N_2$ elution times after the $CO_2$. (The molecular sieve 5A[2]

[1] Available from F & M Scientific Corp. as a replacement column for the Model 450 Blood Gas Analyzer.

[2] Trade name of American Carbide Corp.

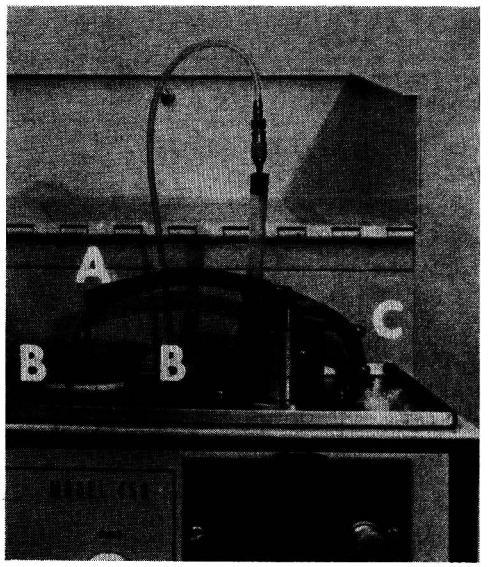


*Fig. 2*—Sample and reagent measuring system. *A*, reagent reservoir; *B*, He control valve; *C*, sampling valve.

column is used to separate $O_2$ and $N_2$ in blood gas analysis and was left in the instrument for this determination, but it was not needed to obtain $CO_2$ content.)

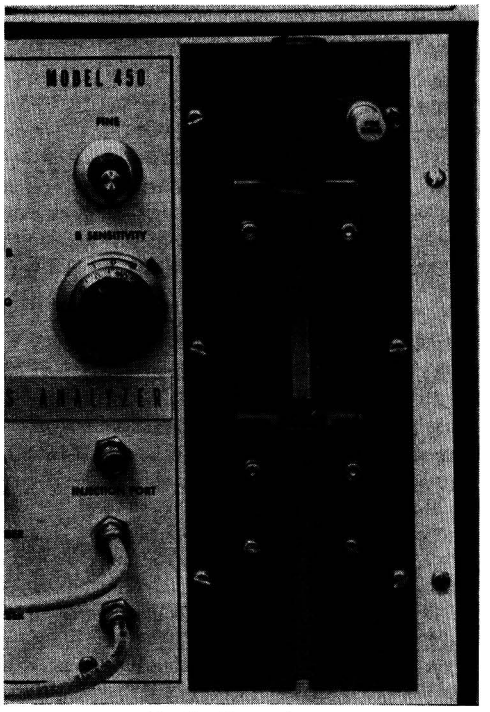


*Fig. 3*—Gas stripper.

(c) *Sampling system (Fig. 2).*—Used to measure the wine sample and the gas releasing reagent into a reaction vial; consisting of a 2" long × ¼" o.d. nylon tube with serum vial caps enclosing both ends.

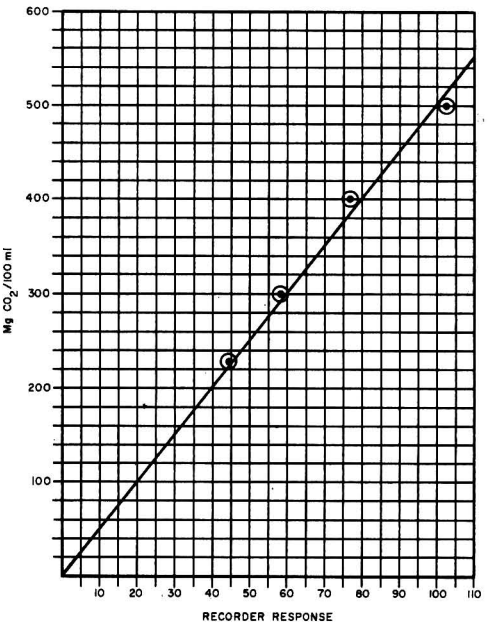


*Fig. 4*—Typical calibration curve for $CO_2$.

The sample and reagent measuring system (Fig. 2) consists of the following sub-assemblies: *A*, reagent reservoir containing sufficient gas releasing reagent for 8 hr operation; *B*, valve to control He flow to reservoir for degassing reagents and pressurizing reservoir and reagent metering valve; *C*, sampling valve to perform following functions without atmospheric contamination of sample: purge reaction vial with He, measure a 50 µl sample of liquid, control the reagent flow from the reservoir through reagent metering valve, and deliver sample and reagent to reaction valve; and *D*, spring-loaded diaphragm valve (not shown in Fig. 2, but located under sampling valve) designed to deliver reproducible quantity of gas-releasing reagent through sample port of sampling valve into reaction wall.

(d) *Gas stripping mechanism (Fig. 3).*—Used to hold reaction vial firmly in vertical position, while the following operations are performed in an automatically-timed sequence:

1. Areas between ends of reaction vial and sealed needles are purged with He for 20 sec. to allow a needle to be inserted into each end of reaction vial in He atmosphere.

2. After needles are inserted via pneumatic actuator and pressure in reaction vial has equilibrated with chromatograph inlet pressure, He carrier gas is passed through reaction vial for 25 sec., thereby stripping gases from liquid into chromatograph.

3. Needles are extracted by a spring return on pneumatic actuator, and are sealed in silicone rubber in extracted position.

4. He purge to ends of capsule is turned off; the extracted gases, after being swept into chromatograph, are separated and quantitated in normal manner.

***Procedure***

Standardize gas chromatograph daily at one point only with a duplicate sample of aqueous bicarbonate standard, (**c**). A typical $CO_2$ calibration curve is shown in Fig. 4.

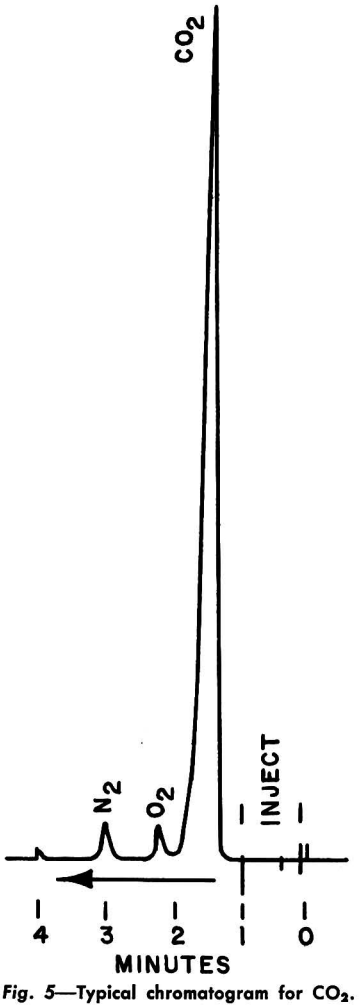


*Fig. 5*—Typical chromatogram for $CO_2$.

After reaction capsule has been placed on sampling valve and purged with He for at least 15 sec., inject about 0.25 ml sample into sampling valve from 2 ml syringe. Then, inject 150 $\mu$l $H_2SO_4$-antifoam reagent into reaction vial along with 50 $\mu$l sample via sampling valve. Agitate reaction vial in shaker for 4 min., as determined by the analytical time of chromatograph, not by reaction kinetics. Allow sample to remain in shaker to keep work flow organized. All $CO_2$ should evolve in 2 min. Rinse sampling valve with $H_2O$ between samples. Place capsule in gas stripping and injection system (**d**), and start automatic analytical program. Measure height of $CO_2$ peak on a 0–100% chart scale. (The $O_2$ and $N_2$ peaks were not quantitated for this determination.) A typical chromatogram is shown in Fig. 5.

Determine $CO_2$ content of wine as follows: mg $CO_2$/100 ml wine = (110 mg $CO_2$/100 ml $H_2O$) ($CO_2$ peak height of wine sample)/($CO_2$ peak of $H_2O$ std).

**Results and Discussion**

Wines may contain up to 300 ppm sulfur dioxide, which can interfere with the results obtained by the analytical methods now in general use. This interference is eliminated by the gas chromatographic system described in this paper, since the charcoal column does not elute $CO_2$ and $SO_2$ at the same time.

Results of duplicate determinations (Table 1) indicate that the chromatographic determinations are generally higher than the manometric method, but application of the

**Table 1. Comparison of $CO_2$ content of wine by gas chromatographic and manometric methods**

| | | mg $CO_2$/100 ml | |
|---|---|---|---|
| Sample | Amount Added | Gas Chromatography | Manometric Method |
| 1 | 225 | 221 | 230 |
| 2 | 250 | 257 | 252 |
| 3 | 275 | 291 | 284 |
| 4 | 300 | 313 | 304 |
| 5 | 350 | 387 | 371 |
| 6 | 400 | 421 | 411 |
| 7 | 450 | 452 | 444 |
| 8 | 500 | 490 | 497 |
| 9 | 550 | 555 | 538 |
| A | 0 | 257 | 260 |
| B | 0 | 258 | 261 |

"t" test reveals no significant difference between the two methods ($P > 0.1$). Samples A and B were wine samples from the same batch and were analyzed by both methods.

Since $O_2$ and $N_2$ content of the wine is not needed, the analytical time of the chromatograph could be reduced to approximately 2 minutes by removing the molecular sieve column and using the second detector filament as another reference filament.

This method should be applicable to other determinations involving the chemical release of gases from liquids.

**Acknowledgments**

The standard wine solutions were prepared and the manometric determinations were performed at the Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington 25, D.C. The wine standards were then shipped to F & M Scientific Corp., Avondale, Pa. for chromatographic determinations.

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## EXTRANEOUS MATERIALS

# Removal of Urea from Corn by the Steeping Process of Wet Corn Milling

By J. F. NICHOLSON* and KENTON L. HARRIS (Division of Microbiology, Food and Drug Administration, Washington, D.C. 20204)

**To test the removal of urine from corn by the corn steeping process, urea containing C-14 was added to corn in four different ways. Radiation present after steeping demonstrated that some of the added C-14 remained in the corn, indicating that some urine persists through the corn steeping process. When added to the insect exit hole, the greatest amount remained unremoved by steeping (average of 2.9%). Amounts above radiation normal to leached corn (0.2 counts/min.) also remained in one instance when the radioactive urea was placed on the endosperm and in one or two instances when it was allowed to enter at the germ end.**

Corn, like all cereal grains, is subject to rodent contamination, consisting of fecal pellets, urine, gnawing, hairs, and other debris. These contaminants not only defile the grain, but, since rodents urinate where they feed, the contaminants are also indexes of the presence of rodent urine. Available methods of identification are applicable only to excreta pellets, damaged kernels, and hairs. Since the urine remains undetected, the ab-

* Deceased.

sence of pellets could not be considered proof that the corn had been satisfactorily cleaned.

Ferrigan, *et al.* (1) used autoradiographs to measure the sorption of soluble solids into wheat. Laakso, *et al.* (2) used urease and Nessler's reagent to detect ammonia from urine urea. Keppel (3) has reported and perfected a method for the identification of urine-contaminated wheat. Since all these procedures deal with wheat, they cannot be used to give more than a general indication of urine in corn.

The problem of suitable methods for urine in corn is partly due to the absence of adequate sorting procedures to isolate contaminated kernels for subsequent confirmation, and partly to a lack of reliable confirmatory tests for urine. The situation is complicated by the fact that water added to corn will carry soluble solids in at the same time it leaches them out.

Regulatory agencies have been reluctant to approve the use of rodent-contaminated grain even though the pellets have been removed, since the urine remains on the grain. Similarly, the salvage of rodent-contaminated grain has necessitated the removal of both the surface urine and that which penetrated beneath the surface layers. In the case of wheat, this has been done by abrading off the surface layers. This process seemed to be commercially impractical for corn. However, much corn is milled through the so-called "wet milling" process, in which the corn is steeped in large quantities of water, and it has been suggested that any urine contamination present would be removed in the steep water. The current study was undertaken to determine the fate of urine during the steeping operation.

Since only a small quantity of urine would be expected to be present after the steeping operation, the fate of the urea was followed by treating the corn with urea tagged with carbon-14.

Radioactivity was measured before and after a laboratory-simulated steeping operation.

### *Contamination of Corn with C-14 Urea*

When 0.1 $\mu$c of C-14 urea (220,000 emissions/min.) was dissolved in about 11 ml $H_2O$, 0.03 ml of the resulting solution gave 666 counts/min.[1] Seventeen corn kernels were each injected with 0.03 ml of this solution from a hypodermic syringe at an insect emergence hole; another 17 kernels were similarly contaminated on the intact germ surface. The same kernels were then treated with 0.01 ml (222 counts/min.) added to the exposed endosperm where rodents had blazed the kernels. Another 0.03 ml was added to each of several small depressions in modeling clay. The attachment end of each kernel was then dipped in the liquid until all the liquid had been absorbed by the corn and/or water evaporated. Counts on the material remaining in the depression averaged 113/min. This corn contained not over 553 counts/min., since some of the solution may have been absorbed into the clay itself.

### *Corn Treatment by a Laboratory-Simulated Steeping Process*

The commercial wet milling of corn to produce corn starch includes a steep in which the corn is soaked for about 40 hours in a counter current of water slightly acidified with $SO_2$. The $SO_2$ prevents undesirable bacterial growth and helps loosen certain physical and chemical bonds within the kernels. The counter-current flow of about 40 gallons per bushel of corn per 40 hours flushes the freshest water over the corn that has been in the steep for the longest time. This process was simulated in the laboratory by passing the water through successively small batches of corn in wide-mouth jars (Fig. 1). The rate of flow was 1 gallon per hour, an excess over that used commercially.

The industrial corn steep is a *continuous* counter-current process in which the used steep water is passed over the newly introduced corn. To parallel this condition in the laboratory, the step-wise steeping was begun 40 hours before the corn containing contaminated kernels was added, so that the soaking would be started and continued on a normal steep-water basis.

Contaminated kernels were placed in nylon

[1] Total radiation present, not the amount that could be counted. Some soft C-14 radiation is absorbed by the corn, and the total amount present can not be measured.

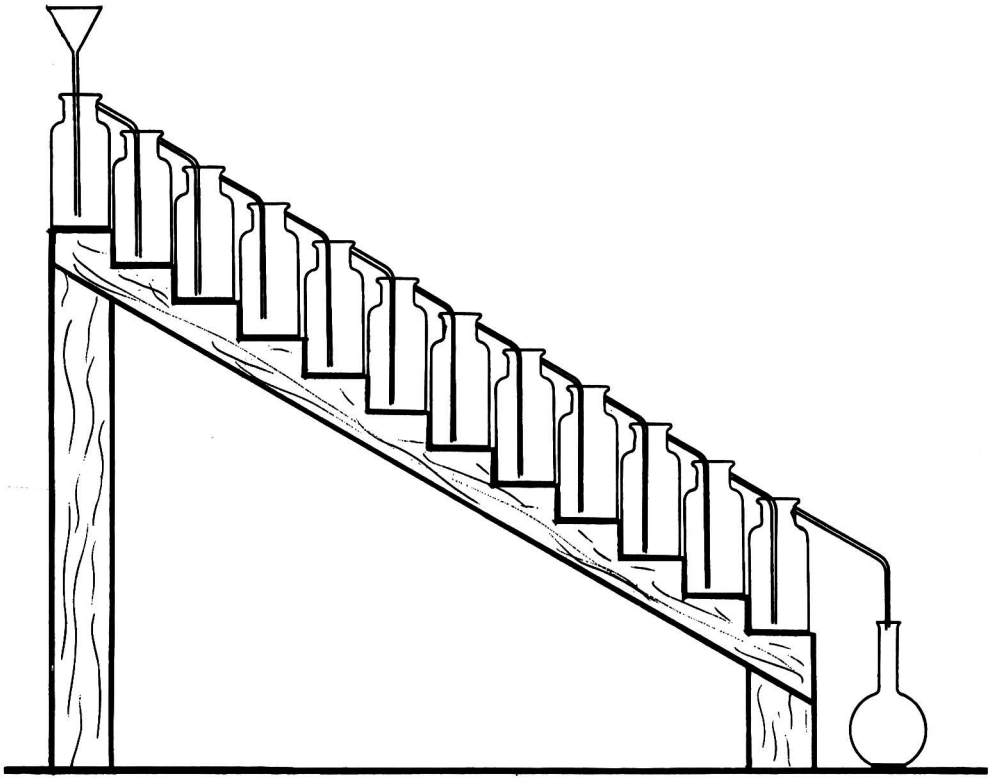

*Fig. 1*—Laboratory-simulated corn steeping operation. (Corn not shown.)

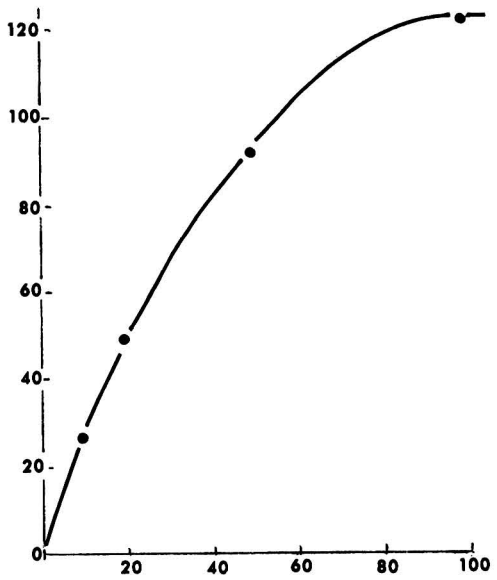


*Fig. 2*—Measurable radioactivity from corn containing C-14 urea.

**Table 1. Radioactivity from pulverized corn containing C-14 urea (background radiation not included)**

| | Counts/min. | | | |
|---|---|---|---|---|
| | 10 mg Corn | 20 mg Corn | 50 mg Corn | 100 mg Corn |
| | 30.7 | 50.4 | 94.3 | 114.8 |
| | 22.7 | 50.5 | 93.3 | 112.0 |
| | 28.6 | 46.4 | 92.1 | 117.3 |
| Av. | 27.3 | 49.1 | 93.2 | 114.7 |

tulle bags beneath the surface of the corn and allowed to steep with the uncontaminated bulk of the material. After 40 hours the jars were emptied, and the small bags of contaminated corn were recovered.

### *Measurement of C-14 Urea*

C-14 is a beta emitter with a weakly penetrating ray and a half life of about 5500 years. Since the emission has so little pene-

**Table 2. Radiation counts of corn kernels contaminated with C-14 urea and steeped for 40 hours (original contamination added, 93.2 counts/min.; leached, uncontaminated corn, 0.2 counts/min.)**

| Methods of Adding C-14 Urea | Counts/Minute Above Background | | | |
|---|---|---|---|---|
| | Run 2 | Run 3 | Run 6 | Run 12 |
| Injected into exit hole | 2.22[a] | 1.77 | 3.72 | 3.25 |
| Injected on exposed endosperm | 1.51 | 0.23 | 0.28 | 0.04 |
| Injected on unbroken germ surface | −0.02 | 0.48 | 0.01 | 0.12 |
| Germ end dipped into solution | 0.38 | 0.10 | 1.19 | 0.72 |
| Control (av. of 3 kernels): 0.2 | | | | |

[a] Re-counts of same material gave 3.24 and 2.69.

tration, small amounts of plant tissues stop the radiation, causing self-absorption.

To determine the self-absorption of the corn, an individual kernel containing C-14 at the rate of 666 counts/min. was finely ground, and various amounts were weighed into the planchette and counted on a carbon Geiger tube. Results are given in Table 1 and Fig. 2. The curve in Fig. 2 is flattened, indicating that an increase in sample size does not give a proportional increase in radioactive counts. For example, the increase between 50 and 100 mg samples is only 21 counts/min.

On the basis of this curve it was decided to use 50 mg samples. While this is not the most sensitive point on the curve, it does give a sufficiently high count.

Five grains of uncontaminated, unsteeped corn were ground, and 50 mg of each grain was counted. The average of these five determinations was 0.4 counts/min. above background.

Three grains of uncontaminated, steeped corn were dried and ground, and 50 mg of each grain was counted as before. The average of these three counts was 0.2 counts/min. above background. Thus, the difference between the unleached and leached corn was insignificant.

Three unleached kernels containing 0.03 ml of the urea C-14 solution were analyzed. A 55 mg sample gave 92.1 counts above background, while two 50 mg portions gave 94.3 and 93.3 counts above background.

Next portions of corn treated with C-14 urea were taken from four of the nylon bags and the kernels were separated according to the area of urea treatment. These samples were then dried, ground individually in a mortar, and examined. The radiation was measured by determining the elapsed time required to obtain 600 counts from 50 g of corn, with the results shown in Table 2. The original portions of corn were taken from different jars of steeped material so as to be representative of the entire run.

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## FEEDS

# Determination of Water in Silage by Fischer Titration of Concentrated Ethanolic Suspensions

By MILAN F. DUBRAVČIĆ[1] (Fodder Conservation Section, Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia)

**A method is described for rapid determination of water in ensiled fodder materials. The procedure is based on a brief homogenization of 10–20% suspensions of samples in ethanol followed by titration of gravimetric aliquots with Fischer reagent. It has been found that the concentration of water in the alcoholic phase quickly reaches an equilibrium that is linearly related to the proportion of sample in the mixture and, accordingly, equal to the concentration of water in the suspended tissue. The uniformity of distribution of water simplifies both the aliquoting and the calculation. No interfering reactions have been encountered and the recovery of added water is close to 100%. Ethanol as solvent gives less variable results than either methanol or ethylene glycol. Incorporation of a dry box in the titration apparatus considerably improves the precision of the titration. Coefficient of variation of the titration is about 0.2%.**

The quantitative evaluation of changes brought about by ensiling processes depends on the accuracy of analytical methods for determination of dry matter, and conversely of water. Volatile compounds, such as fermentation products in silage, interfere with the determination of water by oven drying or by distillation methods (1–4). Corrections based on separate estimation of volatile acids and bases can be used to improve the accuracy, but the determination is thereby lengthened.

The reagent discovered by Karl Fischer (5) has been extensively used to determine water in various organic and inorganic materials (6). It is reasonably specific for water, and the titration can be carried out in about 10 minutes. To insure a practically complete extraction of different biological materials prior to Fischer titration, the ratio of water in sample to solvent (methanol) is usually kept small, e.g., 1:100 or less (7–9). However, with inhomogeneous material of high moisture content, such as silage, sample weights of from 10 to 100 g are usually considered necessary and the direct application of such ratios would involve excessively large volumes of solvent. Extraction of water, followed by aliquoting, appeared desirable. If more concentrated mixtures could be used, less solvent would be handled and more tolerance in water content of the blank could be permitted. On the other hand, considering the affinity for water of biocolloids (10), in particular those forming the structural elements of plant tissue, there was some doubt whether water could be quantitatively extracted by a single treatment with a small volume of alcohol. Distribution of water between the tissue and the ethanolic phase appeared, therefore, to be a major physical problem when dealing with concentrated suspensions.

## METHOD

### *Apparatus*

(a) *Titration cell.*—Because of the fibrous nature of the material in suspensions, a two-piece titration cell was used which permitted pre-weighing of test portions in detachable titration flasks (Fig. 1). The "electrode head" (type D4201 by Radiometer Co., Copenhagen, Denmark), modified by addition of spherical joints, is firmly clamped to the back (stand not shown). The lower part of the cell is the detachable flask connected by a Ʇ 34/45 joint to the "head." A series of flasks with stoppers, preferably of polyethylene, is required for routine work.

[1] Present address: 1080 Gilford St., Vancouver 5, B.C., Canada.

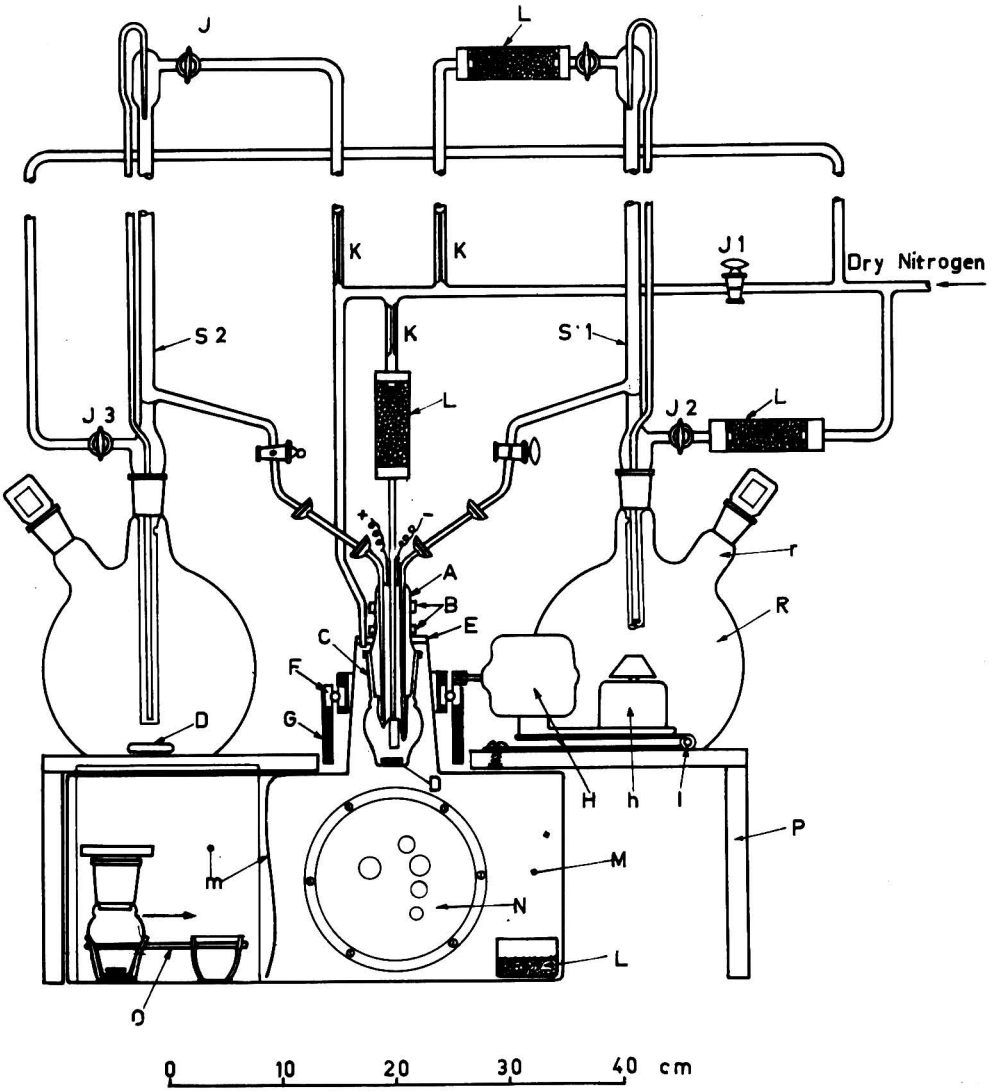


*Fig. 1*—Apparatus for titration and exchange of flasks in a controlled atmosphere. *A*, electrode head. *B*, clamp. *C*, titration flask. *D*, stirring bar, Teflon-coated. *E*, rubber washer (tight fit around A). *F*, ball bearing. *G*, magnet. *H*, motor with speed control (h). *I*, fulcrum of the hinge. *J*, stopcocks. *K*, capillaries joining vents to nitrogen train. *L*, desiccant (Drierite). *M*, dry box with flap doors (m). *N*, rubber glove. *O*, circular rack with flasks. *P*, wooden bench. *R*, 3 L reservoir with opening for filling (r). *S*. 50 ml burets: 1—Fischer reagent; 2—water-in-methanol.

(b) *Dry box and stirring.*—To prevent the absorption of atmospheric moisture during the exchange of titration flasks, the cell is enclosed in a dry box. The contamination can thus be reduced to less than 0.1 mg per operation. The box, about 18 × 18 × 40 cm, is made from a clear 0.1 mm P.V.C. film by sealing the overlapping edges with a transparent adhesive tape. The front wall of the box is reinforced by a sheet of Lucite, about 20 × 25 cm, which supports a rubber glove stretched and sealed over a 13 cm opening. The box is extended upwards to enclose the cell, and the conical extension is encircled by a ring-shaped device (11) for magnetic stirring of the flask contents. A small motor with speed control is horizontally hinged on the bench top and rotates the stirring device by friction. Satisfactory mixing was obtained at speeds of 200 rpm and over, at which point a reproducible relationship between the concentration of the Fischer reagent in the cell and current was shown.

(c) *Galvanometer.*—A spot galvanometer (Cambridge Instrument Co., Ltd., London) with a sensitivity of 30 mm per $\mu$a was used to detect the end point by the electrometric "dead stop" technique (6). It was connected in series with the platinum electrodes of the cell between which a potential drop of not more than 15 mv was provided. Since in the vicinity of the equivalence point an excess of only 5 $\mu$l of Fischer reagent caused a permanent deflection of about 10 mm on the galvanometer scale, this small positive reading was taken as the end point.

(d) *Homogenizer.*—A Sorvall homogenizer (Ivan Sorvall, Inc., Norwalk, Conn.) was used with 1 or 2 pint Mason jars having lids with rubber seals. The jars could be attached to the homogenizer by a leak-proof joint.

### *Reagents*

(a) *Karl Fischer reagent.*—Water equivalence 4–5 mg $H_2O$/ml.

(b) *Water-in-methanol solution.*—About 3 mg $H_2O$/ml.

(c) *Ethanol.*—Commercial absolute grade, usually without further treatment; when necessary, dry over Drierite and distill.

(d) *Standard solution of water in ethanol.*—About 80 mg $H_2O$/g.

(e) *Nitrogen.*—Commercially dried to not more than 0.02 mg $H_2O$/L, as supplied.

(f) *Drierite* (granular anhydrous calcium sulfate).—Regenerate at 230°C overnight.

### *Procedure*

Weigh suitable quantities of silage (e.g., 25 g) and ethanol (about 120 g) in dry 1 pint Mason jars and homogenize for 2 min. Avoid any unnecessary exposure to the atmosphere. Have sets of 4–5 titration flasks, previously dried under vacuum at 60–70°C, ready on circular racks in desiccators. Using dry dropping pipets (wide opening), transfer 0.5–1 g portions of suspensions (about 80 mg water) to flasks, stopper, and weigh. Using forceps, insert a dry polyethylene or Teflon coated stirring bar.

Before titration, thoroughly mix the reagents in reservoirs by placing a conventional magnetic stirrer underneath the wooden bench. Rinse and fill the burets by operating stopcocks J-1 and J-2 (or J-3) and purge the dry box with nitrogen for 1 hr (at 20–30 L/hr). Renew the desiccant in the box daily. Maintaining the flow of nitrogen, insert the rack with flasks through the flap door first into the left hand side of the box, and then through a similar door into the main chamber. After about 10 min. replace the flask protecting the electrodes by the first flask from the rack. Add first the Fischer reagent in a small excess (about 0.5 ml), maintaining the excess with vigorous stirring for about 30 sec. Then, back-titrate with water-in-methanol to the end point. This should remain steady, indicating freedom from any slow side-reactions or any delayed extraction of water from plant tissue.

### *Standardization and Calculation*

Determine the water equivalence of the Fischer reagent at least once a week by titrating several 1 g portions of the standard solution. Obtain the strength of the water-in-methanol solution by balancing it against the Fischer reagent.

Correct for the volume of the excess of Fischer reagent added and for water contributed by ethanol, and calculate the water content of the material, assuming a uniform distribution of water throughout the mixture.

## Results and Discussion

*Distribution of Water in Suspensions.*—The 2 minute treatment in the high-speed homogenizer disintegrated the material effectively and simultaneously insured an equilibrium distribution of water between the alcoholic liquid and the suspended tissue. The water content of the tissue-free liquid was not changed by prolonging the homogenization time from 2 to 6 minutes, or by extending the contact time after the homogenization from 10 minutes to 24 hours.

Although an equilibrium was attained between the phases, tissue particles tended to settle to the bottom of the jar, and representative aliquots were difficult to obtain. Nevertheless, the concentration of water in the sample can be related to the concentration of water in the liquid phase. Knowing that total water is the sum of water in the two phases, the following equation has been derived:

$$\frac{W}{S} = \frac{U}{M-T}\left[\frac{M}{S} + \frac{T}{S}\left(\frac{1}{P} - 1\right)\right] \qquad (1)$$

where S = g sample; W = g water in sample; U = g water in liquid phase; M = g total mixture (sample plus alcohol); T = g tissue phase; M − T = g liquid phase; and $P = \frac{U}{M-T} : \frac{W-U}{T}$ = partition ratio.

Equation (1) can also be expressed as:

$$C = y\,(x + K) \qquad (2)$$

where C = concentration of water in sample; $y$ = concentration of water in liquid phase (dependent variable); $x = \frac{M}{S}$ (independent variable); and $K = \frac{T}{S}\left(\frac{1}{P} - 1\right)$.

If $\frac{T}{S}$ and P are assumed constant within the concentration range used, the unknown parameters C and K of the equation (2) can be estimated by the experimental determination of $y$ for two different values of $x$.

It should be noted, if P = 1, K disappears, leaving

$$y = C \cdot \frac{1}{x} \qquad (3)$$

or, in words, the concentration of water in the liquid phase is linearly related to the proportion of sample in the mixture. This linear relationship was, in fact, found to hold for all samples tested, e.g., samples of ryegrass and seven widely different silages (Fig. 2). Consequently, for any of these samples, preparation of a single mixture with ethanol is sufficient for determining water content.

*Effect of Solvent.*—Methanol and ethylene glycol were compared with ethanol. The results are summarized in Table 1. The significantly higher mean value and the higher "method" variance with methanol as compared with ethanol is thought to be due to its higher vapor pressure and greater tendency to volatilize. A similar apparent increase in water content was obtained when known mixtures of water and methanol were tested in absence of silage.

Some difficulties in sampling of silages occurred during the tests with ethanol and ethylene glycol, resulting in a large sampling variance. However, even if this variation had not existed, there is no evidence of any significant difference between means. The agreement of means for ethanol and ethylene glycol, whose vapor pressure is considerably lower than that of ethanol, is assurance that the loss of ethanol is negligible. Also, the low "method" variance with ethanol in both series indicates that, of the three, ethanol is the preferred solvent for use with this method.

*Precision and Accuracy.* — The precision obtainable in the use of apparatus was first examined independently from deviations caused by samples (such as sampling errors or possibly any physical or chemical interferences by constituents). The estimate of the "within batch" coefficient of variation in standardization of Fischer reagent was 0.195 with 95% confidence range of ±0.043; most of the variation probably arises from the errors in measuring the volume of the reagent.

The coefficient of variation for the determination of water in silage, when estimated for a single titration, was usually of the order of 0.3% (0.25% was calculated from the first ethanolic series given in Table 1, and 0.31% from the titrations summarized in Table 4). This was only slightly larger than that for the standardization. Owing to a higher sampling variance in some cases, an increased coefficient of variation was shown (e.g., 1.15% was calculated from the second ethanolic series of Table 1). This indicates that the largest potential source of error is in sampling. The chance of sampling error can be reduced by increasing the number of samples or their size. Only single 20 g lots were used in the above experiment but the recommended technique permits a fair degree of flexibility.

In absence of an independent method giving absolute values, the standard-addition technique was used to check the accuracy of the proposed method. Water was added (Table 2) to analyzed ethanolic suspensions of silage and, after a 24 hour contact period with intermittent shaking, the mixture was re-analyzed. Since the material contained approximately 80 parts of water in a hundred, only about half that quantity was added in order to avoid abnormal dilution. The recovery, obtained by difference, is therefore subject to summation of the absolute errors of both determinations, and the total error, when expressed as the percentage of *added* water, may amount to about 5 times the normal error of titration. However, the mean recovery (100.54%) did not differ significantly from 100 and, accordingly, the presence of the silage appeared to have little over-all effect, if any. In a further experi-

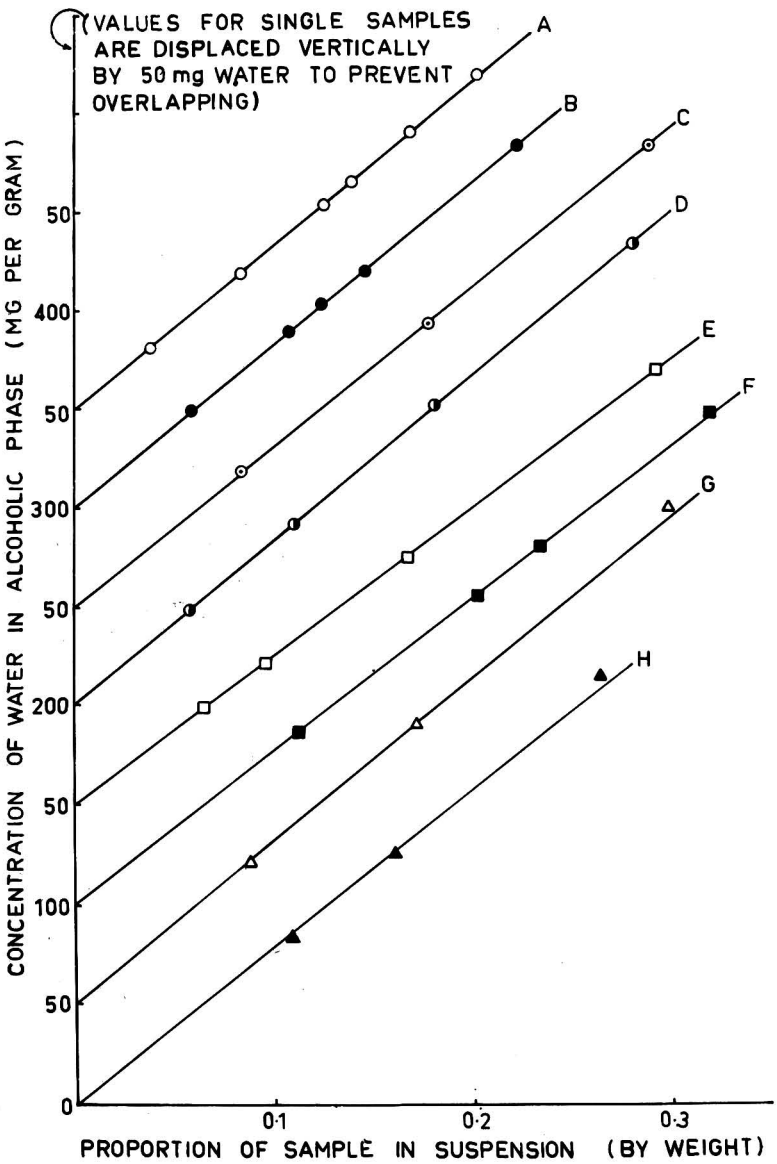


*Fig.* 2—Linear relationship of the proportion of silage in suspension and the concentration of water found in alcoholic phase. *A*, fresh ryegrass (short-rotation species, precut to about 4 cm lengths). *B*, *C*, *D*, ryegrass silages (pH 4.9, 5.7, 5.9). *E*, *F*, Lucerne silages (pH 5.1, 5.9). *G*, *H*, legume silages (G from *Lotononis bainesii*, pH 4.2; *H* from *Phaseolus atropurpureus*, pH 5.6). Prior to ensiling, all materials were cut to about 2 cm lengths.

ment (Table 3), residues from ryegrass silages dried at 80°C were used and water was added to correspond to "silages" with 67–95% water. Here the individual recoveries were within limits expected by the precision of the method and the mean was close to 100%.

No chemical interferences have been observed. From previous work (6) it appears unlikely that organic acids or bases would interfere in concentrations that normally occur in fresh or ensiled pasture materials. Total amounts of organic acids in silages normally do not exceed 2–3%, lactic acid usually being the most abundant. De Freitas (12) added lactic acid to fresh plant material

**Table 1. Effect of solvent**

| No. of Silages | Solvent[a] | Mean Water, % | Sampling Variance | Method Variance[b] | Difference between Means for Solvents |
|---|---|---|---|---|---|
| 6 | Ethanol | 84.32 | 0.0244 | 0.0210 | Highly |
| | Methanol | 85.24 | 0.0244 | 0.2126 | significant |
| 8 | Ethanol | 79.24 | 0.8033 | 0.0291 | Not |
| | Ethylene glycol | 79.28 | 0.8033 | 0.1430 | significant |

[a] One mixture per solvent and silage was prepared.
[b] Duplicate titrations were carried out on each mixture; variance was calculated from the difference within duplicates.

**Table 2. Recovery of water added to silages**

| Type of Silage | pH | Sample, g | Initial Water,[a] g | Added Water, g | Total Water | | Recovery | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Calcd, g | Found,[b] g | g | % |
| Ryegrass | 4.0 | 23.30 | 17.73 | 9.66 | 27.39 | 27.57 | 9.84 | 101.86 |
| | 4.9 | 23.98 | 19.13 | 9.91 | 29.04 | 28.92 | 9.79 | 98.79 |
| | 5.7 | 28.25 | 22.53 | 9.84 | 32.37 | 32.37 | 9.84 | 100.00 |
| | 6.6 | 32.62 | 27.11 | 9.85 | 36.96 | 37.24 | 10.13 | 102.84 |
| | 7.3 | 27.67 | 22.98 | 9.77 | 32.75 | 32.86 | 9.88 | 101.13 |
| Lucerne | 5.1 | 24.98 | 18.15 | 12.34 | 30.49 | 30.57 | 12.42 | 100.65 |
| *Lotononis bainesii* | 4.2 | 21.40 | 17.40 | 11.20 | 28.60 | 28.61 | 11.21 | 100.09 |
| *Phaseolus atropurpureus* | 5.6 | 20.96 | 16.32 | 11.28 | 27.60 | 27.48 | 11.16 | 98.94 |
| | | | | | | | Mean | 100.538 |

[a] Includes about 0.3 g of water from ethanol.
[a, b] Based on titration in duplicate.

**Table 3. Recovery of water added to dried ryegrass silages**

| Sample, g | Water in Sample,[a] g | Added Water,[b] g | Total Water | | Recovery | |
|---|---|---|---|---|---|---|
| | | | Calcd, g | Found,[c] g | g | % |
| 0.85 | 0.0117 | 7.2265 | 7.2382 | 7.2035 | 7.1918 | 99.52 |
| 2.48 | 0.0341 | 7.8539 | 7.8880 | 7.8883 | 7.8542 | 100.00 |
| 3.30 | 0.0454 | 6.7809 | 6.8263 | 6.8516 | 6.8062 | 100.37 |
| 0.61 | 0.0020 | 11.6399 | 11.6419 | 11.6494 | 11.6474 | 100.06 |
| 2.44 | 0.0079 | 13.5596 | 13.5675 | 13.5852 | 13.5773 | 100.13 |
| 3.52 | 0.0114 | 10.3249 | 10.3363 | 10.3534 | 10.3420 | 100.17 |
| | | | | | Mean | 100.042 |

[a] Determined separately on a larger quantity of sample.
[b] A 7% solution of water in ethanol was added.
[c] Based on titration in duplicate, after a 24 hour contact period.

to correspond to about 4% of lactic acid in silage and found no effect on Fischer titration.

*Comparative Methods.*—Table 4 shows the magnitude of differences obtained when 16 silages were analyzed concurrently by the titration method and by oven drying at 80°C. The loss of weight by drying was generally higher than the water content by titration, even after correction for volatilized

**Table 4. Water content by oven drying and Fischer titration methods, per cent**

| Ryegrass Silage | pH | Oven Drying[a] | | Fischer Titration[d] | Difference |
|---|---|---|---|---|---|
| | | Uncorrected[b] | Corrected[c] | | |
| 1 | 3.9 | 80.36 | 79.87 | 79.09 | 0.78 |
| 2 | 4.0 | 74.57 | 74.30 | 73.41 | 0.89 |
| 3 | 4.1 | 78.95 | 78.44 | 77.56 | 0.88 |
| 4 | 4.1 | 79.73 | 79.43 | 78.87 | 0.56 |
| 5 | 4.2 | 79.42 | 78.96 | 78.51 | 0.45 |
| 6 | 4.3 | 81.27 | 80.81 | 80.35 | 0.46 |
| 7 | 4.5 | 80.27 | 79.69 | 78.81 | 0.88 |
| 8 | 4.5 | 81.97 | 81.69 | 81.36 | 0.33 |
| 9 | 4.5 | 79.87 | 79.22 | 79.03 | 0.19 |
| 10 | 4.9 | 79.11 | 78.43 | 78.20 | 0.23 |
| 11 | 5.3 | 81.62 | 81.13 | 81.12 | 0.01 |
| 12 | 5.6 | 82.30 | 81.64 | 82.22 | −0.58 |
| 13 | 6.1 | 80.85 | 79.85 | 79.85 | 0.00 |
| 14 | 7.0 | 83.50 | 82.24 | 81.67 | 0.57 |
| 15 | 7.0 | 83.51 | 82.71 | 83.40 | −0.69 |
| 16 | 7.0 | 84.04 | 82.82 | 82.52 | 0.30 |

[a] Dried for 16 hours at 80°C with forced draft.
[b] Mean of three determinations of the loss of weight.
[c] Correction for the loss of volatile fatty acids (as acetic acid) and of volatile bases (as ammonia) was obtained by taking the difference in amounts of these constituents in the silage and in the dry residue.
[d] Mean from two suspensions, each titrated in duplicate.

acids and bases. However, the correction for volatile acids, regarded as acetic, was probably underestimated (in most cases, when a chromatographic separation of these acids had been carried out, the average molecular weight was found to be somewhat higher than that of acetic acid). Furthermore, no correction was made for the volatilization of any neutral constituents, their presence having been reported in many silages (1, 13). On the other hand Fischer titration indicated that the oven-dried material still contained about 0.2% water (on fresh silage basis).

It can be stated in conclusion that with physical methods of determining water in silage, in particular with oven drying, an element of doubt often remains owing to the presence of variable quantities of a wide range of volatile constituents (1, 13, 14). The specificity for water of the Fischer reagent in conjunction with the described technique permits the analysis of silage samples of a large quality spectrum with improved speed and accuracy.

**Acknowledgments**

The author thanks G. W. Lanigan for supplying silage samples and data other than water contents by titration; R. Birtwistle for the help in statistical analyses; and Mrs. J. F. Melvin for suggestions in the preparation of this paper.

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## VITAMINS AND OTHER NUTRIENTS

# Hydrolysis of Proteins for Amino Acid Analysis. II. Acid Hydrolysis in Sealed Tubes of Mixtures of ß-Lactoglobulin and Starch

By WILLIAM G. GORDON and JAY J. BASCH (Eastern Regional Research Laboratory,[1] Philadelphia, Pa. 19118)

**Experiments with a mixture of β-lactoglobulin and starch, simulating the composition of carbohydrate-rich foods and feeds, have been carried out to determine conditions for acid hydrolysis that will permit maximal recovery of amino acids in hydrolysates of such materials. When a large excess of 6*N* HCl is used for hydrolysis, good recoveries of most amino acids are obtained. However, about one-quarter of the tyrosine present is destroyed under the conditions investigated. Some destruction of methionine and cystine may also be attributed to the presence of carbohydrate, but a special method for the determination of these amino acids is available.**

A previous report (1) described the problem of preparing acid hydrolysates of foods and feeds, which represent as accurately as possible the amino acid composition of the original material, and outlined an experimental approach to its possible solution. In brief, it was planned to hydrolyze under different conditions highly purified β-lactoglobulin AB, the amino acid composition of which is well-known (2), in the presence of 20 times as much starch and to select those conditions that brought about the least destruction of labile amino acids. Some promising results have now been obtained and it is likely that a useful method can be proposed in the near future for collaborative test. Meanwhile, some of the pertinent data are summarized in this report.

### Experimental

β-Lactoglobulin AB was a lyophilized sample, five times recrystallized, that had been analyzed previously for its amino acid content (2). Potato starch was a commercial product; it was washed thoroughly first with dilute HCl, then with water, alcohol, and ether, and dried in the air.

To accurately weighed samples (2–4 mg) of β-lactoglobulin of known moisture content and 20-fold quantities of starch in 16 × 150 mm test tubes, 2,000-fold quantities (4–8 ml) of 6*N* HCl were added. The tubes were drawn out at the neck, immersed in a Dry Ice bath until the contents were frozen, evacuated to a pressure of less than 1 mm, and sealed under vacuum. After they reached room temperature they were heated for the desired periods of time at 110 ± 1° in an oil bath. In the experiments to be reported here, 24, 72, and 96 hour periods of hydrolysis were used. Because serine and threonine decompose progressively, values for these amino acids are obtained by extrapolation to zero time; because valine and isoleucine are not completely liberated in 24 hours, longer periods of hydrolysis are required.

After hydrolysis, the tubes were cooled and opened. To remove humin the contents of each tube were filtered through a glass fiber filter disk fitted in a coarse, sintered-glass Büchner funnel, and the filtrate was collected in a 50 ml round-bottom, long-neck flask. Five 1 ml portions of hot 1*N* HCl were used as washes for quantitative transfer. The filtrate and washes were concentrated to a sirup in a rotary evaporator, a few ml of water were added, and the evaporation was repeated to remove excess HCl. The residue was dissolved in pH 2.2 buffer (3) containing 5 ml per L of thiodiglycol. It was then either transferred quantitatively to

[1] Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

the 150 cm column of the amino acid analyzer, or it was made to volume (5 ml) and suitable aliquots were applied to both 15 and 150 cm columns. Analyses were run in an automatic analyzer of the type described by Spackman, Stein, and Moore (4). Many of the preceding operations were patterned on the procedures described by Moore and Stein (5), to whom we are indebted for details prior to publication.

**Table 1. Amino acid composition of β-lactoglobulin as determined in the absence and presence of starch following hydrolysis in sealed tubes[a]**

| | g Amino Acid per 100 g Dry Protein | |
|---|---|---|
| | β-Lactoglobulin (Reference 2) | β-Lactoglobulin plus Starch |
| Aspartic acid | 11.2 | 11.3 |
| Threonine | 4.94 | 4.90 |
| Serine | 3.64 | 3.81 |
| Glutamic acid | 19.3 | 19.7 |
| Proline | 5.09 | 5.11 |
| Glycine | 1.41 | 1.36 |
| Alanine | 6.98 | 6.92 |
| Valine | 6.03 | 6.07 |
| Methionine | 3.16 | 2.94 |
| Isoleucine | 6.89 | 7.08 |
| Leucine | 15.2 | 15.5 |
| Tyrosine | 3.90 | 2.92 |
| Phenylalanine | 3.55 | 3.53 |
| Lysine | 11.6 | 11.9 |
| Histidine | 1.57 | 1.65 |
| Arginine | 2.79 | 2.61 |

[a] The figures are average values for all determinations regardless of time of hydrolysis with the following exceptions: values for threonine and serine are extrapolated values by the method of least squares from analyses of 24, 72, and 96 hour hydrolysates; valine and isoleucine values are averages of analyses from 72 and 96 hour hydrolysates.

## Results and Discussion

In Table 1 a comparison is made of the amino acid composition of β-lactoglobulin as determined both in the absence of starch and in its presence. The results of the present analyses are either averaged or extrapolated values from two separate hydrolysates at each time of hydrolysis in the case of the basic amino acids (15 cm column) and at least three different hydrolysates at each time for the other amino acids (150 cm column). The analyses made in the presence of starch have been corrected for small quantities of amino acids found in control hydrolysates of starch alone.

With few exceptions good recoveries of amino acids were obtained. The presence of carbohydrate clearly had no deleterious effect on aspartic acid, threonine, serine, glutamic acid, proline, alanine, valine, isoleucine, leucine, phenylalanine, lysine, and histidine. The small apparent destruction of glycine is thought to be not significant, but the somewhat larger destruction of arginine, though unexpected, may be significant and will bear closer scrutiny.

Cystine values have been omitted because of considerable destruction in both the absence and presence of starch. It is anticipated that in future studies it will be possible to obtain reliable values for cystine (plus cysteine) following performic acid oxidation to cysteic acid by the method of Moore (6). It also will be noted from Table 1 that some destruction of methionine occurred in the present experiments. It may be possible to obtain satisfactory recoveries of this amino acid as well, after similar oxidation to methionine sulfone.

The most serious destruction of an amino acid brought about by the addition of starch to β-lactoglobulin was that of tyrosine, whose recovery was only about 75% of the amount previously found. The lability of tyrosine during acid hydrolysis of protein, especially in the presence of carbohydrate, has been recognized for some time. The expedient of stabilizing tyrosine by adding excess tryptophan before acid hydrolysis was proposed by Lyman, *et al.* (7), and the method was applied to the analysis of various classes of materials used or potentially useful in feeds for poultry and swine. In a few experiments with β-lactoglobulin-starch mixtures to which tryptophan was added, better recoveries of tyrosine were indeed obtained; the amount of additional humin

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The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. See *This Journal*, 47, 121 (1964).

formed, however, is substantial and presents special problems, especially when the sealed tube technique is used. Further study of this method is planned. For a number of reasons it seemed desirable to find out whether hydrolysis under reflux, in even more dilute solution than that used in sealed tubes, might not give as good or better results with greater convenience than those obtained in the present series of experiments. Experiments along these lines are in progress and indications are that hydrolysis under reflux may well be the method of choice.

It is recommended that study be continued.

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# Collaborative Study of a Chemical Method for Vitamin D

By E. L. HOVE, G. M. SHUE, S. W. JONES, and J. B. WILKIE (Division of Nutrition, Food and Drug Administration, Washington, D.C. 20204)

**A modification of the USP chemical method for vitamin D has been studied collaboratively. Included in the study were four samples, varying from 150 to 100,000 International Units per gram: a fortified chick feed, a fortified evaporated milk, a high potency concentrate, and a fish liver feeding oil. Results by the chemical estimation are compared with those on the same samples by the AOAC rat bioassay, and, when applicable, by the AOAC chick bioassay. The mean results of the chemical assays were 75–100% of the bioassay results. Further modifications of the chemical method to ensure applicability to feeds and foods in the range of 5–95 units per gram should be studied collaboratively.**

The chemical method for vitamin D described in the USP XVI (1960) was designed for analyses of pharmaceutical products, and specified sample sizes to contain about 10,000 I.U. of vitamin D. The method involves the estimation of the orange compound formed when the purified unsaponifiable fraction of the sample reacts with $SbCl_3$ in dichloroethane. In preparing the sample for assay, the unsaponifiable fraction is passed through two chromatographic columns to remove vitamin A and other interfering materials.

The USP method has been modified by Osadca and DeRitter (1) to make it applicable to feed and food materials of lower potency. The significant variant involves the use of a third column (alumina) in the purification process in those samples where the blank may be expected to be high. The high blanks are thought to be due to carotene and related components which the alumina removes. Minor variants of the method include change in some of the solvents and omission of the internal standard.

It is hoped that modifications and refinements will eventually result in a routine chemical method for samples of potency as low as four I.U. vitamin D per gram. At this sensitivity it could replace the biological assay on samples for which it is suited. Before refinements were considered however, it was decided to subject the modified method to collaborative study.

## Collaborative Study

*Methods.*—The chemical method of Osadca and DeRitter (1) was used, except that the internal standard technique as given for the

**Table 1. Summary data on collaborative comparison of vitamin D methods[a] (average of reported results ± variability coefficient)**

| Sample | Chemical I.U./g | Rat Bioassay I.U./g | Chick Bioassay I.U./g |
|---|---|---|---|
| 1 | 216 ± 23% | 205 ± 33% | 252 ± 15% |
| 2 | 3,864 ± 24% | 4,187 ± 19% | — |
| 3 | 88,200 ± 18% | 108,700 ± 15% | 103,600 ± 15% |
| 4 | 484 ± 27% | 446 ± 21% | 514 ± 14% |

[a] The average values were obtained from the reported results of 10 and 11 laboratories for the chemical method, 5 and 6 laboratories for the rat assay method, and 5 laboratories for the chick assay method (See Tables 2 and 3 for omitted values).

USP XVI method (2) was retained. Collaborators were asked to perform rat and chick bioassays as described in *Official Methods of Analysis* (1960), using two or more assay levels.

*Samples.*—Four samples of widely differing nature and potency were prepared and sent to laboratories that had agreed to take part in the study. The samples were:

(*1*) Fortified chick feed: about 200 I.U. vitamin $D_3$/g.

(*2*) Fortified evaporated milk: about 5,000 I.U. vitamin $D_2$/ml.

(*3*) Gelatin-coated $D_3$ concentrate: about 100,000 I.U. vitamin $D_3$/g.

(*4*) Vitamin A–D feeding oil: about 400 I.U. vitamin $D_3$/g.

## Results

The summary of the data, as given in Table 1, shows that the chemical method is somewhat more variable than the bioassays on the samples tested. However, the mean values of the chemical determination were reasonably close to the results from the biological assays. The low chemical value for Sample 3 is thought to be related to inadequate instructions for dissolving the gelatin coating. The collaborators had difficulty with the evaporated milk, Sample 2; the wide variations in results indicate that the vitamin D concentrate was inadequately homogenized into the sample.

The variability coefficient of the chick biological assay was about ± 15%. The rat biological assay was somewhat more variable at about ± 20%. The chemical method showed a variability of about ± 24%.

The individual results are given in Table 2 (chemical) and Table 3 (bioassays). The

**Table 2. Collaborative results by the chemical vitamin D method (as I.U. vitamin D per gram)**

| Lab | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
|---|---|---|---|---|
| A | 215 | 5,320 | 82,760 | 849 |
| | 202 | 4,710 | 95,300 | 711 |
| B | 250 | 2,570 | 65,300 | 634 |
| | 260 | 3,750 | 73,500 | 605 |
| C | 220 | 3,496 | 78,950 | 409 |
| | 233 | 3,334 | 85,030 | 563 |
| D | 194 | 2,970 | 86,470 | 519 |
| | 296 | 1,795 | 87,920 | 456 |
| E[a] | 163 | 2,510 | 66,300 | 537 |
| | 204 | — | — | – |
| F[a] | 164 | 1,912 | 12,250 | 262 |
| G | 180 | 3,780 | 54,000 | 353 |
| | 190 | 2,760 | 65,700 | 345 |
| H | 197 | 4,770 | 112,000 | 461 |
| | 190 | 4,830 | 113,500 | 443 |
| I[a] | 245 | 4,150 | 115,000 | 570 |
| J | 240 | 3,400 | 80,300 | 470 |
| | 207 | 3,150 | 86,900 | 493 |
| K | 286 | 5,040 | 113,000 | 543 |
| | 298 | 4,800 | 113,000 | 543 |
| L | 192 | (1,960)[a] | 87,240 | 393 |
| | 254 | (1,950) | 91,930 | 406 |
| M | (375)[a] | 3,118 | 106,300 | 275 |
| | (388) | 4,440 | 81,100 | 384 |
| N | 102 | 4,670 | 90,090 | 404 |
| | 114 | 4,575 | 90,000 | 394 |

[a] Omitted from mean.

**Table 3. Collaborative results by the bioassay methods for vitamin D (as I.U. vitamin D per gram)**

| Lab | Sample 1 | Sample 2 | Sample 3 | Sample 4 |
|---|---|---|---|---|
| | | Chicks | | |
| A | 209 | — | 89,500 | 449 |
| H | 287 | — | 113,000 | 600 |
| I | 215 | — | 85,600 | 506 |
| J | 258 | — | 107,000 | 579 |
| K | 290 | — | 122,900 | 436 |
| | | Rats | | |
| A | 140 | 3,870 | 102,600 | 410 |
| F | 178 | 4,160 | 104,300 | 393 |
| H | 240 | 3,330 | 88,400 | 350 |
| I | 320 | 5,500 | 134,000 | 540 |
| J | 150 | 4,080 | 122,900 | 583 |
| L | 200 | — | 100,000 | 400 |

instructions to collaborators had requested that chemical analyses be made in duplicate; those laboratories reporting single values were omitted from the summary. The results from Laboratory F were outside the acceptable range. Omission of data was based in two cases on the analyst's statements of specific trouble, such as formation of a blue compound.

The chemical method calls for color development time of exactly 45 seconds. Some earlier experience in our laboratory had indicated that a better result was obtained when the color development was limited to 20 seconds. We asked our collaborators to check this point. The differences are very small and not significant.

### Recommendation

The chemical method for vitamin D has been found to be approximately as satisfactory as the official rat and chick assays for a set of four samples of widely different nature and potency. The lowest potency was 200 I.U. of vitamin D per gram. However, the collaborative study has shown the need for certain changes in the proposed method.

It is recommended that collaborative studies on chemical vitamin D methods be continued, especially on modifications which permit the application of the method to low potency samples.

### Collaborating Laboratories

Kenneth Morgareidge, Food and Drug Research Laboratories, Maspeth, N.Y.

Lawrence Kogan, The Fleischman Laboratories, Stamford, Conn.

T. K. Murray, Food and Drug Directorate, Ottawa, Canada

R. E. Booth, The Upjohn Company, Kalamazoo, Mich.

Leonard Herriman, Purdue University, Lafayette, Ind.

H. W. Howard, The Borden Company, New York, N.Y.

E. DeRitter, Hoffmann-LaRoche, Inc., Nutley, N.J.

O. F. Hixson, Rosner-Hixson Laboratories, Chicago, Ill.

Earl Richter, Wisconsin Alumni Research Foundation, Madison, Wis.

E. L. Hove, Food and Drug Administration, Division of Nutrition, Washington, D.C.

J. C. Fritz, Dawes Laboratories, Inc., Chicago, Ill.

F. G. McDonald, Mead Johnson Research Center, Evansville, Ind.

R. J. Martin, Food and Drug Administration, Los Angeles, Calif.

W. R. Clark, Food and Drug Administration, Chicago, Ill.

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This report of the Associate Referee, E. L. Hove, was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. See *This Journal*, **47**, 121 (1964).

# Components of Vitamin $B_6$ in Grains and Cereal Products

By MARILYN MACARTHUR POLANSKY, ELIZABETH W. MURPHY, and EDWARD W. TOEPFER (Human Nutrition Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.)

**Extracts of 56 grains and cereal products were chromatographically separated on Dowex-50 ion exchange columns into pyridoxine, pyridoxal, and pyridoxamine fractions. These fractions were assayed microbiologically by using *S. carlsbergensis*. Procedures used and values obtained by the assays are presented, along with values for total vitamin $B_6$ in unchromatographed extracts and proximate composition values for each sample.**

Components of vitamin $B_6$ in hydrolyzed food extracts have been separated on Dowex 50 columns into individual fractions containing pyridoxine, pyridoxal, and pyridoxamine, and the microbiological procedure using *Saccharomyces carlsbergensis* has been described for the determination of the components in the eluted fractions (1–3). Data obtained by these procedures compare well with those obtained by rat bioassay for total vitamin $B_6$ in a few selected food samples (4). Atkin, *et al.* (5) found lower values for vitamin $B_6$ in wheat and wheat products by extraction with 0.055*N* than with 0.44*N* acid, when autoclaved at 15 pounds steam pressure for 5 hours.

This paper presents information on extraction and assay procedures in addition to reporting values for pyridoxine, pyridoxal, and pyridoxamine of grains and cereal products. Values are also reported for total vitamin $B_6$ in unchromatographed extracts (calculated as pyridoxine). Supporting proximate composition data are included to make it possible to relate these vitamin $B_6$ data with data from other sources on similar samples.

## Experimental Studies

### *Samples*

Six samples of whole wheat, and one each of barley and rye, representing composites from many lots of the same variety of grain, were obtained from the Grain Division, Agricultural Marketing Service, U.S. Department of Agriculture. Two samples of whole kernel corn, representing single lots of hybrid corn, were obtained through the Cereal Crops Research Branch, Agricultural Research Service, U.S. Department of Agriculture. For other grain and cereal samples, several available brands of each item were purchased in the Washington, D.C. area, and composited on an equal-weight basis. The composites were ground to pass a 20-mesh screen, thoroughly mixed by tumbling, and stored at 32°F in push-top cans or amber glass jars. Although soybeans are a legume, soya flour was included in this study as a flour because of its manner of use.

### *Procedures*

Details of procedures for preparation of reagents, chromatographic separation of food extracts, and the microbiological assay have been previously reported (3). Preparation of the Dowex AG-50-WX8 resin for use in the chromatographic separation has been changed slightly from the earlier procedure as follows:

After heating with 3*N* HCl and rinsing with water until neutral washings were obtained, the resin was stirred for 1 hour in about 300 ml of 6*N* KOH, washed with water until neutral, suspended in 2*M* potassium acetate, and stored under refrigeration. Before use, the resin was washed with water until neutral washings were obtained.

For the extraction of vitamin $B_6$ components, finely divided subsamples (about 1 g) were placed in 500 ml wide-mouth Erlenmeyer flasks, suspended in 200 ml 0.44*N* HCl, and autoclaved at 15 pounds steam pressure for 2 hours. For the effect of acid concentration on the amounts of $B_6$ components hydrolyzed from whole wheat, extractions were made: with water, autoclaving 15 minutes; with 0.055*N*, 0.44*N*, and 1.0*N* HCl, autoclaving 5 hours; and with 0.44*N*

HCl, autoclaving 2 hours. When cool, the extracts were adjusted to pH 4.5 with KOH, diluted to 250 ml, and filtered through Whatman No. 40 filter paper. Chromatographic and microbiological procedures were carried out as previously described.

Procedures for proximate composition determinations were those of the AOAC (6), modified only as described: section **13.003** for total solids, modified by drying to constant weight at 50°C; section **22.033** for crude fat or ether extract, modified by using ethyl ether for whole grains, uncooked cereals, flours and meals, and petroleum ether for soybean flour; section **13.019** for fat by acid hydrolysis in breads, ready-to-eat cereals, precooked rice, precooked baby cereals, macaroni and similar foods; section **38.010** for Kjeldahl nitrogen, modified by using 0.02*N* HCl as the collectant and back-titrating with 0.02*N* NaOH and methyl red indicator; section **13.006** for total ash.

## Results and Discussion

Comparisons of the amounts of vitamin $B_6$ in extracts obtained with increasing concentrations of acid were made on a total of 19 subsamples of whole wheat. The extracts were chromatographed on Dowex 50 columns in order to observe the effect of the extrac-

**Table 1. Values for vitamin $B_6$ components and proximate composition of grains and cereal products**

| Sample Description | Proximate Composition Values, % | | | | Vitamin $B_6$ Values | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Chromatographed, μg/g | | | |
| | Total Solids | Fat | Kjeldahl Nitrogen | Ash | Unchromatographed, μg/g | Sum | Pyridoxine | Pyridoxal | Pyridoxamine |
| Grains: | | | | | | | | | |
| Barley | | | | | | | | | |
| Malting, western, 1961 | 90.2 | 1.7 | 1.95 | 2.49 | 3.87 | 3.87 | 2.18 | 0.49 | 1.20 |
| Pearled | 90.8 | 0.7 | 2.21 | 0.89 | 2.13 | 2.24 | 1.19 | 0.37 | 0.68 |
| Corn, whole kernel yellow | | | | | | | | | |
| Iowa, 1962 | 90.6 | 4.4 | 1.39 | 1.22 | 3.62 | 4.91 | 0.43 | 3.14 | 1.34 |
| Mississippi, 1962 | 92.9 | 5.1 | 1.72 | 1.29 | 6.11 | 7.53 | 1.33 | 3.53 | 2.67 |
| Popcorn | | | | | | | | | |
| White, unpopped | 87.8 | 4.3 | 1.82 | 1.31 | 3.36 | 3.18 | 0.35 | 1.80 | 1.03 |
| Yellow, unpopped | 87.6 | 3.9 | 1.73 | 1.25 | 3.38 | 3.65 | 0.41 | 1.99 | 1.25 |
| Rice | | | | | | | | | |
| Brown, long grain | 89.8 | 2.7 | 1.34 | 1.49 | 5.56 | 5.37 | 4.21 | 0.62 | 0.54 |
| Converted | 91.7 | 0.3 | 1.15 | 0.64 | 5.38 | 4.25 | 3.64 | 0.29 | 0.32 |
| White, long grain | 89.8 | 0.5 | 1.25 | 0.55 | 1.80 | 1.78 | 1.17 | 0.34 | 0.27 |
| White, precooked | 90.5 | 0.4 | 1.24 | 0.23 | 0.48 | 0.32 | 0.14 | 0.10 | 0.08 |
| White, short grain | 88.6 | 0.5 | 1.19 | 0.58 | 2.24 | 2.04 | 1.27 | 0.38 | 0.39 |
| Rye, 1961 | 88.2 | 1.4 | 2.04 | 1.85 | 2.98 | 2.94 | 1.74 | 0.59 | 0.61 |
| Wheat | | | | | | | | | |
| Dark hard winter, 1961 | 89.3 | 1.5 | 2.17 | 1.72 | 3.84 | 3.78 | 2.78 | 0.57 | 0.43 |
| Dark northern spring, 1961 | 90.8 | 1.8 | 2.76 | 1.73 | 4.29 | 4.14 | 2.92 | 0.50 | 0.72 |
| Dark northern spring, 1957 | 89.4 | 1.8 | 2.68 | 1.75 | 4.22 | 4.07 | 2.96 | 0.48 | 0.63 |
| Hard amber durum, 1961 | 91.2 | 1.9 | 2.73 | 1.64 | 4.07 | 3.99 | 2.67 | 0.58 | 0.74 |
| Red winter, 1961 | 88.6 | 1.4 | 1.91 | 1.68 | 3.68 | 3.49 | 2.63 | 0.48 | 0.38 |
| Soft white, 1961 | 90.6 | 1.5 | 2.07 | 1.70 | 3.63 | 3.86 | 2.97 | 0.49 | 0.40 |
| Flours and meals: | | | | | | | | | |
| Cornmeal | | | | | | | | | |
| White | 90.5 | 2.2 | 1.22 | 0.76 | 3.04 | 3.17 | 0.37 | 1.56 | 1.24 |
| Yellow | 90.4 | 1.7 | 1.32 | 0.60 | 2.28 | 2.39 | 0.27 | 1.27 | 0.85 |
| Flour | | | | | | | | | |
| All-purpose | 90.4 | 0.9 | 1.85 | 0.47 | 0.76 | 0.58 | 0.32 | 0.14 | 0.12 |
| Buckwheat | 90.3 | 3.3 | 2.17 | 1.90 | 6.80 | 5.78 | 3.07 | 1.32 | 1.39 |
| Rye | 91.3 | 1.3 | 1.86 | 1.62 | 3.36 | 3.15 | 2.03 | 0.68 | 0.44 |
| Soya | 94.6 | 21.9 | 6.19 | 5.23 | 6.81 | 5.75 | 3.62 | 1.43 | 0.70 |
| Whole wheat | 91.1 | 1.8 | 2.17 | 1.41 | 3.24 | 3.12 | 2.28 | 0.49 | 0.35 |

(*Continued*)

**Table 1.** (*Continued*)

| Sample Description | Proximate Composition Values, % | | | | Vitamin $B_6$ Values | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total Solids | Fat | Kjeldahl Nitrogen | Ash | Unchromatographed, μg/g | Chromatographed, μg/g | | | |
| | | | | | | Sum | Pyridoxine | Pyridoxal | Pyridoxamine |
| Breads: | | | | | | | | | |
| Cracked wheat | 63.8 | 2.1 | 1.66 | 1.90 | 1.31 | 0.92 | 0.47 | 0.23 | 0.22 |
| French | 69.0 | 1.7 | 1.94 | 1.94 | 0.74 | 0.53 | 0.20 | 0.15 | 0.18 |
| Rye | 63.2 | 2.9 | 1.71 | 2.27 | 1.06 | 0.91 | 0.55 | 0.22 | 0.14 |
| Wheat | 64.2 | 2.8 | 1.85 | 2.29 | 1.24 | 1.12 | 0.59 | 0.31 | 0.22 |
| White enriched | 62.9 | 2.3 | 1.52 | 2.03 | 0.64 | 0.43 | 0.14 | 0.16 | 0.13 |
| Whole wheat | 60.9 | 1.3 | 1.79 | 2.59 | 1.99 | 1.94 | 1.35 | 0.38 | 0.21 |
| Cereals to be cooked: | | | | | | | | | |
| Corn grits, enriched, quick-cooking | 89.5 | 0.8 | 1.14 | 0.36 | 1.67 | 1.47 | 0.18 | 0.70 | 0.59 |
| Oats, rolled | | | | | | | | | |
| Quick-cooking | 92.2 | 6.1 | 2.58 | 1.77 | 1.60 | 1.42 | 0.25 | 0.57 | 0.60 |
| Regular cooking | 91.7 | 6.2 | 2.49 | 1.87 | 1.66 | 1.19 | 0.14 | 0.59 | 0.46 |
| Wheat | | | | | | | | | |
| Refined, quick-cooking | 90.7 | 0.7 | 1.83 | 1.46 | 0.90 | 0.74 | 0.42 | 0.16 | 0.16 |
| Refined, regular cooking | 89.6 | 0.6 | 1.95 | 0.36 | 0.65 | 0.42 | 0.18 | 0.13 | 0.11 |
| Whole, instant cooking | 91.3 | 2.1 | 2.33 | 1.64 | 4.24 | 4.07 | 3.23 | 0.43 | 0.41 |
| Whole, regular cooking | 92.2 | 2.3 | 2.20 | 1.72 | 3.61 | 3.75 | 2.98 | 0.43 | 0.34 |
| Cereals ready-to-eat: | | | | | | | | | |
| Corn | 95.7 | 0.2 | 1.11 | 2.94 | 0.98 | 0.75 | 0.16 | 0.35 | 0.24 |
| Oats | 96.1 | 3.3 | 2.05 | 4.26 | 1.14 | 0.86 | 0.21 | 0.32 | 0.33 |
| Rice | 94.4 | 0.3 | 0.99 | 2.82 | 1.53 | 1.25 | 0.96 | 0.15 | 0.14 |
| Rice, puffed | 92.5 | 1.1 | 0.94 | 0.47 | 0.95 | 0.75 | 0.48 | 0.12 | 0.15 |
| Wheat | | | | | | | | | |
| Bran, 100% | 96.6 | 1.2 | 1.86 | 6.16 | 8.96 | 8.20 | 7.08 | 0.71 | 0.41 |
| Bran flakes, 40% | 95.7 | 1.4 | 1.59 | 3.86 | 4.34 | 3.84 | 3.46 | 0.23 | 0.15 |
| Whole wheat | 96.3 | 1.6 | 1.53 | 3.48 | 3.39 | 2.92 | 2.59 | 0.20 | 0.13 |
| Shredded | 92.4 | 1.2 | 1.72 | 1.46 | 2.83 | 2.44 | 2.03 | 0.26 | 0.15 |
| Puffed | 93.3 | 1.0 | 2.52 | 1.36 | 2.28 | 1.70 | 1.38 | 0.17 | 0.15 |
| Cereals precooked, infants: | | | | | | | | | |
| Barley | 91.9 | 2.0 | 2.32 | 4.93 | 4.20 | 3.87 | 2.50 | 0.46 | 0.91 |
| High protein | 92.5 | 3.2 | 5.63 | 6.90 | 6.31 | 5.31 | 3.06 | 1.04 | 1.21 |
| Mixed | 92.8 | 3.1 | 2.42 | 4.77 | 2.78 | 2.34 | 0.90 | 0.50 | 0.94 |
| Oatmeal | 92.3 | 6.6 | 2.55 | 4.62 | 1.94 | 1.81 | 0.31 | 0.53 | 0.97 |
| Rice | 91.4 | 1.8 | 1.08 | 4.30 | 2.53 | 2.58 | 1.82 | 0.32 | 0.44 |
| Other grain products, uncooked: | | | | | | | | | |
| Egg noodles | 90.2 | 4.5 | 2.36 | 0.74 | 1.16 | 0.88 | 0.38 | 0.37 | 0.13 |
| Macaroni | 89.3 | 1.5 | 2.17 | 0.56 | 0.79 | 0.67 | 0.35 | 0.16 | 0.16 |
| Spaghetti | 89.4 | 1.4 | 2.15 | 0.56 | 0.80 | 0.68 | 0.32 | 0.21 | 0.15 |
| Wheat germ | 95.4 | 10.4 | 5.02 | 4.79 | 13.26 | 13.10 | 9.23 | 1.16 | 2.71 |

tion on the individual $B_6$ components. The amounts of the components and total vitamin $B_6$ released in the whole wheat extracts were identical for 0.44 and 1.0*N* HCl when autoclaved 5 hours. The values for pyridoxal were the same for 0.055, 0.44, and 1.0*N* HCl. Pyridoxine and pyridoxamine were incompletely freed by 0.055*N* HCl. Autoclaving with 0.44*N* HCl for 2 hours was found to be equivalent to a 5-hour autoclaving period, and was the procedure adopted for use in the analyses of grains and cereal products.

The values for the vitamin $B_6$ components of grains and cereal products as determined microbiologically are given in Table 1. Vitamin $B_6$ values are averages of the results obtained from at least three independently run assays. Proximate composition data for these grains and cereal products, included in

Table 1, are averages of the results obtained from triplicate determinations made in at least two separate assay periods.

Vitamin $B_6$ values obtained for the unchromatographed extracts of samples were somewhat greater than the totals of the individual components of the chromatographed fractions for most of the samples. The data from the chromatographed extracts may be considered representative of actual or true values. Previously published work (4) showed agreement between values obtained by chromatography and by rat bioassay.

For wheat and rice, over 70% of the vitamin $B_6$ was pyridoxine; for corn, over 50% was pyridoxal. Only barley and corn appeared to contain as much as 30% of the vitamin $B_6$ as pyridoxamine. The 1957 dark northern spring wheat sample had essentially the same values for the vitamin $B_6$ components as the 1961 sample from the same geographical area. The large difference in vitamin $B_6$ content of the two whole kernel corn samples may be due to variety as much as to location. Brown rice, buckwheat flour, soya flour, and the whole kernel corn sample from Mississippi had the highest vitamin $B_6$ content among the flours, whole grains, and whole grain products analyzed.

This study did not permit a direct comparison between the vitamin $B_6$ values of whole grains and of their processed products because of the way the samples were obtained. However, those food samples containing less than 1 $\mu$g/g of total vitamin $B_6$ were highly processed or refined products—precooked white rice, all-purpose flour, white breads, refined wheat cereal, certain ready-to-eat cereals, and macaroni products. In contrast, wheat bran (8.20 $\mu$g/g) and wheat germ (13.10 $\mu$g/g) contained the most vitamin $B_6$ of all of the foods analyzed. Refining and processing thus appear to cause considerable loss in the vitamin $B_6$ content of foods as available to the consumer. Precooked baby cereals in general are fairly good sources of vitamin $B_6$ not only because of the vitamin content of the individual grain itself, but also some of the other added products such as dried yeast and malt.

Most whole grains and whole grain products contained 2–4 $\mu$g of vitamin $B_6$/g, while other cereal products, such as all-purpose flour, white bread, precooked rice, noodles, macaroni, and spaghetti, generally contained less than 1 $\mu$g/g.

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# Potentiometric Determination of Vitamin C in Highly Colored Products

By WILLIAM S. HARDESTY (Food and Drug Administration, 1141 Central Parkway, Cincinnati, Ohio 45202)

**The method of Barakat and co-workers for determination of vitamin C in colored products has been modified for potentiometric use. The procedure is simple and rapid: An aliquot of sample containing 1–3 mg of ascorbic acid is titrated in a 3% acetic acid medium in the presence of KI with previously standardized 0.01% N-bromosuccinimide. The potentiometric titration uses two Pt electrodes with a polarizing current. Results were compared with the USP XVI method and the AOAC 2,6-dichlorophenol indophenol method. Recoveries, obtained from seven commercial products, four of which were vitamin C-enriched, ranged from 96 to 101%. The method also works well on drug mixtures containing ascorbic acid. It is recommended that the method be studied collaboratively.**

It is often necessary to determine vitamin C (ascorbic acid) in products not adaptable to the USP and AOAC methods. These products may contain extensive coloration or chemically interfering ions. Excessive coloration or pigmentation excludes any method relying on an internal indicator.

Barakat, El-Wahab, and El-Sadr (*Anal. Chem.*, 27, 536 (1955)) have described an N-bromosuccinimide titration, applicable to a variety of substances, in which the reaction of liberated iodine with an internal starch indicator produces a visual end point. This starch-iodide end point, however, is masked by coloring matter in many products. The procedure of Barakat, *et al.* was modified by the Associate Referee for potentiometric use.

Recovery experiments were made on seven commercial products, four of which were enriched with vitamin C. To obtain recovery data, the product was first assayed for ascorbic acid content; then a known amount of the vitamin was added to an aliquot and this aliquot was again assayed. The difference in content before and after addition of the vitamin was considered to be the ascorbic acid recovered. Recovery values ranged from 96 to 101% (Table 1).

## METHOD

### *Special Apparatus*

(a) *Titrating apparatus.*—Fisher Titrimeter, Model 35, or equivalent, equipped with a constant stirring assembly.

(b) *Two platinum wire electrodes.*

### *Reagents*

(a) *Acetic acid.*—3% (v/v). Dilute 15 ml glacial acetic acid to 500 ml with $H_2O$.

(b) *Potassium iodide solution.*—4% (w/v).

**Table 1. Recovery experiments of ascorbic acid from commercial juices and drinks[a]**

| Product | mg Ascorbic Acid per 5 ml Prepared Sample | | Ascorbic Acid Recovered | |
|---|---|---|---|---|
| | Present | Added | mg | % |
| Orange juice | 1.68 | 0.86 | 0.86 | 100 |
| Enriched cranberry juice | 1.46 | 1.52 | 1.48 | 97 |
| Enriched vegetable juice | 0.55 | 2.96 | 2.99 | 101 |
| Enriched grape juice | 1.23 | 2.00 | 1.98 | 99 |
| Concentrated grape juice | 0.08 | 2.24 | 2.25 | 100 |
| Tomato juice | 1.37 | 2.00 | 1.92 | 96 |
| Enriched vegetable juice | 0.76 | 2.33 | 2.30 | 99 |

[a] Ascorbic acid content of various commercial products was determined before and after the addition of known amounts of the vitamin. Submitted data represent averages of duplicate and triplicate determinations.

Dissolve 4 g reagent grade KI in $H_2O$ and dilute to 100 ml.

(c) *N-bromosuccinimide reagent.* — 0.01% (w/v). Add 200 mg N-bromosuccinimide and about 100 ml $H_2O$ to a 200 ml volumetric flask. Warm and shake until dissolved. Cool and dilute to 200 ml. This stock solution (0.1%) is stable for a few days at 4°C. Dilute stock solution (1+9) just before use. 1 ml of this 0.01% reagent is approximately equivalent to 0.1 mg ascorbic acid.

(d) *Standard ascorbic acid solution.*—About 1 mg/ml. Weigh accurately about 50 mg Reference Standard Ascorbic Acid. Transfer to a 50 ml volumetric flask and dilute to mark with 3% acetic acid. Standardize 0.01% N-bromosuccinimide at once as described below.

### *N-Bromosuccinimide Standardization*

Transfer 2.0 ml freshly prepared ascorbic acid standard (about 2 mg) to a 100 ml beaker. Add 5 ml 4% KI solution and dilute with 3% acetic acid to about 45 ml (or enough to cover electrodes). Titrate to point of maximum inflection, using 2 platinum electrodes with polarizing current.

Stir at maximum rate without creating a strong vortex or splashing. After each addition of the titrant, re-adjust the voltage. Determine the volume of titrant that was used to reach equivalence point. Using this value, express concentration of standard reagent in terms of its ascorbic acid equivalent. (Example: 1 ml 0.01% N-bromosuccinimide = 0.0979 mg ascorbic acid.)

### *Determination*

Assay sample immediately after opening container. Filter through cotton if necessary. Transfer an aliquot equivalent to 1–3 mg ascorbic acid to a 100 ml beaker (do not take aliquot greater than 25 ml). Add 5 ml 4% KI solution and dilute with 3% acetic acid to about 45 ml (or enough to cover electrodes). Titrate with standard 0.01% N-bromosuccinimide as in *N-Bromosuccinimide Standardization.* ml titrant × equivalent = mg ascorbic acid in aliquot.

### Discussion and Recommendation

N-bromosuccinimide is a highly selective oxidizing agent. In the titration process of Barakat, *et al.,* free iodine is liberated by a second selective oxidation of KI after ascorbic acid has been totally oxidized to the dehydroascorbic acid stage.

Barakat, El-Wahab, and El-Sadr report that a number of substances that might be expected to interfere, including reductones, reductic acid, and iron salts, have no influence on the titration process.

The method described has been used for drug mixtures with good results. Sample and standard were prepared by the method in USP XVI, p. 67. Assays on three such mixtures agreed with the 2,6-dichlorophenol indophenol method within 2%. The method has also been used for routine analysis of drinks enriched with vitamin C, with apparently good results.

By changing the concentration of the N-bromosuccinimide reagent to 0.001%, amounts as low as 100 μg of ascorbic acid have been determined. This range has been tried only with the pure substance and not with sample material.

It is recommended that the method be studied collaboratively.

### Acknowledgments

The author is indebted to George Schwartzman and George Keppel, Cincinnati District, for their helpful suggestions.

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. See *This Journal,* 47, 121 (1964).

# Assay of Oil-Soluble Vitamins. Part I. Vitamin A and Carotene: Suggestions for Improving Assay

By M. J. DEUTSCH, S. W. JONES, J. B. WILKIE, D. DUFFY,[1] and H. W. LOY (Division of Nutrition, Food and Drug Administration, Washington, D.C. 20204)

**The adaptation of official methods to the assay of vitamin A in many commercial products has required modifications in the apparatus used and improvements in assay techniques. These modifications increased the number of routine samples that are screened per day from three to eight, with a corresponding increase in per cent recovery and degree of reproducibility. The most important modifications were installation of equipment to permit handling of more samples at a time, changes in volumes and concentrations of solutions to improve separations, and use of an internal standard in the antimony trichloride method for the assay of vitamin A.**

Official methods are not suitable for assay of certain vitamin products that are tested by this laboratory, and have had to be modified.

One analyst using regular laboratory techniques as described in *Official Methods of Analysis* (1) or the USP (2) usually completes only two or three vitamin A assays in an eight-hour workday. A number of improvements and modifications have been developed in this laboratory which enable an analyst to complete as many as eight samples a day. All saponifications, extractions, and column work are performed in the hood.

The concentration of KOH used for saponification is 100% stronger than that used in the AOAC vitamin A method. This stronger solution has greater utility in assaying a wide diversity of samples that may contain appreciable amounts of interfering substances.

For routine screening assays of pharmaceutical preparations the absorbance of the vitamin is determined on the nonchromatographed solution. Chromatography is suggested when distortion of the vitamin A curve is indicated, carotene is present, or samples may contain extraneous interfering material. Likewise, the antimony trichloride colorimetric procedure with either the chromatographed or nonchromatographed assay solution is presented as an alternative where greater specificity is desired.

The methods are set forth so that the analyst can apply a variety of approaches to a single sample extract. Often, however, this entails a transfer of the vitamin from one solvent to another and subsequent adjustment of the concentration of the vitamin to conform with the limits of the procedure. In all assays where solvents must be transferred, the solvent containing the vitamin must be cautiously and expeditiously evaporated under a stream of nitrogen. This may be conveniently done in a small beaker held in the palm of the hand or by placing a centrifuge tube in a clamp on the steam bath post high above the top of the bath, followed by dilution in the proper solvent.

It is suggested throughout the presented methods that absorbance readings be made at 300, 310, 325, 334, and 355 mμ. Although not all of these readings have applicability in all calculations, they either serve as an index of curve distortion or are applied in the Morton-Stubbs calculation of the USP.

A standard graph of units of vitamin A standard or μg carotene standard vs. absorbance may be plotted and the concentration of the vitamin in the sample interpolated from the curve. In preparing the standard graph, the standard vitamin A or carotene solutions must be carried through the same assay procedure that is applied to the sample.

The nature of the product being assayed may warrant the addition of standard vitamin A or standard carotene to the sample and subsequent assay to obtain per cent recovery or verification of assay results.

[1] Now with the Division of Food Standards and Additives.

***Apparatus***

Use apparatus stated in **39.002(a)**, **(b)(1)**, and **(d)**, and the following:

**(a)** *Photoelectric colorimeter.* — Beckman Model B, Bausch & Lomb Spectronic 20, or equivalent instrument, with direct reading deflection-type galvanometer.

**(b)** *Colorimetric tubes.* — 20 mm standardized test tubes.

**(c)** *Chromatographic tubes.*—*(1)* Tube about 30 cm long, 2.3 cm i.d., with fritted glass disk and stopcock at bottom. *(2)* Tube 30 cm long and 0.6 cm i.d., with lower 6 cm pulled out to form constricted exit 0.2 cm i.d. Plug about 0.5 cm of upper part of constricted section with glass wool. Fuse flared tube 10 cm long and 2.4 cm i.d. to top of 0.6 cm i.d. section. Mark tubes at specified reagent segment levels.

**(d)** *Precision portable steam bath.* — Four-hole, copper, steam. Modified by adding four slip-fit removable side-arm clamp posts (Fig. 1).

**(e)** *Hood rack.* — Custom-made for holding separatory funnels for conducting extractions (Fig. 2).

**(f)** *Vacuum gauge.*—Vacuum gauge **39.002 (c)**, or a manifold (Fig. 3) composed of a closed 1.5 by 7" brass pipe provided with several jet cocks at convenient angles and with needle valve for bleeder action.

**(g)** *Condenser.*—Cold finger, Pyrex, 91300, size 4, or equivalent.

***Reagents*** [2]

Use reagents stated in **39.003(b)**, **(c)**, **(d)**, **(f)**, **(k)**; **39.009 (b)**, **(c)**, **(j)**; and the following:

**(a)** *Potassium hydroxide solution.*—Dissolve about 454 g KOH in 223 ml $H_2O$.

**(b)** *Isopropanol.*—Spectral grade.

**(c)** *Celite 545.*—Johns-Manville.

**(d)** *Aluminum oxide.*—Reagent grade suitable for chromatographic adsorption (80–200 mesh). Heat for 3 hours at 600° and let come to room temperature in tightly closed screw-cap bottle.

**(e)** *Magnesium oxide-Celite mixture.*—Heat Sea Sorb 43 (MgO) for 4 hours at 600°. Cool, and mix with equal weight of Celite 545 in tightly closed jar.

**(f)** *1,2-Dichloroethane.*—Spectral grade.

**(g)** *Antimony trichloride (Carr-Price) reagent.*—Dissolve ¼ lb of $SbCl_3$ crystals in 1,2-dichloroethane and dilute to 500 ml with 1,2-dichloroethane. Store in tightly stoppered brown bottle.

[2] The following reagents have been found satisfactory; other commercial sources may be equally satisfactory.

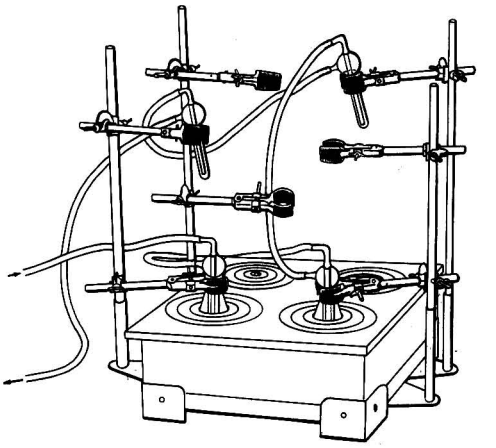

*Fig. 1*—Precision portable steam bath.

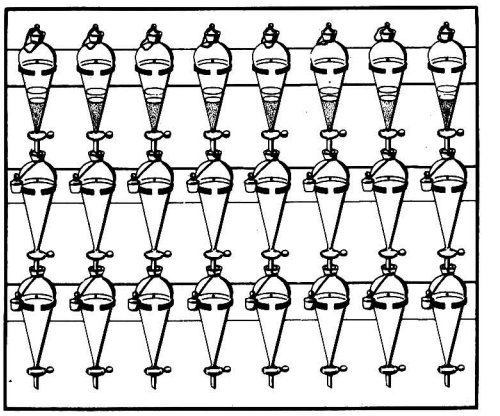

*Fig. 2*—Hood rack.

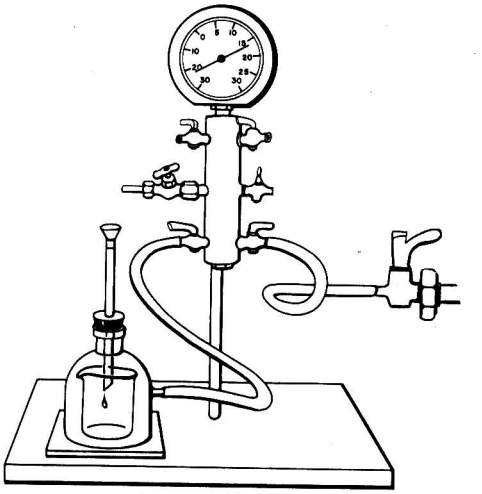

*Fig. 3*—Manifold arrangement.

(h) *Stock standard vitamin A solution.*—Use 1 capsule of USP Vitamin A Reference Standard (25,000 units/capsule, equivalent to 100,000 units/g) and proceed as in Method III, *Preparation of assay solution,* through "Immediately dissolve residue in small volume of petroleum ether." Transfer to 250 ml volumetric flask and dilute to volume with petroleum ether. 1 ml = 100 units vitamin A. Store in refrigerator.

(i) *Procedural standard vitamin A solution.*—Transfer 1 ml *Stock standard vitamin A solution* to small beaker and evaporate to dryness under nitrogen. Immediately add a small volume of isopropanol. Transfer to 10 ml volumetric flask and dilute to volume with isopropanol. Determine absorbance at 300 and 325 mμ, using spectrophotometer. $A_{300}/A_{325}$ should be about 0.62. Calculate concentration of vitamin A by formula: $C_s = A_{325} \times 18.30$ where $C_s$ is units vitamin A/ml. (This establishes potency of *Standard vitamin A solution* to be used in formula in Method IV, *Colorimetric procedure.*)

(j) *Standard vitamin A solution.*—Transfer 1 ml *Stock standard vitamin A solution* to small beaker and evaporate to dryness under nitrogen. Immediately add a small volume of 1,2-dichloroethane. Transfer to 10 ml volumetric flask and dilute to volume with 1,2-dichloroethane.

(k) *Iso-octane.*—2,2,4-trimethylpentane, 99.5%.

(l) *Polyethylene glycol 600.*—Commercial grade, Carbide and Carbon Chemical Co.

(m) *Nitrogen.*—Compressed-oil pumped grade.

(n) *Acetic anhydride.*—ACS.

## METHODS

### *I. Screening Method for Pharmaceutical Preparations*

*Preparation of assay solution.*—Place sample (5–10 tablets) in 300 ml Erlenmeyer flask and heat for several minutes with enough water to cover sample. If tablets do not disintegrate readily, crush remaining solids with a glass rod. Add 50 ml 95% ethanol and 10 ml KOH solution, reflux for 20 minutes on steam bath, rapidly cool flask to room temperature, using an ice-water bath, and transfer solution to 500 ml separatory funnel with 50 ml $H_2O$. Add 45 ml ethyl ether and shake several times. Let separate and draw off aqueous layer. Extract aqueous phase again with 45 ml ethyl ether. Repeat procedure twice, for a total of four extractions.

To the combined ether extracts in a separatory funnel, add 150 ml $H_2O$, draw off bottom layer, and discard. Add 10 ml $H_2O$, shake, let separate, and draw off bottom layer. Repeat with one 10 ml portion $H_2O$ and then with two 50 ml portions $H_2O$.

Finally add 100 ml $H_2O$, let separate, and draw off aqueous layer. Transfer ether solution to 300–400 ml tall-form beaker containing 1–2 g anhydrous $Na_2SO_4$, and stir. Transfer solution to another beaker and wash residual $Na_2SO_4$ with several portions of ether. Combine ether extract and washings. If necessary to concentrate solution to desired volume (usually 100 or 200 ml), evaporate on steam bath, cool rapidly, transfer to volumetric flask, and dilute to volume with ethyl ether. Transfer an aliquot containing 100–200 units vitamin A to small beaker. Evaporate to dryness under nitrogen. Immediately dissolve residue in 3–4 ml isopropanol. Transfer to 10 ml volumetric flask and dilute to volume with isopropanol. Make further dilutions, if necessary, so that 1 ml of final dilution contains 10–20 units vitamin A.

*Spectrophotometry.*—Determine absorbance of vitamin A isopropanol solution in 1 cm cell at 300, 310, 325, 334, and 355 mμ on spectrophotometer.[3] $A_{300}/A_{325}$ should not exceed 0.73 (3). If the ratio is 0.73 or higher, proceed as in Method III.

*Calculation.*—If $A_{300}/A_{325}$ is satisfactory, calculate vitamin A by the formula:

Units vitamin A per g, tablet, or capsule = $A_{325} \times 18.30 \times$ dilution factor/g, no. of tablets, or no. of capsules.[4]

### *II. Method for Pharmaceutical Preparations Found Deficient in Vitamin A*

Proceed as in Method I, except decrease reflux time in saponification step to 5 minutes. If $A_{300}/A_{325}$ is below 0.73, calculate as in

[3] The vitamin $A_1$ alcohol or ester generally derived from salt water fish has a maximum absorbance at 325 mμ in isopropanol. The vitamin $A_2$ alcohol and ester derived from fresh water fish have maxima at higher wavelengths. Also, anhydrovitamin A or other derivatives of the vitamin that do not have biological activity will peak at the higher wavelengths. It is therefore suggested that absorbance at 355 mμ be routinely determined to complete the vitamin A curve. If the curve is greatly distorted, the necessity for chromatography, Method III, is indicated.

$A_{310}$ and $A_{334}$ are utilized in the USP calculation set forth in Method VII.

[4] It is the present trend to express final results in terms of μg or mg per sample unit. (Units of vitamin A × 0.3 = μg vitamin A or 1 mg vitamin A × 3333 = units of vitamin A.)

Method I. If $A_{300}/A_{325}$ exceeds 0.73, proceed as in Method III.

Samples remaining deficient should be assayed by the colorimetric procedure. Evaporate aliquot of solution obtained in Method I, *Preparation of assay solution,* at "transfer to volumetric flask and dilute to volume with ethyl ether," in small beaker and take to dryness under nitrogen. Immediately dissolve residue in small volume of 1,2-dichloroethane. Transfer to volumetric flask and dilute to volume with 1,2-dichloroethane so that 1 ml of final dilution contains 8–15 units vitamin A. Proceed as in Method IV, beginning with *Colorimetric procedure.*

### *III. Method for Pharmaceutical Preparations for Which* $A_{300}/A_{325}$ *Exceeds 0.73*

*Preparation of assay solution.*—Proceed as in Method I, *Preparation of assay solution,* through "Combine ether extract and washings." Partially reduce volume by evaporation on steam bath. Continue evaporation to dryness under nitrogen. Immediately dissolve residue in small volume of petroleum ether. Transfer to volumetric flask and dilute to volume with petroleum ether so that 1 ml of final dilution contains 200–400 units vitamin A.

*Preparation of column packing.*—Place 20 g Celite 545 in wide-mouth, about 200 ml, screw-cap bottle. Add 100 ml iso-octane and shake until thin slurry is formed. Add 10 ml polyethylene glycol dropwise with vigorous shaking.

*Preparation of column.*—Place about half of column packing in chromatographic tube, (c)(*1*), without using vacuum. Then apply 2" vacuum and add remainder of slurry in small increments, packing with disk plunger. (Height of column should be about 7 cm). As soon as solid surface is formed, remove vacuum and add 2 ml iso-octane.

*Chromatography.*—Before iso-octane disappears into surface of column, transfer onto column 5 ml sample extract containing 1000–2000 units vitamin A. As soon as meniscus of sample extract begins to disappear into surface, add three 2 ml portions of petroleum ether. Let each portion disappear into surface of column before next addition. Add 100 ml petroleum ether in 5–10 ml increments. Discard first 20 ml of eluate from column and collect subsequent eluate. Elution rate should be between 3 and 6 ml per minute (1–2 drops per second). It should not be necessary to use either vacuum or pressure to obtain this elution rate. However, if column needs more speed, apply about 2 lb/sq. in. pressure to top of tube through a rubber stopper.

Follow movement of vitamin A down column by periodic observation with UV light during elution. Stop elution when lowest tip of vitamin A blue fluorescent band is about 0.5 cm above fritted disk. Use eluate just preceding vitamin A (if colored) to estimate carotene. Evaporate carotene eluate to dryness under nitrogen. Immediately dissolve residue in isopropanol. Transfer to volumetric flask and dilute to volume with isopropanol, so that 1 ml final dilution contains 1.5–3.0 μg carotene.

*Spectrophotometry and calculation of carotene.*—Determine absorbance on spectrophotometer at 450 mμ.[5] $A_{450} \times 6.95$ = units carotene/ml of final dilution. Alternatively, read at 436 mμ and calculate concentration of carotene by formula in 39.017. mg carotene × 1667 = units vitamin A activity.

*Determination of vitamin A.*—Continue eluting vitamin A fraction with petroleum ether until all vitamin A is in eluate, as shown by UV fluorescence. Add 10 ml iso-octane to column to elute any residual vitamin A. Evaporate vitamin A eluate to dryness under nitrogen. Immediately dissolve residue in isopropanol. Transfer to volumetric flask and dilute to volume with isopropanol so that 1 ml final dilution contains 10–20 units of vitamin A. Determine absorbance in 1 cm cell at 300, 310, 325, 334, and 355 mμ. If $A_{300}/A_{325}$ is below 0.73, proceed as in Method I, *Calculation.* The vitamin A activity of carotene present in sample may be added to vitamin A content to obtain total vitamin A activity of sample.

### *IV. Method for Feed Samples*

*Preparation of assay solution.*[6]—Weigh an amount (preferably not > 20 g) of sample containing about 1000–2000 units of vitamin A. Proceed as in Method III, *Preparation of assay solution,* through "Immediately dissolve residue in small volume of petroleum ether," except in the saponification step use volume of alcohol equal in ml to 5 times dry weight of sample in g, and volume of KOH equal in ml to dry weight of sample in g. Transfer dissolved residue to volumetric flask and dilute to volume with petroleum ether so that 1 ml of final dilution contains 30–100 units vitamin A.

[5] Calculation based on $A_{450}$ usually gives answers 3–4% higher than that based on $A_{436}$.

[6] If sample slurry stops the passage of the aqueous solution through the stopcock bore of the separatory funnel, insert a copper wire through the top opening of the funnel. With the stopcock in open position, manipulate the wire so that free flow of the aqueous layer is obtained.

*Preparation of column.*—Use column described in **39.002(b)(1)**. Prepare column packing of 3% $H_2O$ mixture of preheated $Al_2O_3$. Mix thoroughly. Prepare column as in **39.010**, except fill column by gravity, without vacuum, and then pass petroleum ether through column, using vacuum.

*Chromatography.*—Pass aliquot of sample solution, containing 150–200 units vitamin A, through column. (Caution: Do not let liquid level fall below top of alumina.)

Collect carotene fraction by eluting with 2 ml 16% ethyl ether in petroleum ether followed by 5 ml petroleum ether. Repeat addition of 2 ml 16% ethyl ether in petroleum ether and 5 ml petroleum ether. Collect fraction and evaporate to dryness under nitrogen. Immediately dissolve residue in isopropanol. Transfer to volumetric flask and dilute to volume with isopropanol so that 1 ml of final dilution contains 1.5–3.0 μg carotene. Proceed as in Method III, *Spectrophotometry and calculation of carotene.*

With different receiver under column, elute vitamin A by passing 25% ethyl ether in petroleum ether through column in 5 ml increments. Continue elution until all the vitamin A has been eluted from column as indicated by inspection with UV light. Evaporate vitamin A eluate to dryness under nitrogen. Immediately dissolve residue in small volume of 1,2-dichloroethane. Transfer to volumetric flask and dilute to volume with 1,2-dichloroethane so that 1 ml of final dilution contains 8–15 units vitamin A. Designate as sample solution.

*Colorimetric procedure.*—Prepare three colorimetric tubes, designated A, B, and C as follows: Transfer 2 ml 1,2-dichloroethane to tube A, 1 ml of 1,2-dichloroethane and 1 ml sample solution to tube B, and 1 ml of *Standard vitamin A solution* and 1 ml sample solution to tube C. To each tube add 1 drop of acetic anhydride. (The following steps are performed at colorimeter to avoid delay.) To tube A, rapidly add 5 ml antimony trichloride (Carr-Price) reagent by inverting graduated cylinder containing reagent directly into colorimeter tube.[7] Immediately place assembly into colorimeter set at 620 mμ. Adjust to zero absorbance.

To tube B, rapidly add 5 ml antimony trichloride (Carr-Price) reagent and determine maximum absorbance. (Color fades in 3–5 seconds.) Repeat this procedure on tube C.

Calculate vitamin A content by formula, $C = C_s/(A_C - A_B) \times A_B$, where $C_s$ is concentration of *Standard vitamin A solution* (units/ml); $A_B$ and $A_C$ are absorbances of tubes B and C, respectively; and $C$ is concentration of vitamin A (units/ml). Multiply by dilution factor to obtain vitamin A content of original sample.

### *V. Method for Liquid Milk Samples*

Add 150 ml 95% ethanol and 50 ml KOH solution to 100–200 ml sample. Reflux on *top* of steam bath for 30 minutes. (Sample should turn dark brown.) Cool flask to room temperature and transfer saponified sample to separatory funnel with 50 ml $H_2O$. Extract with one 150 ml and one 100 ml portion of ethyl ether. Combine ether extracts. Discard aqueous phase.

Add 20 ml ethanol to combined ether extracts and heat on steam bath until ether is evaporated. Add 70 ml additional ethanol and 40 ml KOH solution. Reflux on *top* of steam bath for 10 minutes. Proceed as described in Method I, *Preparation of assay solution,* beginning, ". . . rapidly cool flask to room temperature, using ice water bath," through, ". . . transfer to volumetric flask, and dilute to volume with ethyl ether." Transfer an aliquot containing 150–200 units vitamin A to small beaker and take to dryness under nitrogen. Immediately dissolve residue in 1–2 ml petroleum ether. Using this as sample aliquot, proceed as in Method IV, beginning *Preparation of column.*

Alternatively, transfer an aliquot of solution obtained in Method I, *Preparation of assay solution* at "transfer to volumetric flask, and dilute to volume with ethyl ether," to small beaker and take to dryness under nitrogen. Immediately dissolve residue in small volume of 1,2-dichloroethane. Transfer to volumetric flask and dilute to volume with 1,2-dichloroethane so that 1 ml of final dilution contains 8–15 units vitamin A. Proceed as in Method IV, beginning at *Colorimetric procedure.*

### *VI. Method for Solid Milk Products*

To 20 g sample add 100 ml 95% ethanol and 40 ml KOH solution. Proceed as in Method V, beginning "Reflux on *top* of steam bath for 30 minutes."

### *VII. Method for USP or NF (4) Products—Modified*[8]

Proceed as in Method I, through "Units of vitamin A per g, tablet, or capsule = $A_{325} \times$

[7] The 5 ml antimony trichloride is best added from a 10 ml graduate cut off at the 6 ml mark.

[8] Different amounts of materials are involved in handling samples in this modification.

18.30 × dilution factor/g, no. of tablets, or no. of capsules."

$A_{325}$ must have a value not $<$ $[A_{325}]/1.030$ and not $>$ $[A_{325}]/0.970$, where $[A_{325}]$ is the corrected absorbance at 325 mμ and is given by the equation:

$$[A_{325}] = 6.815\ A_{325} - 2.555\ A_{310} - 4.260\ A_{334}$$

in which $A$ designates the observed absorbance at the wavelength indicated by the subscript.

If $[A_{325}]$ has a value $< A_{325}/1.030$, use $[A_{325}]$ in place of observed absorbance in appropriate equation to calculate vitamin A content.

### *VIII. Method for Cod Liver Oil Samples*

To 1 g sample, accurately weighed, add 50 ml 95% ethanol and 10 ml KOH solution. Reflux for 30 minutes on *top* of steam bath. Proceed as in Method I, *Preparation of assay solution,* beginning, "rapidly cool flask to room temperature."

### *IX. Method for Oleomargarine Samples*[9]

*Preparation of alumina for columns.*—Prepare standard $Al_2O_3$ as under Reagent (d). To prepare alkaline $Al_2O_3$ proceed as in **39.003(i)**.

*Determination of adsorption index of alumina.*—Proceed as in **39.004**. The adsorption index of the standardized $Al_2O_3$ should be 30–40 and that of alkaline $Al_2O_3$, 7–12.

*Preparation of assay solution.*—Proceed as in **39.007(b)** through **39.007(d)**, except as follows: in (b), heat sample on a steam bath rather than on an electric hot plate; in (c) delete "Then add ca 10 ml 0.02*N* KOH, shake vigorously, and discard after sepn."; and after "Combine rinses with ext." add: To combined ether extracts add 100 ml $H_2O$. *Do not shake.* Draw off bottom layer and discard. Repeat once.

*Alumina chromatography.*—Prepare chromatographic column, (c)(*2*), by packing each of the following adsorbants with gravity and slight tapping of tube. Add standardized $Al_2O_3$ to height of 4 cm, then an 8 cm segment of alkaline $Al_2O_3$, and another segment of standardized $Al_2O_3$ 10 cm high. Add 1 cm layer of $Na_2SO_4$ to top of column.

Proceed as in **39.007(e)**, beginning "Apply 5″ of vac.", through "when carotene color cannot be seen in last 1 ml fraction observed." Save eluate for determination of carotene.

Discard eluate that collects after carotene and before vitamin A. Change beakers and continue elution with 25% petroleum ether. Follow movement of vitamin A down column with UV light. Elution is complete when no fluorescence can be detected on column.[10] Concentrate vitamin A eluate on steam bath to about 2 ml. Evaporate to dryness under nitrogen. Immediately dissolve residue in 3–4 ml isopropanol, transfer to 10 ml volumetric flask, and dilute to volume with isopropanol. This is vitamin A assay solution. Concentrate carotene solution on steam bath to about 2 ml. Evaporate to dryness under nitrogen. Immediately dissolve residue in about 5 ml petroleum ether, transfer to 10 ml volumetric flask, and dilute to volume with petroleum ether.

*Spectrophotometric measurements and calculations.*—Proceed as in **39.007(g)**(*1*) and (*2*), using petroleum ether as reference solution for carotene absorbance and isopropanol as reference solution for vitamin A absorbance.

$A_{310}/A_{325}$ of the vitamin A solution is usually 1 or less. If ratio is greater than 1, read absorbance at 332.5 and 355 mμ. $(A_{332.5} - A_{355}) \times 1.812$ should give value in close agreement with $A_{325}$. Calculate potency, substituting this value for $A_{325}$ in equation.

#### Notes

With the modified steam bath (Fig. 1), four samples can be saponified simultaneously. The second set of four samples is saponified while the first set is being rapidly cooled to room temperature in an ice water bath. The clamp modification enables the analyst to easily carry out the saponification in an Erlenmeyer flask with a reflux cold finger condenser held in the neck of the flask by another clamp. This arrangement of clamps also permits use of 50 ml Pyrex centrifuge tubes (pour-out, heavy-duty, plain) for the evaporation of solvents prior to transferring the vitamin to a different solvent.

Installation of a hood rack (Fig. 2) makes it feasible to use twenty-four 500 ml or 1000 ml separatory funnels during the extraction step. The rack holds three horizontal rows of eight Tygon-covered aluminum rings so located that each separatory funnel can drain into the one below it. This results in easier handling of assay solutions; thus fewer mishaps occur in which samples may be lost and hazards arise.

[9] Most oleomargarines contain approximately 15,000 units of vitamin A activity/lb. This is present as about ⅔ vitamin A and ⅓ vitamin A as carotene.

[10] In rare cases, the vitamin A fraction may be colored. If so, rechromatograph on magnesium oxide column as in **39.007 (f)**.

The manifold arrangement (Fig. 3) is also equipped with a combination vacuum-pressure gauge to read vacuum from 30" of mercury to 30 lb per sq. in. pressure. The needle valve permits Vernier action in both vacuum and pressure ranges which serves to expedite elution. This arrangement may be used to control many types of chromatographic columns.

### Discussion

The most important modifications presented in this paper are briefly summarized as follows:

1. Improvements in facilities used in the handling of assay solutions.
2. Changes in volumes and concentration of solutions to promote better separation, better handling of samples, and reduction in assay time.
3. Use of an internal standard colorimetric method for certain samples.

Use of these modifications is recommended for routine analyses. For deficient regulatory samples or samples of questionable composition the official methods of the AOAC, USP, or NF should be followed exactly, regardless of whether the assay results are lower than those obtained by these nonofficial modifications.

### References

(1) *Official Methods of Analysis*, 9th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1960, sec. **39.001** *et seq.*
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# Assay of Oil-Soluble Vitamins. Part II. Vitamin A and Vitamin D Assays from a Common Saponification and Extraction

By M. J. DEUTSCH, D. DUFFY,[1] and C. E. WEEKS (Division of Nutrition, Food and Drug Administration, Washington, D.C. 20204)

**Combining the assays of vitamin A and vitamin D facilitates the completion of analyses of products containing both vitamins. Methods are given for typical pharmaceuticals, feeds, and oils. Samples not containing vitamin A or similar compounds may be assayed for vitamin D without chromatography.**

Many samples of pharmaceutical products, feeds, and oils are submitted for both vitamin A and vitamin D analyses. Since the saponification and extraction procedures for the AOAC (1) assay for vitamin A and the USP (2) assay for vitamin D are similar, it is suggested that these steps be combined for screening assays of both vitamins. This technique permits aliquots for the two vitamin assays to be taken from the same extraction solution of the saponified sample.

Typical pharmaceutical products may contain about 5000 USP units of vitamin A and 500 USP units of vitamin D per tablet or capsule, or other similar ratios facilitating the application of this method. This is also frequently the case for feed samples and oil-type products.

Samples that do not contain vitamin A or carotene may be assayed for vitamin D directly from the stock extraction solution without chromatography.

Use apparatus and reagents listed in Part I, *This Journal*, p. 756, and the following:

[1] Now with the Division of Food Standards and Additives.

***Reagents***

(o) *Stock standard vitamin D solution.*—Accurately weigh 25 mg USP Vitamin $D_2$ or Vitamin $D_3$ Reference Standard. Dissolve in petroleum ether and dilute to 200 ml with petroleum ether to give a known concentration of 125 μg (5,000 units)/ml. Store in refrigerator.

(p) *Procedural standard vitamin D solution.*—Transfer 1 ml *Stock standard vitamin D solution* to small beaker and evaporate to dryness under nitrogen. Immediately dissolve residue in small volume of 1,2-dichloroethane. Transfer to 25 ml volumetric flask and dilute to volume with 1,2-dichloroethane. Determine absorbance at 270 mμ, using spectrophotometer. Calculate concentration of vitamin D by formula: $C_s = A_{270} \times 860$ where $C_s$ is units of vitamin D/ml. (This establishes potency of *Procedural standard vitamin D solution* to be used in Method X, *Colorimetric procedure.*) Prepare daily.

(q) *Fuller's earth (Florex XXS).*—Moderately coarse, chromatographic grade.

(r) *Antimony trichloride reagent for vitamin D determination.—Solution A:* Dissolve ¼ pound of $SbCl_3$ crystals in dichloroethane and dilute to 500 ml with 1,2-dichloroethane. To remove moisture, add about 2 g anhydrous alumina. Mix thoroughly. Filter through paper into clear glass reagent bottle. *Solution B:* Working in hood, add 100 ml acetyl chloride to 400 ml 1,2-dichloroethane, with mixing. Store in glass-stoppered bottle. *Color Reagent:* At least 30 minutes before use, mix 45 ml solution A and 5 ml solution B. Keep in tightly stoppered glass flask. It is preferable to prepare fresh color reagent daily.

## METHODS

### X. Method for Pharmaceutical Preparations

*Preparation of stock vitamin A and vitamin D assay solution.*—Proceed as directed in Method I, *Preparation of assay solution,* Part I, *This Journal,* p. 756, through "Combine ether extract and washings."

Concentrate ether solution by evaporation on steam bath and take to dryness under nitrogen. Immediately dissolve residue in a small volume of petroleum ether and transfer to 25 ml volumetric flask. Dilute to volume with petroleum ether.

This is stock extraction solution for both vitamin A and vitamin D.

*Assay for Vitamin A*

Proceed as in Method I, *Preparation of assay solution,* Part I, *This Journal,* p. 756, beginning, "Transfer an aliquot containing 100–200 units vitamin A," through "Units of vitamin A per g, tablet, or capsule = $A_{325} \times 18.30 \times$ dilution factor/g, no. of tablets, or no. of capsules."

*Assay for Vitamin D*

*Preparation of the first column.*—Proceed as in Method III, *Preparation of column packing,* Part I, *This Journal,* p. 756.

*Chromatography on first column.*—Before iso-octane disappears into surface of column, transfer onto column an aliquot of stock extraction solution containing 1000–2000 units vitamin D.[2]

Proceed as in Method III, *Chromatography,* Part I, *This Journal,* p. 756, beginning, "As soon as the meniscus of the sample extract" through "Stop elution when lowest tip of vitamin A blue fluorescent band is about 0.5 cm above fritted disk." Transfer eluate to beaker and partially reduce volume by evaporation on steam bath. Continue evaporation to dryness under nitrogen. Immediately dissolve residue in 25 ml petroleum ether.

*Preparation on second column.*—Pack midsection of chromatographic tube (c)(2) with 3 g Fuller's earth (Florex XXS) with aid of gentle suction.

*Chromatography on second column.*—Transfer petroleum ether solution of first column eluate onto second column. Rinse evaporation vessel with 10 ml petroleum ether in small increments, transfer each rinse to second column, and allow it to flow through column. Discard eluate. When about 1 ml petroleum ether remains above surface of column, change receiver, add 75 ml benzene, and elute with aid of gentle suction. Collect eluate. Evaporate eluate to an oily residue under nitrogen. Immediately dissolve residue in a small volume of 1,2-dichloroethane, transfer to 10 ml volumetric flask, and dilute to volume with 1,2-dichloroethane. Make further dilutions, if necessary, so that 1 ml of final dilution contains 100–200 units vitamin D. Designate as vitamin D assay solution.

*Colorimetric procedure.*—To each of three colorimetric tubes, designated 1, 2, and 3, transfer 1 ml vitamin D assay solution. Then to

[2] There should be 10,000 to 20,000 units vitamin A present. If less than 3,000 units of vitamin A are present, an aliquot of *Stock standard vitamin A solution* must be added to trace the vitamin D through column. The upper limit of vitamin A content on column is 35,000 units. Above this limit the vitamin A will influence the assay and two or more absorption steps are necessary.

tube 1 add 1 ml *Procedural standard vitamin D solution;* to tube 2 add 1 ml 1,2-dichloroethane; and to tube 3 add 1 ml of a mixture of equal volumes of acetic anhydride and 1,2-dichloroethane.

To tube 1 rapidly add 5 ml antimony trichloride reagent for vitamin D determination, and mix. Forty-five seconds after addition of antimony trichloride reagent, place tube in colorimeter and determine absorbance at 500 mμ against a zero setting for 1,2-dichloroethane blank. Repeat procedure for tubes 2 and 3. Forty-five seconds after determining absorbances of tubes 2 and 3 at 500 mμ, determine absorbance of these tubes at 550 mμ. Designate absorbances as $A^1_{500}$, $A^2_{500}$, $A^3_{500}$, $A^2_{550}$, and $A^3_{550}$, in which the superscript indicates number of tube and the subscript indicates wavelength.

Calculate vitamin D content as follows: $C = (C_s \times A_u)/A_s$ in which $C_s$ is concentration of *Procedural standard vitamin D solution* (units/ml), $A_u = (A^2_{500} - A^3_{500}) - 0.67\ (A^2_{550} - A^3_{550})$, $A_s = A^1_{500} - A^2_{500}$, and $C$ is the concentration of vitamin D (units/ml). Multiply by dilution factor to obtain vitamin D content of original sample.

### *XI. Method for Feed Samples*

*Preparation of stock vitamin A and vitamin D assay solution.*—Weigh an amount of sample (preferably not > 20 g) containing 10,000–20,000 units of each vitamin. If potency of the feed is low and these concentrations are not possible, 2,500–5,000 units of each vitamin may be sufficient for assay.

Proceed as in Method X, *Preparation of vitamin A and vitamin D assay solution,* through "This is stock extraction solution for both vitamin A and vitamin D," except in the saponification step use volume of alcohol equal in ml to 5 times dry weight of sample in g and volume of KOH equal in ml to dry weight of sample in g.

*Preparation and assay of vitamin A solution.*—Dilute an aliquot of the stock extraction solution for both vitamin A and vitamin D to a measured volume that contains 30–100 units vitamin A/ml. Proceed as in Method IV, beginning at *Preparation of column,* Part I, *This Journal,* p. 756.

*Preparation and assay of vitamin D solution.*—Take an aliquot of stock extraction solution containing 1000–2000 units vitamin D, and proceed as in Method X, beginning at *Assay for vitamin D* through "Evaporate eluate to an oily residue under nitrogen." Immediately dissolve residue obtained from second column eluate in a small amount of petroleum ether.

*Preparation of column.* — Proceed as in Method IV, *Preparation of column,* Part I, *This Journal,* p. 756.

*Chromatography.*—Transfer petroleum ether sample solution onto column. Add solution of 2 ml ethyl ether and 6 ml petroleum ether to elute decomposed vitamin A. Discard eluate. Replace receiver and add 25 ml 5% ethanol in petroleum ether to elute vitamin D. Collect vitamin D eluate and evaporate to dryness under nitrogen. Immediately dissolve residue in 1,2-dichloroethane. Transfer to 10 ml volumetric flask and dilute to volume with 1,2-dichloroethane. Make further dilutions, if necessary, so that 1 ml final dilution contains 100–200 units vitamin D. Proceed as in Method X, beginning at *Colorimetric procedure.*

### *XII. Method for Oil Products*

For oil-type products containing concentrations of vitamin A and vitamin D adequate for chemical assay, proceed as in Method X, except increase reflux time in the saponification step to 30 minutes.

### *XIII. Method for Samples Containing No Vitamin A or Carotene*

Take amount of sample containing 10,000–20,000 units vitamin D and proceed as in Method X, *Preparation of stock vitamin A and vitamin D assay solution,* through "This is stock extraction solution for both vitamin A and vitamin D." Transfer aliquot containing 1000–2000 units vitamin D to small beaker and evaporate to dryness under nitrogen. Immediately dissolve residue in a small volume of 1,2-dichloroethane. Transfer to 10 ml volumetric flask and dilute to volume with 1,2-dichloroethane. Make further dilutions, if necessary, so that 1 ml final dilution contains 100–200 units vitamin D. Proceed as in Method X, beginning at *Colorimetric procedure.*

## Discussion

Vitamin A and vitamin D can be assayed on many products from the stock extraction solution obtained from one saponification, separation, and dehydration. Methods are presented for typical pharmaceuticals, feeds, and oils.

These methods are recommended for the routine screening analysis of samples. For deficient regulatory samples or samples of questionable composition, the official meth-

ods of the AOAC, USP, or NF should be followed exactly.

**Acknowledgment**

The authors of Part I and Part II express their appreciation to Miss Helen Reynolds for her editorial assistance on these papers.

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(1) *Official Methods of Analysis*, 9th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1960, sec. **39.001** *et seq.*

(2) *Pharmacopeia of the United States of America*, 16th Rev., Mack Printing Co., Easton, Pa., 1960, p. 910.

# Collaborative Study of Microbiological Assay for Folic Acid in Food

By LAURA M. FLYNN (Department of Agricultural Chemistry, University of Missouri, Columbia, Mo.)

**The extraction and microbiological determination of folic acid in foods was studied collaboratively. Four samples and four extraction procedures were used. Extracts were assayed for folic acid activity with *L. casei* and *S. faecalis*, and chick assays were also made on the samples. The four extraction procedures were: (A) Incubating 4 hours with chick pancreas at 37°C after autoclaving 15 min. at 15 lb in 0.2*M* pH 7.2 phosphate buffer and cooling; (B) autoclaving 30 min. at 15 lb in potassium ascorbate (pH 6.0, 6 mg/ml); (C) incubating 4 hours with chick pancreas enzyme after autoclaving 15 min. at 15 lb in 0.2*M* pH 8 phosphate buffer, cooling, and adding potassium ascorbate (20 mg/ml, pH 6) in volume equal to the phosphate buffer; and (D) autolyzing in incubator 4 hours at 37°C in mixture of equal volumes of potassium ascorbate (20 mg/ml, pH 6) and 0.2*M* phosphate buffer, pH 8.0. The highest values were obtained with *L. casei* in extracts containing potassium ascorbate. A rough estimation of the types of folic acid derivative in each extract could be made by comparing values obtained in the various extracts with each organism. Collaborators' results by chick assay agreed fairly well with those by microbiological assay, but further work is needed to standardize the extractions and both types of assays.**

Microbiological methods for the assay of folic acid with *S. faecalis* and *L. casei* were studied collaboratively by the AOAC in 1947, 1948, and 1949. Following these studies *S. faecalis* was adopted as the test organism, and the extraction procedure specified incubation with chick pancreas powder, after autoclaving the sample 15 minutes at 15 pounds, in 0.2*M* pH 7.2 phosphate buffer. Research work in various laboratories during the last 14 years has revealed the multiplicity of folic acid-active compounds found in nature and the necessity for use of new extraction procedures in the assay of folic acid activity in foods and feeds. When an extract is assayed with each of three organisms (*Pediococcus cerevisiae, L. casei,* and *S. faecalis*), results often differ widely from that found if only *S. faecalis* is used. Since the response of the test organism to a compound in the folic acid family may differ both quantitatively and qualitatively, microbiological assays of foods or feeds must be paralleled by assays with chicks or small animals.

Materials chosen for assay during the past year were commercial alfalfa meal; navy beans, cooked by autoclaving 40 minutes at 10 lb before drying in a forced air oven at 60°C and grinding; commercial spray-dried egg yolk solids (flash process); and lyophilized raw bovine liver.

Materials sent to the participants included crystalline folic acid for the standard solution, agar stab cultures of *L. casei* and *S. faecalis,* and a small amount of desiccated

chicken pancreas for the enzyme hydrolysis of the samples. The medium specified was that adopted for folic acid with *S. faecalis* (*Official Methods of Analysis*, 7th Ed. (1950)); it was adapted in this laboratory from the Tepley and Elvehjem method and was used in 1948 and 1949 collaborative assays of folic acid with *L. casei* and *S. faecalis*. It is easily prepared from commercially available materials, it is reproducible, and by altering the buffer it can be used successfully with *L. casei*, *S. faecalis*, or *P. cerevisiae*. Responses of each of the three organisms to folic acid can be evaluated turbidimetrically.

The concentration of B-vitamins in the medium was changed in the directions sent to collaborators during the past year. The new directions for vitamin solution III (folic acid-free) specify a concentration of each vitamin twice that listed in the 9th edition of *Official Methods of Analysis*; 5 μg cyanocobalamin (vitamin $B_{12}$) per 100 tubes (500 ml basal medium) was added to the medium, also. The medium for *S. faecalis* is adapted for *L. casei* by omitting the 26 g of Na citrate dihydrate and 3.1 g of $K_2HPO_4$ (per 500 ml basal medium) and substituting 20 g of anhydrous Na acetate, and 0.5 g of $KH_2PO_4$ plus 0.5 g of $K_2HPO_4$. This same medium gives excellent results for *P. cerevisiae* by either turbidimetric or acidimetric measurements.

The extraction procedures studied for the microbiological assay are listed below. All samples were processed in 125 ml Erlenmeyer flasks plugged with absorbent cotton.

(A) Sample was suspended in 20 ml 0.2*M* $PO_4$ buffer, 2-octanol (methyl hexylcarbinol) was added as an antifoam agent, and the mixture was autoclaved 15 min. at 15 lb. After cooling to room temperature 5 mg of chick pancreas acetone powder was added to the buffer-sample mixture, and it was incubated under toluene 4 hr or overnight at 37°. (The chick pancreas preparation was reconstituted in a small mortar before use by adding a drop or two of glycerine as a wetting agent, and grinding the pancreas powder with a few ml of water; then it was added to the sample mixture quantitatively, and the rinsings from the mortar were added to the mixture.) The incubated mixture was autoclaved 5 min. at 15 lb, cooled, brought to volume, clarified by filtration or centrifugation, and diluted if necessary.

(B) Sample was suspended in 20 ml potassium ascorbate (6 mg/ml, pH 6) with 0.1 ml 2-octanol added as an antifoam agent. The mixture was autoclaved at 15 lb for 30 min., cooled, brought to volume, clarified by filtration or centrifugation, and diluted if necessary with potassium ascorbate (4 mg/ml, pH 6).

(C) Similar to extraction (A), but the sample was autoclaved in 0.2*M* phosphate buffer, pH 8, and cooled, and 20 ml potassium ascorbate solution (20 mg/ml, pH 6) was added prior to the enzyme preparation. After incubation with chick pancreas enzyme the mixture was autoclaved 5 min. at 15 lb, cooled, brought to volume, clarified, and diluted with potassium ascorbate (4 mg/ml, pH 6).

(D) The sample was suspended in 10 ml 0.2*M* phosphate buffer, pH 8; then 10 ml potassium ascorbate (20 mg/ml, pH 6) was added. It was allowed to autolyze under toluene 4 hr or overnight, then autoclaved 5 min. at 15 lb, cooled, brought to volume, clarified, and diluted with potassium ascorbate (4 mg/ml, pH 6).

($A_2$) The sample was extracted like (A) but brought before clarification to half the volume for (A). An aliquot of the clarified extract was adjusted to pH 4.5 with acetate buffer, autoclaved 30 min. at 15 lb, cooled, adjusted to pH 6, brought to volume, and clarified.

($C_2$) The sample was extracted like (C) but brought before clarification to half the volume for (C). An aliquot of the clarified extract was adjusted to pH 4.5 with acetate buffer, autoclaved 30 min. at 15 lb, cooled, adjusted to pH 6, brought to volume, and clarified.

## Results

Although some results were disappointing, the Associate Referee feels that the difficulties encountered can be eliminated. Unfortunately the directions sent the collaborators were not adequate for good assays with *L. casei* in most of these laboratories.

Table 1 shows the composition of the basal folic acid-free ration in the chick test. Folic acid was added to the ration in the "Standard Rations" at levels of 5, 10, 20, 40, and 200 μg per 100 g of ration. The samples were added in amounts estimated to contain 5, 10, 20, and 40 μg of folic acid. Casein levels were lower in rations containing beans and eggs to correct for added nitrogen in the samples. Soybean oil levels were lower in rations containing eggs to correct for fat added in the egg yolks.

**Table 1. Basal ration for 1962 chick assay for folic acid[a] (Use male broiler strain chick in a four-week trial. Growth and hematocrit may be employed as criteria of adequacy.)**

| | % |
|---|---|
| Casein (ANRC, Sheffield) | 25.0 |
| Gelatin | 10.0 |
| DL-Methionine | 0.5 |
| Arginine HCl | 0.36 |
| Glucose (Cerelose) | 46.74 |
| Salts (Fox Briggs Salts N)[b] | 6.0 |
| Soybean oil | 9.0 |
| ADEK in soybean oil[c] | 1.0 |
| B-Vitamin mix in Cerelose[d] | 1.0 |
| Choline Cl[e] | 0.2 |

[a] Assay rations planned by Boyd L. O'Dell and J. E. Savage.
[b] *J. Nutrition*, 72, 243 (1960); no selenium or molybdenum added.
[c] Supplies per 100 g diet: 2,000 I.U. of A, 433 I.C.U. of D, 2.5 mg menadione, 2.5 mg alpha-tocopherol, and 0.0125 g ethoxyquin.
[d] Supplies in mg per 100 g diet: Thiamine HCl, 1.0; riboflavin, 1.0; pyridoxine HCl, 1.0; Ca pantothenate, 3.0; niacin, 5.0; inositol, 50.0; biotin, 0.02; cyanocobalamin, 0.003.
[e] Added to the ration as a 40% solution in $H_2O$ after all other ingredients are mixed. Purchased as a 70% water solution from Hoffman-Taff, Inc., Springfield, Mo.

The collaborators' results are summarized in Tables 2–6. To facilitate comparisons, arithmetic means and ranges are listed in the tables. In the calculation of these means and ranges only one figure was used from each laboratory, an average of the results of individual tests from that laboratory.

Table 2 shows disappointing variations in results, particularly in data for egg yolk solids and liver. Results of microbiological assays in Tables 3–6 show effects of varying the extraction procedure or the test organism.

*P. cerevisiae (Leuconostoc citrovorum)* responds to 5-formyl tetrahydrofolic acid (folinic acid, citrovorum factor, Leucovorin) and to tetrahydrofolic acid.

*S. faecalis* responds to 5-formyl tetrahydrofolic acid, free folic acid (folacin, pteroylglutamic acid, vitamin $B_c$), and the diglutamate form of folic acid that results from treating samples containing polyglutamates of folic acid with chick pancreas enzymes.

*L. casei* responds to 5-formyl tetrahydrofolic acid, free folic acid, the diglutamate form of folic acid, 5-methyl tetrahydrofolic acid (monoglutamate, Prefolic A), polyglutamates of folic acid, and other folic acid-active compounds.

Tetrahydrofolic acid and 10-formyl tetrahydrofolic acid are relatively unstable; 5-methyl tetrahydrofolic acid is less stable to heat than 5-formyl tetrahydrofolic acid; and 5-formyl tetrahydrofolic acid is relatively stable.

The addition of potassium ascorbate protects the reduced forms of folic acid during extraction (either by autoclaving in potassium ascorbate or by using chick pancreas) and storage.

**Table 2. Folic acid-active compounds in foods and feeds (μg folic acid/g D.M. by chick assays)[a]**

| Lab. | Alfalfa Meal | Navy Beans | Egg Yolk Solids | Lyophilized Raw Liver (Baby Beef) |
|---|---|---|---|---|
| A | (2) 4.3 | (1) 0.40 | (1) 0.70 | (2) 9.3 |
| B | (2) 6.2 | (1) 1.87 | (1) 3.32 | (2) 19.0 |
| C | (2) | (3) | (2) | (3) |
| D | (2) 4.55 | (2) 0.90 | (1) 1.33 | (2) 18.75 |
| E | (2) 5.00 | (2) 1.21 | (1) 1.40 | (2) 8.46 |
| Mean | 5.0 | 1.05 | 1.78 | 15.68 |
| Range | 4.3–6.2 | 0.4–1.87 | 0.70–3.32 | 9.3–19.0 |

[a] Data submitted by collaborators in 1962 AOAC collaborative chick assay. Rations and design of chick assay planned by Dr. Boyd L. O'Dell, Agricultural Chemistry Department, and Dr. J. E. Savage, Poultry Husbandry Department, University of Missouri. Data summarized by the Associate Referee. Data are results calculated by collaborators who supervised chick assays in the several laboratories. Sample number is shown in parentheses above each result.

**Table 3. Folic acid activity in alfalfa (μg/g D.M. by microbiological assay)[a]**

| | Extraction Method | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | |
| Lab. | S.f. | L.c. | S.f. | L.c. | S.f. | L.c. | S.f. | L.c. |
| Sample I: Lyophilized fresh alfalfa | | | | | | | | |
| 1 | 3.1 | 3.4 | | | 3.75 | 4.15 | 4.2 | 4.0 |
| Sample II: Alfalfa meal | | | | | | | | |
| 3 | 3.46 | — | | | 3.25 | | | |
| 4 | — | | 1.66 | 4.16 | 3.51 | 7.49 | | |
| 6 | 2.33 | | | | | | | |
| 7 | 4.26 (9) | | | | 3.89 (9) | | | |
| Range | 2.33–4.26 | | | | 3.25–3.89 | | | |
| Mean | 3.35 (3) | | 1.66 | 4.16 | 3.55 (3) | 7.49 (1) | | |
| Folic acid activity by chick assays: Sample II, Range 4.3–6.2 Mean 5.0 (4) | | | | | | | | |

[a] No. in parentheses is number of values averaged.

**Table 4. Folic acid activity in navy beans (cooked) (μg/g D.M. by microbiological assays)[a]**

| | Extraction Method | | | | | |
|---|---|---|---|---|---|---|
| | A | | B | | C | |
| Lab. | S.f. | L.c. | S.f. | L.c. | S.f. | L.c. |
| Sample I: Navy beans autoclaved 40 min. at 10 lb, dried, and ground | | | | | | |
| 1 | | | | 0.31 | 1.15 | |
| 4 | | | 0.116 | 7.75 | 1.3 | 2.32 |
| 7 | 1.41(6) | | | | 1.41(9) | |
| | | | | 0.31–7.75 | 1.15–1.41 | |
| | 1.41 | | 0.116 | — | 1.28(3) | |
| Sample II: Navy beans (different lot), same treatment | | | | | | |
| 3 | 1.37 | | | | 1.40 | |
| 6 | 0.85 | | | | — | |
| Range | 0.85–1.37 | | | | — | |
| Mean | 1.11(2) | | | | | |
| Folic acid activity by chick assays: Sample I, Range 0.40–1.87 Mean 1.13 Sample II, Range 0.90–1.21 Mean 1.05 | | | | | | |

[a] No. in parentheses is number of values averaged.

**Table 5. Folic acid activity in egg yolk solids[a] (μg/g D.M. by microbiological assays)[b]**

| | Extraction Method | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | |
| Lab. | S.f. | L.c. | S.f. | L.c. | S.f. | L.c. | S.f. | L.c. |
| 1 | | | | | | | 1.04 | |
| 3 | | | | | 0.548(2) | | 0.640(2) | |
| 4 | | | 0.816 | 3.94 | 0.75 | 3.74 | | |
| 6 | 0.37(3) | | | | | | | |
| 7 | | | | | 0.54(6) | | 10.5(6) | |
| Range | | | | | 0.54–1.55 | | 0.64–105 | |
| Mean | 0.37 | | 0.816 | 3.94 | 0.61(3) | | 0.91(3) | 4.0 |

Folic acid activity by chick assays:
Range 0.71–3.32
Mean 1.78 (4)

[a] Commercial spray-dried (flash process).
[b] No. in parentheses is number of values averaged.

**Table 6. Folic acid activity in lyophilized raw liver (baby beef) (μg/g D.M. by microbiological assays)[a]**

| | Extraction Method | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | |
| Lab. | S.f. | L.c. | S.f. | L.c. | S.f. | L.c. | S.f. | L.c. |
| | | | | Sample I | | | | |
| 1 | 6.4 | 7.4 | | | 12.4 | 9.4 | 12.0 | 13.0 |
| | | | | Sample II | | | | |
| 1 | | | | | | | 10.8 | 20.6 |
| 3 | | | | | 8.5(2) | | 11.5(2) | |
| 4 | | | 7.5 | 16.38 | 7.01 | 18.0 | | |
| 6 | 9.3 | | | | | | | |
| 7 | | | | | 12.03(9) | | 19.2(9) | |
| Range | | | | | 7.01–12.03 | | 10.8–19.2 | |
| Mean | 9.3 | | 7.5 | 16.38 | 9.51(3) | 18.0 | 13.83(3) | 20.6 |

Folic acid activity by chick assays:
Range 9.3–19.0
Mean 15.68

[a] No. in parentheses is number of values averaged.

The above statements, although somewhat over-simplified, aid in interpreting the data compiled in Tables 2–7.

Table 7 presents data from recent tests in the laboratory of the Associate Referee. The materials assayed were similar to but not identical with those reported in Tables 2–6. Table 6 presents data for lyophilized raw liver, Table 7 for liver ground at 0°, autoclaved 30 minutes at 15 lb, and dried overnight in a forced air oven at 60°. Results in Table 7 are reported as percentages

**Table 7. Results of extractions expressed as percentages of highest values found[a]**

| | Alfalfa Meal | Egg Yolk | Bovine Liver |
|---|---|---|---|
| | | L. casei | |
| A | 67.4 | A 85.7 | A 47.5 |
| | | $A_2$ 12.3 | $A_2$ 26.5 |
| B | 84.8 | — | — |
| C | 104.3 | C 94.9 | C 99.7 |
| | | $C_2$ 79.2 | $C_2$ 51.7 |
| | | S. faecalis | |
| A | 81.2 | A 31.3 | A 60.3 |
| | | $A_2$ 27.2 | $A_2$ 23.6 |
| B | 30.9 | — | — |
| C | 85.7 | C 21.1 | C 27.7 |
| | | $C_2$ 22.9 | $C_2$ 17.0 |
| | | P. cerevisiae | |
| A | 29.3 | A 3.7 | A 9.7 |
| | | $A_2$ 1.7 | $A_2$ 5.5 |
| B | 9.8 | — | — |
| C | 29.3 | C 9.0 | $C_1$ 9.8 |
| | | $C_2$ 2.5 | $C_2$ 6.6 |

[a] Highest values as found with *L. casei:*
Alfalfa meal, 4.69 μg/g D.M.
Egg yolk solids, 2.48 μg/g D.M.
Bovine liver (cooked baby beef), 6.7 μg/g D.M.

of the highest values found, because *L. casei* gave results as high as or higher than *S. faecalis* in all assays.

In Table 3 several values close to 4 μg/g D.M.. found with *L. casei* and with *S. faecalis* check fairly well with the values found by Laboratories A and D in the chick assay of alfalfa meal. Assays of extracts prepared by procedures A, C, and D (lyophilized samples) gave approximately equal values with the two test organisms. For extract B without enzyme treatment, the result with *S. faecalis* was only 40% of that with *L. casei; S. faecalis* cannot use polyglutamates or 5-methyl tetrahydrofolic acid.

With the alfalfa meal used (Table 7) the addition of potassium ascorbate in the chick pancreas treatment (Treatment C) gave higher values with both *L. casei* and *S. faecalis* than were found in Extract A, lacking ascorbate. In Extract B, however, assay results with *S. faecalis* were about 36% of that found with *L. casei*. This suggests again the presence of polyglutamates or the 5-methyl compound which *S. faecalis* cannot use. Assays with *P. cerevisiae* indicate that about 29% of the highest activity in Extracts A and C is in the form of 5-formyl tetrahydrofolic acid, while extract B contains only about a third as much as Extracts A and C in a form available to *P. cerevisiae*.

In regard to the limited data on the folic acid activity of beans (Table 4), Extracts A and C produced approximately the same results with *S. faecalis*, and Extract B contained folic acid activity available to *L. casei* but not to *S. faecalis*. Data for cooked navy beans are not included in Table 7.

Values for egg yolks from *L. casei* assays, reported in Table 5, are higher than those for *S. faecalis*. We assume that the egg yolks contain the 5-methyl form not used by *S. faecalis*. Table 7 shows that Extract C yields a higher assay value than Extract A from egg yolks. The results of assays with the two extracts with *S. faecalis* suggest the need for further study. The results of assays of Extracts A and C with *P. cerevisiae* indicate that only a small amount of the folic acid activity of dry egg yolk comes from 5-formyl tetrahydrofolic acid. Autoclaving Extracts A and C at pH 4.5 for 30 min. appeared to have little effect on the folic acid activity as measured by *S. faecalis*, but had a large effect

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on the activity as assayed in Extract A with *L. casei*. A large amount of 5-methyl tetrahydrofolate may be present in the egg yolk.

The presence of potassium ascorbate markedly protected the activity from 5-formyl tetrahydrofolic acid and from 5-methyl tetrahydrofolic acid, as would be expected. Unfortunately the chick assays were not completely satisfactory; the *L. casei* values of about 4 μg/g may be high but are not completely out of line with the folic acid value of egg yolk for the chick. A value of 2 or 2.5 μg/g may be a good estimate for the chick value.

Table 6, liver, indicates that autolysis may give the highest results if the sample proteins are not denatured. For Sample II, *L. casei* gives higher results for Extracts B, C, and D than *S. faecalis* does. The higher value may show the presence of 5-methyl folic acid in high amounts or other forms of folic acid to which *S. faecalis* can not respond. Since our chick test values do not check each other, we cannot verify the results of our microbiological assays.

Table 7 shows an expected decrease in folic acid activity of Extracts A and C when they are adjusted to pH 4.5 and autoclaved 30 min. at 15 lb. The highest value for this liver, 6.7 μg/g D.M., was found with *L. casei* but was much lower than expected. In processing, the liver was subjected to autoclaving 30 min. at 15 lb followed by drying at 60° in a forced air oven. The cooked liver probably lost folic acid activity in the form of 5-methyl folate and possibly 5-formyl folate or other forms. Less than 10% of the total folic acid activity was in the form of 5-formyl folate in Extract A or C at the time of assay. The presence of potassium ascorbate protected the activity of 5-methyl tetrahydrofolic acid in assays with *L. casei*.

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### Recommendation

It is recommended that work on the extraction and assay of bound folic acid be continued.

This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. *See This Journal*, 47, 121 (1964).

### Collaborators

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A. Chick assays:

P. M. Derse, Wisconsin Alumni Research Foundation, Madison, Wis.

J. C. Fritz, Dawe's Laboratories, Inc., Chicago, Ill.

B. L. O'Dell, D. L. Ruggles (Dept. of Agricultural Chemistry), and J. E. Savage (Dept. of Poultry Husbandry), Univ. of Missouri, Columbia, Mo.

E. L. R. Stokstad, Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.

B. Microbiological assays:

P. H. Derse and Maria Burger, Wisconsin Alumni Research Foundation, Madison, Wis.

L. M. Flynn, N. K. Das, and Herman Brown (Dept. of Agricultural Chemistry), University of Missouri, Columbia, Mo.

C. L. Graham, The Upjohn Co., Kalamazoo, Mich.

C. A. Luhman and W. C. Green, Division of Chemistry, Dept. of Agriculture, State of California, Sacramento, Calif.

Edward Martin and Dorothy L. Mueller, Nutritional Research Laboratories, Ralston Purina Laboratories, St. Louis, Mo.

# Chemical Assay of Chick Edema Factor in Fats and Fatty Acids

By G. R. HIGGINBOTHAM (Division of Food Chemistry, Food and Drug Administration, Washington, D.C. 20204)

**A microcoulometric gas chromatographic method for detecting chick edema factor in fats was studied collaboratively by six laboratories. The method involved extracting unsaponifiable material from fat and fractionating it on an activated alumina column, then examining the third alumina fraction by microcoulometric gas chromatography. Results did not warrant adoption of the method. Initial studies with electron capture gas chromatography for this determination showed promise. A better cleanup procedure needs to be developed. It is planned to continue the study, and to combine acetonitrile extraction and cleanup on an acid-Celite column with electron capture determination.**

Chick edema disease, first encountered in 1957, is caused by a group of chlorinated aromatic hydrocarbons of unknown origin. Wootton and co-workers (1) have isolated three of these compounds from a toxic fat. It was estimated that 5 μg of one of these materials was enough to kill one chick. At present, the detection and assay of chick edema factor in fats is carried out by a bioassay procedure (2). Because chromatographic procedures were found to be more rapid and sensitive than the bioassay, a rapid screening test was proposed for detecting chick edema factor in fats and oils (3). This test consisted of adsorption chromatography of extracted unsaponifiables on alumina and analysis of a specific fraction of the eluate by microcoulometric gas chromatography, which is sensitive only to halogens.

This report describes results of a collaborative study of the microcoulometric method and, in addition, presents results of preliminary study of the use of electron capture gas chromatography for this determination.

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

## Collaborative Study of Microcoulometric Method

Six laboratories participated in a study of the microcoulometric method. Each collaborator was furnished a copy of the method, an instruction sheet, a report form, 0.5 g of unsaponifiable matter from a toxic fat (positive control), and 4 test samples. Test samples 1, 2, and 3 contained 0, 1.56, and 6.25% of toxic fatty material, respectively, in nontoxic cottonseed oil. Test sample 4 contained 25% of a toxic oleic acid in nontoxic oleic acid. Collaborators were requested to follow the method and instructions carefully, and to make a single examination of each of the 4 test samples.

Briefly, the microcoulometric procedure involves (*a*) extraction of unsaponifiables from 111 g of fat; (*b*) fractionation of unsaponifiable matter on an activated alumina column by elution with petroleum ether, 10% ethyl ether in petroleum ether, and 25% ethyl ether in petroleum ether; and (*c*) examination of the third alumina fraction (25% ethyl ether eluate) by microcoulometric gas chromatography. Toxic fats yield gas chromatographic peaks with retention times of 5 or more, relative to aldrin.

Three changes were made from the original method (3) for this study. Collaborators were requested to activate the alumina by heating at 200°C for 4 hours, to hold the gas chromatographic column temperature at 245 ± 1°C, and to control the nitrogen flow rate so that aldrin eluted between 2.5 and 3 minutes.

Results were received from all 6 collaborators. However, one collaborator did not use the gas chromatographic column temperature specified, and his results were not included in the summary of data recorded in Table 1. Numbers assigned to collaborators are listed in the first column of Table 1. Weights of material eluted in alumina fraction 3 are shown in the second column. The per cent recovery of aldrin is tabulated in the third column (should be at least 70%)

The amount of alumina fraction 3 injected into the gas chromatograph is recorded in the fourth column.

Collaborators were instructed to inject 0.9 of fraction 3 (equivalent to 100 g of fat) if injection of 0.1 of the sample did not produce peaks indicative of a toxic sample. The retention times of gas chromatographic peaks relative to aldrin ($R_a$) are listed in column 5; only peaks with $R_a$ values equal to or greater than 5 are listed.

No false positives were reported for test sample 1 (the negative sample). However, Collaborators 2 and 5 reported negative results for test sample 2; this sample, containing a low level of toxic fat, gives bioassay results that are at the limit of detection for chick edema factor. Low instrument sensitivity would account for the negatives obtained here. Collaborator 2 obtained an aldrin recovery of less than 70%; Collaborator 5 reported that his instrument was not operating optimally (the instrument was rather insensitive when the recorder pen was close to the zero point).

Collaborator 5 also reported a false negative for test sample 3. However, he obtained 189 mg of material in alumina fraction 3, a sample too large for microcoulometric analysis, and only 0.1 of this fraction was examined. (Generally, about 125 mg of material is the maximum amount of sample which may be injected into the microcoulometric gas chromatograph.) The remaining

**Table 1. Summary of data: 1963 collaborative chemical assay for chick edema factor**

| Coll. | mg Unsaponifiable from 111 g Sample | % Recovery of Aldrin | mg of Alumina Fraction 3 Injected | $R_a$ Values of Peaks in Gas Chromatogram ($R_a \geqq 5$) | Results (Pos. or Neg.) |
|---|---|---|---|---|---|
| Sample 1 (Nontoxic Cottonseed Oil) | | | | | |
| 1 | 23 | 80 | 20 | none | neg. |
| 2 | 37 | 52 | 33 | none | neg. |
| 3 | 21 | 79 | 19 | none | neg. |
| 4 | 58 | 83 | 53 | none | neg. |
| 5 | 62 | 97 | 55 | none | neg. |
| Sample 2 (1.56% Toxic Fat in Cottonseed Oil) | | | | | |
| 1 | 13 | 75 | 12 | 7.0 | pos. |
| 2 | 16 | 68 | 14 | none | neg. |
| 3 | 8 | 81 | 7 | 6.4, 9.7 | pos. |
| 4 | 29 | 93 | 26 | 5.8 8.7 | pos. |
| 5 | 123 | 97 | 110 | none | neg. |
| Sample 3 (6.25% Toxic Fat in Cottonseed Oil) | | | | | |
| 1 | 30 | 77 | 12 | 7.0, 11.0 | pos. |
| 2 | 40 | 68 | 4 | 7.8 | pos. |
| 3 | 6 | 80 | 5 | 6.9, 10.4 | pos. |
| 4 | 53 | 95 | 5 | 5.7, 8.9 | pos. |
| 5 | 189 | 94 | 18 | none | — |
| Sample 4 (25% Toxic Oleic Acid in Nontoxic Oleic Acid) | | | | | |
| 1 | 24 | 88 | 2.4 | 6.0, 10.0 | pos. |
| 2 | 32 | 52 | 3.2 | 6.04 | pos. |
| 3 | 18 | 91 | 16.0 | 6.4, 10.4 | pos. |
| 4 | 45 | 88 | 4.6 | 5.2, 8.5 | pos. |
| 5 | 62 | 95 | 55.8 | 6.2, 9.8 | pos. |

0.9 of alumina fraction 3 from sample 3 was sent to the Associate Referee by Collaborator 5 and the sample was rechromatographed in Division of Food Chemistry with the same alumina originally used by the collaborator. No difficulty was encountered, and 23 mg of material was obtained in alumina fraction 3. When the alumina column was stripped with 400 ml of 100% ethyl ether, an additional 125 mg of material was obtained. Further studies of alumina activation and effect of column flow rate indicated that the high weights in alumina fraction 3 were most probably due to inadequate mixing of the eluting solvents (petroleum ether and ethyl ether) prior to column chromatography. A high concentration of ethyl ether at any instant will deactivate the alumina and cause elution of normally retained materials. No false negatives were reported for sample 4.

In summary, two collaborators reported negative results for a sample containing a low level of toxic fat, apparently due to low instrument sensitivity, and one of the collaborators had difficulty with the alumina chromatography cleanup, obtaining weights of alumina fraction 3 eluates too large for microcoulometric analysis. The other three collaborators reported correct results for all samples analyzed.

### Electron Capture Detection of Chick Edema Factor

The use of electron capture gas chromatography for detection of chick edema factor in fats was investigated in this laboratory. Initial studies were encouraging, and indicated that a rapid analytical method can be developed by using this technique.

The four collaborative test samples were selected for this preliminary work. The alumina chromatography cleanup procedure was used but the greater sensitivity of electron capture detection necessitated removal of background interferences by passing alumina fraction 3 through an acid-Celite column. The acid-Celite cleanup procedure used was a modification of AOAC method **24.111(a)**(*3*). Columns were prepared by grinding 10 g of Celite 545 thoroughly with 3 ml of fuming $H_2SO_4$ and 3 ml of concd $H_2SO_4$. The mixture was transferred to a chromatographic column 25 mm o.d. × 300 mm long with a coarse fritted plate and Teflon stopcock. A 10 ml $CCl_4$ solution of alumina fraction 3 was added to the column, and a total of 400 ml of $CCl_4$ was passed through the column at a rate of about 6 ml per minute. The $CCl_4$ eluate was evaporated to dryness in a tared flask, and the residue, after weighing, was dissolved in redistilled hexane so that the concentration was about 1 mg/ml. Recoveries of chick edema factor from the acid-Celite column were in the range of 70 to 110%, as determined by quantitative microcoulometric analysis of alumina fraction 3 from a toxic reference sample. Per cent recovery was calculated as the ratio of the peak area of $CCl_4$ eluate from a given weight of sample treated with acid-Celite to the peak area of an equivalent weight of sample without acid-Celite treatment. The peaks with $R_a$ values $\geqq 5$ were compared and used for these recovery calculations.

Acid-Celite residues were examined in an Aerograph Hy Fi (Model 600 B) gas chromatography equipped with a tritium source electron capture detector. Pyrex glass columns, 3′ × ⅛″, packed with 5% SE-52 gum rubber were used, and 1–5 μl of hexane solution was injected. The column temperature was held at 207°C, and three different positive standards and several chlorinated pesticides were run under the same conditions as the test samples. Chromatograms from positive standards and carbon tetrachloride eluates from collaborative samples 2, 3, and 4 (positive samples) showed peaks with $R_a$ values equal to or greater than 10. Chromatograms from several chlorinated pesticides and collaborative sample 1 (negative sample) showed peaks with $R_a$ values less than ten.

Results of the analyses are summarized in Table 2. $R_a$ values $< 10$ from test samples and positive control are not listed. The equivalent starting sample analyzed is shown in column 4.

The data show that less than 100 mg of toxic fat will give a satisfactory response when examined by electron capture detection. On the other hand, it is necessary to examine the equivalent of at least 10 g of

**Table 2. Electron capture detection of chick edema factor[a]**

| Sample | Concn (mg/ml) | Nanograms Injected | Equivalent Starting Sample Analyzed, g | Chromatographic Peaks,[b] Retention Time vs. Aldrin ($R_a$) ≧ 10 |
|---|---|---|---|---|
| Collab. 1 | 0.6 | 3,200 | 0.11 | none |
| Collab. 2 | 1.0 | 5,000 | 0.03 | 11.8 (0.45); 21.7 (0.28) |
| Collab. 3 | 1.5 | 7,700 | 0.05 | 11.9 (1.1); 20.8 (0.94); 45.5 (0.35) |
| Collab. 4 | 1.0 | 2,000 | 0.04 | 10.5 (1.5); 19.3 (2.7); 43.6 (0.46) |
| Positive control | 0.8 | 820 | 0.0003 | 12.5 (0.93); 22.1 (0.93); 38.0 (0.05) |
| Aldrin | 0.001 | 0.8 | — | — |
| DDT | 0.0016 | 11.2 | | 3.2 |
| Mirex | 0.002 | 1.6 | | 6.05 |
| α–3.17[c] | 0.002 | 10 | | 13.0 |
| F400[d] | 0.005 | 25 | | 3.5, 6.5, 10.6 |

[a] Aerograph Hy Fi (Model 600 B) with tritium source. Input impedance, $10^7$; attenuator, 16; output sensitivity, 10×; recorder chart speed, 0.25″/min.; column, 3′ × 1/8″ Pyrex with 5% SE 52 gum rubber on acid-washed Chromosorb W (60/80 mesh); column temp., 207°C; $N_2$ flow rate, about 52 ml/min.
[b] Values in parentheses are peak areas in sq. in.
[c] Low melting inactive isomer having the same retention time as a toxic isomer; supplied by Dr. N. R. Artman, Procter and Gamble, Co., Cincinnati, Ohio.
[d] Concentrate from a toxic fat, active at 0.1 ppm.

toxic fat to obtain a comparable response by microcoulometric gas chromatography. The extreme sensitivity of electron capture detection (more than 1000 times that of microcoulometric detection), when combined with a suitable cleanup procedure, should permit the routine determination of 1 g or less of fat for chick edema factor.

### Discussion and Recommendation

Results of the collaborative study of the microcoulometric method and preliminary work on electron capture detection indicate that both procedures can be used to screen fats for the presence of chick edema factor. However, the greater sensitivity of electron capture detection warrants further study, and efforts will be made to develop a rapid method based on this technique.

Cleanup procedures used in the microcoulometric method and in the initial electron capture work are rather time-consuming and are not well suited for routine analysis of large numbers of samples. An efficient cleanup procedure combined with electron capture detection should provide an ideal method for chemical assay of chick edema factor. In pesticide methodology, acetonitrile and dimethyl sulfoxide are currently used as partitioning solvents for extracting chlorinated pesticides from fats and oils (4, 5). Since the substances which cause chick edema disease are chlorinated hydrocarbons, it is reasonable to assume that solvent partitioning would also be applicable to this work and might eliminate the saponification step. If saponification is eliminated, the same samples could be routinely examined for both chick edema factor and pesticides, since labile pesticides would not be altered by an alkali treatment. This laboratory proposes to undertake a study of the applicability of solvent partitioning and other cleanup procedure to the detection of chick edema factor. Our first approach will involve acetonitrile extraction followed by subsequent cleanups on an acid-Celite column and final detection by electron capture gas chromatography.

It is recommended that further work be done on chemical assay of chick edema factor.

This recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A, and was accepted by the Association. See *This Journal*, 47, 121 (1964).

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## OILS, FATS, AND WAXES

# Safflower Oil: Physical and Chemical Properties, and Fatty Acid Composition

By WALID IBRAHIM, JOHN IVERSON, and DAVID FIRESTONE (Division of Food, Food and Drug Administration, Washington, D.C. 20204)

**Some physical and chemical properties, including fatty acid composition by gas chromatography, of 15 commercially refined safflower oils and three crude oils extracted from safflower seed, were determined. The average and standard deviations found were: refractive index, 1.4748 ± 0.0003; specific gravity, 0.9210 ± 0.0005; iodine number, Wijs, 143.4 ± 1.4; squalene, 7.1 mg/100 g ± 1.1; and Bellier test, 7.4° ± 0.9. The major fatty acid components were; palmitic 6.7 ± 0.3, stearic 2.7 ± 0.4, oleic 12.9 ± 0.6, and linoleic 77.5 ± 1.1. Minor fatty acid components detected were myristic, arachidic, and eicosenoic acids.**

Recent consumer interest in oils of high polyunsaturated content has resulted in a large increase in the production and use of safflower oil for edible purposes. This has prompted us to study the chemical and physical characteristics and the fatty acid composition of commercial safflower oil.

Standard reference works (1–3) have reported data on the characteristics and fatty acids of various foreign and domestic safflower oils. More recent data on fatty acid composition obtained using gas-liquid chromatography are also available (4, 5).

This paper reports the analysis of 15 samples of refined safflower oil. In addition, the fatty acid composition of oil extracted from 3 samples of safflower seed is also reported.

### Experimental

*Nature of Samples.*—The 15 samples of refined safflower oil collected in California by Food and Drug Administration inspectors late in 1962 and early in 1963 represent oil extracted from seeds grown in California and the Northern Great Plains. Three varieties of safflower seed were supplied by the Pacific Oil Seed Corporation, Woodland, Calif.

*Extraction of Oil from Seed Samples.*—Pressed oil samples were obtained with a Carver laboratory press by using 2,000 pounds per square inch pressure. Seeds were prepared for pressing and solvent extraction by grinding in a Wiley Mill with sieve removed. The seeds were passed through the mill four times to compensate for the missing sieve.

**Table 1. Chemical and physical characteristics of commercial refined safflower oils**

| Sample No. | Refractive Index,[a] 25°C | Specific Gravity,[b] 25/25°C | Iodine No.,[b] Wijs | Squalene, mg/100 g Oil[b] | Crystallization Temp. (Bellier test), °C[b] |
|---|---|---|---|---|---|
| 1 | 1.4747 | 0.9206 | 144.2 | 7.7 | 7.7 |
| 2 | 1.4749 | 0.9207 | 143.6 | 8.1 | 7.7 |
| 3[c] | 1.4746 | 0.9207 | 143.7 | 8.8 | 7.5 |
| 4 | 1.4746 | 0.9211 | 142.4 | 4.4 | 6.8 |
| 5 | 1.4752 | 0.9221 | 141.7 | 5.7 | 7.5 |
| 6 | 1.4751 | 0.9223 | 140.4 | 5.8 | 6.0 |
| 7 | 1.4747 | 0.9210 | 141.9 | 8.2 | 7.5 |
| 8 | 1.4745 | 0.9206 | 141.8 | 6.4 | 7.9 |
| 9 | 1.4745 | 0.9208 | 143.6 | 7.2 | 6.8 |
| 10[c] | 1.4745 | 0.9207 | 143.7 | 7.1 | 6.8 |
| 11[c] | 1.4746 | 0.9211 | 145.5 | 7.1 | 7.5 |
| 12[c] | 1.4753 | 0.9208 | 144.6 | 7.3 | 7.0 |
| 13 | 1.4750 | 0.9206 | 144.3 | 7.7 | 6.8 |
| 14 | 1.4750 | 0.9206 | 144.4 | 7.7 | 10.0 |
| 15 | 1.4749 | 0.9207 | 144.5 | 7.3 | 7.3 |
| Av. | 1.4748 | 0.9210 | 143.4 | 7.1 | 7.4 |
| Range | 1.4745–1.4753 | 0.9206–0.9223 | 140.4–145.5 | 4.4–8.8 | 6.0–10.0 |
| Std dev. | 0.0003 | 0.0005 | 1.4 | 1.1 | 0.9 |

[a] Average of 3 determinations.
[b] Average of 2 determinations.
[c] Commercial antioxidants added.

Oil was also obtained from ground seeds and from Carver pressed cake by Soxhlet extraction with redistilled petroleum ether. After a 7 hour extraction, Soxhlet extracts were evaporated on a steam bath under nitrogen.

*Preparation of Methyl Esters.*—Oil samples were saponified and the unsaponifiables were extracted with ethyl ether according to AOAC method **26.049** (6). The soaps were acidified with HCl and the fatty acids were extracted with ethyl ether-petroleum ether (1 + 1). The extract was dried with anhydrous sodium sulfate, and the solvent was evaporated under nitrogen on a steam bath. The last traces of solvent and moisture were removed under a vacuum of 6–10 mm of mercury on the steam bath. The fatty acids were converted to methyl esters by esterification with $BF_3$-methanol (7).

*Gas Chromatography.* — An Aerograph 350B gas chromatograph (Wilkins Instrument and Research, Inc., Walnut Creek, Calif.) with thermal conductivity cell was used for qualitative and quantitative analyses of prepared methyl esters on a ¼″ × 6′ aluminum column packed with 25% (w/w) diethylene glycol succinate polyester (DEGS) on Gas-Chrom P (80–100 mesh). The instrument was operated at 190°C with a flow rate of about 72 ml/min. so that methyl stearate was eluted in approximately 6 min.

For quantitative estimation of various components, areas beneath gas chromatographic peaks were determined by the peak height-retention time method (8). Individual peak areas were calculated by multiplying retention time by the peak height. A mixture of fatty acid methyl ester standards (Hormel Institute, Austin, Minn.) was used for peak identification.

*Chemical and Physical Characteristics.*—Methods outlined in Chapter 26 of *Official Methods of Analysis* (6) were used for deter-

**Table 2. Extraction of oil from 3 varieties of safflower seed by Carver press and by Soxhlet extraction with petroleum ether**

| Extraction Method | Seed Variety, % yield | | |
|---|---|---|---|
| | Gilla | US-10 | P-1 |
| 1. Carver press | 16.7 | 14.3 | 9.8 |
| 2. Soxhlet extraction of pressed cake | 17.7 | 20.6 | 21.7 |
| 3. Total of 1 + 2 | 34.4 | 34.9 | 31.5 |
| 4. Soxhlet extraction of ground seed | 35.0 | 33.3 | 30.9 |

mination of refractive index, specific gravity, iodine number (Wijs), squalene, and temperature of crystallization of fatty acids (Bellier test). The squalene values were determined for reference in cases where safflower oil is used in mixtures with olive oil. The Bellier test values are for the detection of peanut oil when used in mixtures with safflower oil.

## Results and Discussion

Chemical and physical characteristics of 15 refined safflower oils are shown in Table 1. The average values obtained are as follows: refractive index at 25°C, 1.4748; specific gravity (25°/25°C), 0.9210; iodine number (Wijs), 143.4; squalene (mg/100 g oil), 7.1; and Bellier test (temperature of crystallization of fatty acids, °C), 7.4. Unsaponifiable matter was not determined on the current samples. However, analysis of 12 samples of refined safflower oil from the same source as the current samples but representing 1961 production, gave the following results for unsaponifiable matter: Average, 0.51%; range, 0.41–0.57%; standard deviation, ± 0.05. The values for refractive index, specific gravity, iodine number, and unsaponifiable matter are in general agreement with those reported by Eckey (3).

Yields of oil obtained from 3 varieties of safflower seed (Gilla, US-10, and P-1) are shown in Table 2. Soxhlet extraction with petroleum ether yielded 31–35% oil. Yields of 10–17% oil were obtained by using the Carver press. Subsequent Soxhlet extraction of the pressed cake with petroleum ether gave a total yield of pressed oil plus extracted pressed cake oil of 32–35%, agreeing closely with the oil yield obtained by straight Soxhlet extraction of the ground seed.

Results of fatty acid analysis of 10 commercial refined safflower oils are listed in Table 3. A typical gas chromatogram is shown in Fig. 1. The average content of

**Table 3. The fatty acid composition of refined safflower oils (% of total fatty acids)**

| Sample No. | Myristic 14:0[a] | Palmitic 16:0 | Stearic 18:0 | Oleic 18:1 | Linoleic 18:2 |
|---|---|---|---|---|---|
| 1 | — | 6.8 | 2.5 | 12.6 | 78.1 |
| 2 | — | 6.7 | 3.5 | 12.7 | 77.1 |
| 4 | 0.02 | 6.8 | 2.6 | 13.3 | 77.2 |
| 6 | 0.10 | 7.6 | 2.8 | 14.3 | 75.2 |
| 7 | 0.09 | 6.8 | 2.4 | 13.5 | 77.0 |
| 9 | 0.03 | 6.4 | 3.1 | 12.5 | 77.9 |
| 11 | — | 6.5 | 2.1 | 12.1 | 79.2 |
| 13 | — | 6.4 | 3.2 | 13.0 | 77.3 |
| 14 | — | 6.7 | 2.5 | 12.7 | 78.0 |
| 15 | — | 6.6 | 2.5 | 12.5 | 78.3 |
| Av. | 0.06 | 6.7 | 2.7 | 12.9 | 77.5 |
| Range | 0.02–0.10 | 6.4–7.6 | 2.4–3.5 | 12.1–14.3 | 75.2–79.2 |
| Std dev. | 0.04 | 0.3 | 0.4 | 0.6 | 1.1 |

[a] First figure refers to number of carbons; second figure to the number of double bonds.

linoleic acid is 77.5%. Other major components are palmitic, 6.7%; stearic, 2.7%; and oleic, 12.9%. These results are in general agreement with published gas chromatographic results (4, 5). About 0.1% of myristic acid and small amounts of arachidic (20:0) and eicosenoic (20:1) acids were also found to be present in safflower oil, but these levels were too low to be detected in the routine gas chromatographic procedure used for analysis of the commercially refined oils.

The fatty acid composition of safflower oil extracted from 3 varieties of safflower seed is shown in Table 4. Each of the varieties had essentially the same fatty acid composition, and average values are as follows: myristic, 0.07%, palmitic, 7.4%; stearic, 2.5%; oleic, 13.2%; and linoleic, 76.4%. In addition, with the aid of overloaded gas chromatograms, about 0.27% of linolenic and/or eicosenoic acid and trace amounts of arachidic acid were found to be present in these laboratory-extracted oils.

Additional analysis of a refined safflower oil using a nonpolar stationary phase (8%

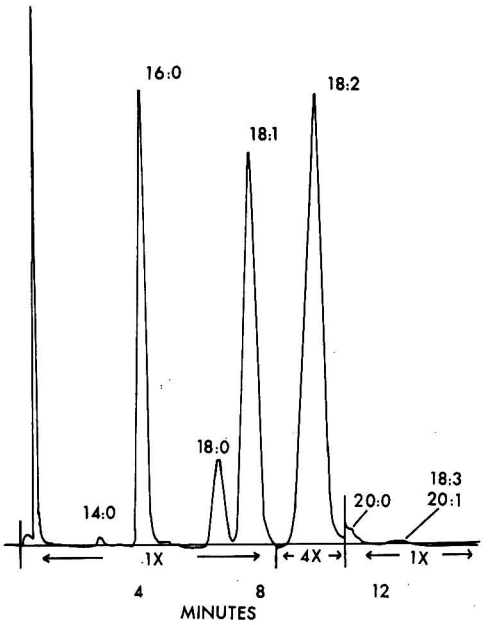


*Fig. 1*—Chromatogram of methyl esters of refined safflower oil. Instrumentation: Aerograph 350B gas chromatograph; 25% diethylene glycol succinate polyester on Gas-Chrom P (80–100 mesh); ¼" × 6' column, 190°C; helium, 72 ml/min.

**Table 4. The fatty acid composition of safflower oil extracted from three varieties of seed (% of total fatty acids)**

| Sample | Myristic 14:0[a] | Palmitic 16:0 | Stearic 18:0 | Oleic 18:1 | Linoleic 18:2 | Linolenic, 18:3 and/or Eicosenoic, 20:1 |
|---|---|---|---|---|---|---|
| Soxhlet-extracted oil from ground seed | | | | | | |
| Gilla | 0.05 | 6.8 | 2.5 | 13.4 | 76.9 | 0.27 |
| US-10 | 0.10 | 8.0 | 2.3 | 12.7 | 76.5 | 0.28 |
| P-1 | 0.04 | 7.6 | 2.7 | 13.8 | 75.5 | 0.36 |
| Pressed oil from ground seed | | | | | | |
| Gilla | 0.10 | 7.6 | 2.6 | 13.2 | 76.2 | 0.18 |
| US-10 | 0.07 | 7.9 | 2.4 | 12.9 | 76.5 | 0.18 |
| P-1 | 0.08 | 6.6 | 2.6 | 13.3 | 77.0 | 0.40 |
| Soxhlet-extracted oil from pressed cake | | | | | | |
| Gilla | 0.08 | 7.3 | 2.6 | 12.8 | 77.0 | 0.19 |
| US-10 | 0.09 | 8.2 | 2.2 | 13.8 | 75.2 | 0.43 |
| P-1 | 0.06 | 6.7 | 2.5 | 13.2 | 77.3 | 0.13 |
| Av. | 0.07 | 7.4 | 2.5 | 13.2 | 76.4 | 0.27 |

[a] First figure refers to number of carbons; second figure to the number of double bonds.

Apiezon L on Gas Chrom P in a ¼ × 9″ aluminum column; column temperature, 216°C) indicated that about 0.5% each of arachidic and eicosenoic acids are present. Arachidic and eicosenoic acids are cleanly separated from linolenic acid by using an Apiezon L column, whereas linolenate and eicosenoate elute together when a Polar DEGS column is used under the conditions described here.

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# Artificial Flavors in Blended Oils: Detection of Ethyl Butyrate by Gas Chromatography

By A. F. RATAY (Food and Drug Administration, 1200 U.S. Appraisers' Stores Bldg., New York, N.Y. 10014)

**A procedure is described for detecting ethyl butyrate in olive oil and other vegetable oils. Volatiles are isolated from the oil by steam distillation, and the distillate is extracted with methylene chloride. The ethyl butyrate in the solution is determined by gas chromatography at a column temperature of 100°C in a thermal conductivity cell on a 6′ × ¼″ i.d. stainless steel column, packed with 20% diethylene glycol succinate polyester on 60–80 mesh, acid-washed Chromosorb W. The method detects 0.001% or less of ethyl butyrate in olive oil and other vegetable oils. Recoveries from known olive oil blends were 66–100%.**

Adulteration of vegetable oil to simulate olive oil continues to be one of the major enforcement problems of state and federal regulatory agencies. With the addition of artificial olive oil flavor, the substitution of cheaper vegetable oils for olive oil in olive oils and olive oil blends is difficult to detect organoleptically since the adulterated oil appears to have an odor resembling olive oil. In addition, the usual physical and chemical methods of analysis are inconclusive.

Imitation olive oil flavors generally contain lower fatty acid esters (such as amyl and ethyl butyrates) that are not normally present in olive oil. Their presence is usually detected by organoleptic examination A procedure is described below for the gas chromatographic detection of ethyl butyrate in vegetable oils and blends.

## METHOD

### *Apparatus*

(a) *Gas chromatograph.*—Beckman GC-2A equipped with a thermal conductivity detector cell contained in a constant temperature oven with the column.

(b) *Column.*—Stainless steel tubing, 6′ long × ¼″ i.d. Packed with diethylene glycol succinate (DEGS), 20% coated on 60–80 mesh, acid-washed Chromosorb W.

### *Procedure*

Place 250 g oil, 100 ml water, 10 ml methylene chloride, and boiling chips into an 800 ml Kjeldahl flask connected to a vertically mounted condenser with glass tube bent into an inverted U-shape. Steam distill, collecting

**Table 1. Recoveries of ethyl butyrate added to olive oil blends**

| Sample No. | Ethyl Butyrate Added, mg | Level in Oil, % | Recovered | |
|---|---|---|---|---|
| | | | mg | % |
| 1 | 5.0 | 0.002 | 4.8 | 96 |
| 2 | 10.0 | 0.004 | 9.9 | 99 |
| 3 | 5.0 | 0.002 | 4.7 | 94 |
| 4 | 5.0 | 0.002 | 5.0 | 100 |
| 5 | 5.0 | 0.002 | 3.3 | 66 |
| 6 | 5.0 | 0.002 | 4.5 | 90 |
| 7 | 5.0 | 0.002 | 3.8 | 76 |

100 ml distillate. Saturate distillate with sodium chloride and shake. Transfer to separator and let stand. Draw off organic phase and retain. Extract aqueous layer again with 5 ml reagent grade methylene chloride. Separate the organic layer as before and combine the two organic phases. Filter through a small pledget of cotton into a suitable container. Allow extract to evaporate spontaneously with a minimum amount of heat to 0.5 ml.

Inject 1.0–2.0 $\mu$l sample from a Hamilton $\mu$l hypodermic syringe through silicone seals into the column (column temperature, 100°C). Pass helium gas through the instrument at the rate of 50 ml/min. Apply current of 250 ma to the detector and set the recorder at −0.05 to +1.05 mv, at a speed of 0.5 inches per minute. Suitable chromatograms should be obtained in about 10 minutes. Determine the quantity of ethyl butyrate present by comparing the chromatographic peak areas obtained from the sample extract with the chromatographic peak area obtained from a solution of pure ethyl

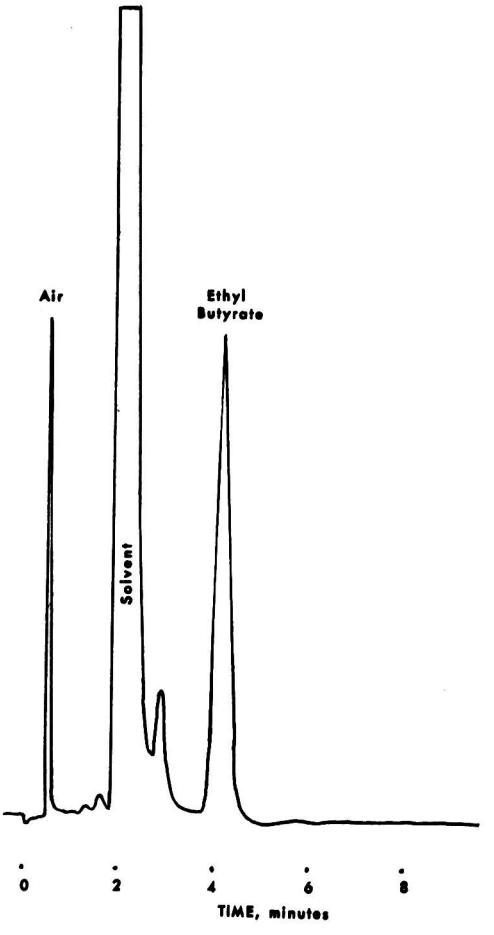


*Fig. 1*—Chromatogram of oil blend with ethyl butyrate on a diethylene glycol succinate column.

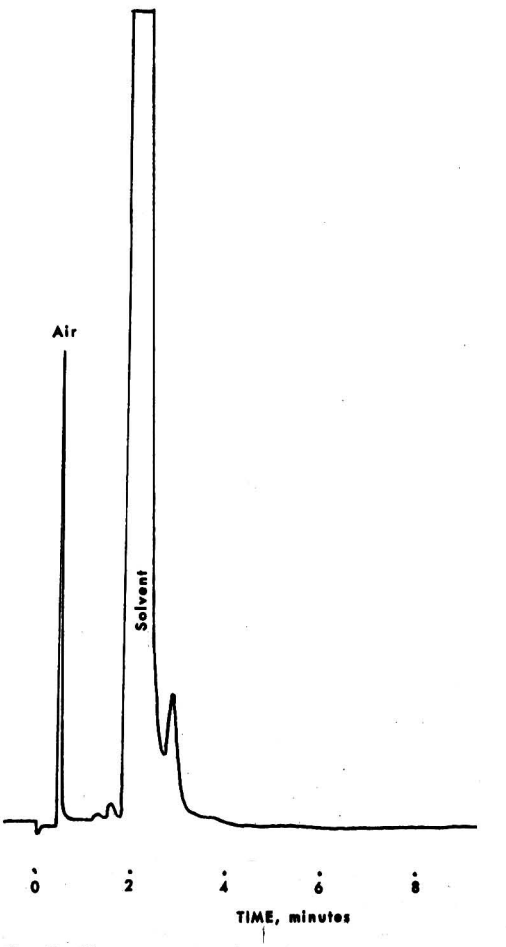


*Fig. 2*—Chromatogram of oil blend with no ethyl butyrate on a diethylene glycol succinate column.

butyrate (b.p., 119–121°C) in methylene chloride (1 μl contains 5 μg ethyl butyrate), injected prior to or following sample injection.

### Results and Discussion

Blends of commercial oils (composed of 25% olive oil and 75% peanut, cottonseed, or corn oil) with added ethyl butyrate were examined by the method. Either 5 or 10 mg of ethyl butyrate (in methylene chloride solution) was added to the oils, which were free of added flavor as determined by gas chromatographic analysis. Recoveries (Table 1) ranged from 66 to 100%. Gas chromatograms of oil extracts with and without added ethyl butyrate are shown in Figs. 1 and 2, respectively.

Eleven commercial samples of vegetable oil blends (25% olive oil and 75% peanut, cottonseed, or corn oil) were also analyzed. No ethyl butyrate was detected in 8 of the samples. Three samples were found to contain 0.003, 0.002, and 0.004% ethyl butyrate, respectively. Analysis of individual samples of refined olive, corn, cottonseed, peanut, and soybean oil indicated the absence of ethyl butyrate.

Two samples of commercial flavoring materials were examined and found to contain 0.049 and 0.041% ethyl butyrate, respectively.

The gas chromatographic method will detect 0.001% or less of ethyl butyrate in vegetable oil. Sensitivity can be increased 10 times or more by using a hydrogen flame ionization detector, or by evaporating the sample extract to a volume of less than the 0.5 ml specified above. Caution must be exercised when evaporating the methylene chloride extract to prevent evaporation to dryness.

Components can be further identified by odor as they emerge from the thermal conductivity cell. The characteristic odor of ethyl butyrate was detected successfully in several instances.

The following column substrates were also used successfully as 20% coatings on acid-washed Chromosorb W: Carbowax 20M, butanediol succinate polyester, sucrose diacetate hexaisobutyrate, and silicone grease DC-11.

### Recommendation

It is recommended that the study of artificial flavors in blended oils be continued.

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This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and Subcommittee C and accepted by the Association. See *This Journal*, 47, 128 (1964).

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## PLANTS

# A Comparison of the Quinoline Molybdate and the Volumetric Method for Total Phosphorus in Food and Food Products

By VOYCE P. WHITLEY (Division of Food Standards and Additives, Food and Drug Administration, Washington, D. C. 20204)

**The quinoline molybdate method for total phosphorus in food and food products was compared with the volumetric method for total phosphorus, 2.020. Values obtained by both methods were in good agreement. Small amounts of tin interfered with the phosphorus determination in fruits and juices; HBr added to the ash prior to HCl extraction yielded a tin-bromine compound that could easily be removed at low temperatures. Studies will be continued to determine the effect of acid concentration on the phosphorus recovery.**

**Table 1. Comparison of the official method for phosphorus, 2.020, with the quinoline molybdate gravimetric method**

| Sample | % P Method 2.020 | % P QM Method | % P Added | Total % P, Method 2.020 | Total % P, QM Method |
|---|---|---|---|---|---|
| Dried whole eggs | 0.77 | 0.74 | 0.46 | 1.21 | 1.22 |
| | 0.76 | 0.74 | | 1.22 | 1.24 |
| Dark rye flour | 0.52 | 0.51 | 0.46 | 1.02 | 0.90 |
| | 0.52 | 0.51 | | 1.02 | 0.92 |
| Ground poultry feed | 1.20 | 1.12 | 0.18 | 1.36 | 1.30 |
| | 1.19 | 1.10 | | 1.36 | 1.26 |
| Raisins | 0.129 | 0.128 | — | — | — |
| | 0.127 | 0.127 | | | |
| Enriched white bread (dried) | 0.19 | 0.18 | — | — | — |
| | 0.22 | 0.19 | | | |
| Nonfat dry milk | 1.07 | 1.00 | — | — | — |
| | 1.10 | 1.03 | | | |
| AAFCO Sample 4A | 2.92 | 2.88 | — | — | — |
| | 2.89 | 2.93 | | | |
| Tomato juice | 0.025 | 0.028 | — | — | — |
| | 0.024 | 0.028 | | | |

The quinoline molybdate method for the determination of total phosphorus was adopted as official, first action for fertilizers (*This Journal*, **45**, 118 (1962)). The application of this simple and straightforward method to other products, i.e., eggs, milk, fruit, salad dressing, feeds, and other similar substances, would result in one method rather than several.

The methods for phosphorus in *Official Methods of Analysis*, 9th Ed. (1960) refer to section **2.017**, gravimetric method, or section **2.020**, volumetric method.

This paper presents a comparison of results obtained by method **2.020** with those by the quinoline molybdate gravimetric test (*This Journal*, **45**, 201 (1962)).

## Phosphorus in Food and Food Products

### *Comparative Study*

For each substance analyzed the sample preparations used were those recommended for the individual class of substances in *Official Methods of Analysis*. In most sections on phosphorus, two techniques of sample preparation are given: the sample is digested in acid; or the sample is ashed and taken up in dilute acid. The ashing procedure was used in all experiments in this study. Results are given in Table 1.

During the course of this study, it was learned that small amounts of tin interfere with the determination of phosphorus, especially in canned fruits and fruit juices with high acid content.

To study this interference, cans of fruit and fruit juices that had been stored for 3 or 4 years were analyzed by the quinoline molybdate method and by method **2.020**. A literature search indicated that from 69 to 250 ppm of tin was often present in some canned products. Experiments were conducted on products containing known added amounts of tin. Results are given in Table 2.

This paper was presented as the report of the Associate Referee at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

**Table 2. % Phosphorus obtained by method 2.020 and the quinoline molybdate gravimetric method on sample with tin present**

| Description of Sample | Method 2.020 | QM Method |
|---|---|---|
| Animal feed 4A | 2.92 | 2.88 |
| | 2.89 | 2.93 |
| Animal feed 4A + 200 ppm $SN^{++}$ | 2.97 | [b] |
| | 2.97 | |
| Animal feed 4A + 200 ppm $SN^{++}$[a] | 2.99 | [b] |
| | 2.97 | |
| Grapefruit juice | 0.013 | 0.011 |
| | 0.013 | 0.017 |
| Grapefruit juice + 200 ppm $SN^{++}$ | 0.011 | 0.016 |
| | 0.011 | 0.016 |
| Grapefruit juice + 200 ppm $SN^{++}$[a] | 0.013 | 0.015 |
| | 0.012 | 0.016 |
| Tomato juice | 0.025 | 0.028 |
| | 0.024 | 0.028 |
| Tomato juice[a] | 0.023 | 0.028 |
| | 0.023 | 0.028 |
| Blackberries | 0.034 | 0.036 |
| | 0.032 | 0.036 |
| Blackberries[a] | 0.032 | Sample |
| | 0.031 | lost |

[a] 2.0 ml HBr added to ash.
[b] Feed Sample 4A had been previously compared in Table 1. The values obtained by method 2.020 indicated negligible interference from tin.

***Discussion***

The values obtained by the two methods were in good agreement for all substances used in this experiment.

To eliminate tin interference in phosphorus determinations, it was suggested that HBr be used to yield a tin-bromine compound that would be volatile and be removed at low temperatures (about 100°C). The HBr was added prior to taking up the ash in dilute HCl. The mixture was evaporated to dryness and redissolved in dilute acid. The procedure was then followed as prescribed in each method.

A white, gelatinous precipitate was observed in solutions that had been separated from the redissolved ash by filtration. This precipitate formed in low pH fruits and juices with and without added quantities of tin. The precipitate did not appear to interfere with the determinations. In the official method, **2.020**, the precipitate persisted through precipitation of the $P_2O_5$ but it is doubtful, from the values obtained, that it interfered with acid-base titration. The precipitate persisted in the quinoline molybdate test only to the point at which the molybdate-treated solution was boiled for the prescribed 3 minutes. At the end of this boiling time, a clear yellow solution was obtained.

The limited evidence does not show any interference due to tin. The proposed use of HBr should eliminate any likely interference.

**Recommendation**

Due to the limited time devoted to this study, the problem of acid concentration versus phosphorus yield was not studied. Thus, it is recommended that such a study be initiated and that a collaborative study be made of the method.

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were accepted by the Association. See *This Journal*, **47**, 119 (1964).

# Aluminum and Iron in Plants

By ELIZABETH I. LINDEN (Department of Biochemistry, Michigan State University, East Lansing, Mich.)

**A review of literature discloses numerous methods applicable to determination of Al and Fe in plants. A comparison of results for Fe by a colorimetric method using 2,2′-bipyridine as chelating agent with those by the colorimetric *o*-phenanthroline and the $TiCl_3$ titrimetric methods in *Official Methods of Analysis* showed that the 2,2′-bipyridine was simpler, less time-consuming, and equally as accurate as the *o*-phenanthroline procedure, and free of the Cu interference encountered in the $TiCl_3$ method. Further study is recommended for accurate and efficient methods for Al and Fe.**

As noted in the General Referee Report on Methods for Analyzing Plants in 1960 (1), a more up-to-date method is needed for Al, and a less time-consuming and equally reliable colorimetric method is needed for Fe to replace the *o*-phenanthroline method. The titanous titrimetric procedure for Fe is convenient but it is subject to interferences from cupric ions and possibly other cations with variable valences.

To confirm Cu interference in the evaluation of Fe by $TiCl_3$ titration, known concentrations of Fe, to which varying levels of Cu had been added, were titrated with dilute $TiCl_3$ solution. Results are given in Table 1. From the comparison of titration results, it is quite apparent that Cu titrates quantitatively along with the Fe. To be completely accurate, therefore, the Cu would have to be evaluated and the appropriate correction made in the Fe determined by the $TiCl_3$ titration.

A brief literature survey was made of methods used to determine Al and Fe to see which of these methods would meet the AOAC's critical standards of accuracy, freedom from interferences, ease of manipulation, availability of reagents, and general practicality. Many of the procedures were used to determine trace quantities of Al and Fe in other metals, alloys, ores, oil, dust fall samples, and so on. Whether such methods are adaptable to plant analysis remains to be decided.

## Literature Survey

### *Iron*

Many approaches have been used in the separation and evaluation of Fe. Reynolds and Monkman (2) described a method of wet-ashing, followed by color development with bathophenanthroline, and extraction into $CHCl_3$ and EtOH for spectrophotometric evaluation. Penner and Inman (3) used bathophenanthroline extracted into amyl alcohol; thiourea was used to mask Cu interference. Davis and Jacobson (4) determined Fe by spectrophotometric EDTA titration, with 5-sulfosalicylic acid as indicator at pH 1.0 to avoid Al interference. Jackson and Phillips (5) extracted Fe with isobutylmethyl ketone (hexone), and then removed Fe from the organic phase with a buffered solution of 1,10-phenanthroline for spectrophotometric determination. A mixed solvent (2:1, hexone-benzene) was used to overcome interference of Cu, Zn, Ni, Co, and Cr. Diehl and Smith (6) discussed in detail varied procedures for evaluating Fe in the ppb range by using bathophenanthroline and kindred iron-chelating agents. Michaelis, *et al.* (7) used Dowex cation and anion exchange resins and a variable HCl elution to separate Ni and Al from Fe, Co, and Mo. Fritz and Hedrick (8) quantitatively separated traces of Fe from $Cu^{++}$ and $Zn^{++}$ on a column packed with Haloport-F to which 2-octonone was sorbed with 6–8*M* HCl. Duswalt and Mellon (9) described a new Fe-chelate with 6-hydroxy-1,7-phenanthroline in 40% 1-propanol as well as a parallel procedure with 8-quinolinol. Khadem and Saad (10) used dinitroresorcinol as a complexing agent in colorimetric evaluation of Fe. Block and Morgan (11) used a fluorescent extinction method based on a reaction between Fe and fluorescent Al-Pontichrome Blue-Black R

**Table 1. Quantitative Cu interference in $TiCl_3$ titrimetric method for Fe**

| Fe, mg | Cu Added, mg | $TiCl_3$ Titrated, ml | Excess $TiCl_3$ Due to Added Cu, ml | |
|---|---|---|---|---|
| | | | Titrated | Calculated[a] |
| – | 2.0 | 2.87 | – | – |
| 2.0 | – | 3.34 | – | – |
| 2.0 | 0.5 | 4.06 | 0.72 | 0.72 |
| 2.0 | 1.0 | 4.78 | 1.44 | 1.44 |
| 2.0 | 1.5 | 5.48 | 2.14 | 2.16 |
| 2.0 | 2.0 | 6.31 | 2.97 | 2.88 |
| 2.0 | 3.0 | 7.75 | 4.41 | 4.32 |
| 4.0 | 0 | 6.66 | – | – |
| 4.0 | 1.0 | 7.96 | 1.30 | 1.44 |
| 4.0 | 2.0 | 9.60 | 2.94 | 2.88 |
| 4.0 | 3.0 | 11.18 | 4.52 | 4.32 |
| 4.0 | 4.0 | 12.53 | 5.87 | 5.76 |

[a]Based on initial titration, 2 mg Cu = 2.87 ml $TiCl_3$.

complex for a very sensitive determination of Fe. Agazzi, *et al.* (12) added sulfur to prevent loss of metals in ashing, and evaluated Fe with 1,10-phenanthroline.

***Aluminum***

Fewer methods for determining Al have appeared in recent literature. Jones and Thurman (13), analyzing soil, ash, and plant material, described modifications of a spectrophotometric determination of Al, using Eriochrome Cyanine RA. Riley and Williams (14) determined Al spectrophotometrically with 8-quinolinol in $CHCl_3$. A titrimetric method with Complexon III and benzidene as indicator was described by de Gomez (15). Freegarde and Allen (16) added excess EDTA to form an Al-EDTA complex, and back-titrated with Cu. Landi and Braicovich (17) recommended stilbazo for use in spectrophotometric determination of Al, with ascorbic acid to mask Fe.

A continuous determination of both Fe and Al by back-titration has been used. Liteanu and Crisan (18) determined Fe with 0.02*M* Complexon III in presence of sulfosalicylic acid at pH 1–2. To determine Al, pH was raised to > 5, and excess Complexon III was added, then back-titrated with 0.02*M* $FeCl_3$ in 0.1*M* HCl. Others used acetyl acetone or Cu-lan (19), or Eriochrome R (20) as indicators.

This is by no means a complete survey of all methods for determining Al and Fe described in recent literature, but rather is representative of procedures that may be adapted to plant analysis.

**Preliminary Studies**

In a preliminary study, the use of 2,2′-bipyridine as a chelating agent (21) seems quite well adapted for use in plant analysis, along with a simpler means of pH adjustment. In brief, the method under investigation is given below, though it may yet be further refined before presentation for collaborative study.

*Procedure.*—Ash samples by accepted wet or dry-ashing technique. Transfer sample to volumetric flask containing small square of litmus paper. Neutralize with concentrated $NH_4OH$, then add concentrated HCl until just acid, plus 2 drops in excess. Pipet aliquot into 25 ml volumetric flask containing sliver of congo red indicator paper. Add 2 ml hydroquinone solution (1% aqueous, made fresh weekly, kept in dark glass, and stored in refrigerator), and swirl. Add 3 ml 2,2′-bipyridine solution (0.1% in 5% acetic acid, kept in dark glass, and stored in refrigerator). Add Na citrate solution (25% aqueous solution) dropwise until indicator just turns red. Make to volume with Fe-free $H_2O$, mix thoroughly, and let stand 1 hour. Read absorbance of samples and blank in spectrophotometer at 525 mμ, or photoelec-

tric colorimeter with suitable filter, zeroed to $H_2O$. Evaluate from standard curve relating absorbance and concentration of Fe (0–60 μg).

*Discussion.*—The standard curve prepared by this procedure is both stable and reproducible, making it unnecessary to repeat a curve with each set of samples analyzed.

The sliver of congo red paper in aqueous solution does not interfere with the clarity of sample or standard solutions, and eliminates the need for carrying a set of samples in parallel purely to determine the amount of buffer needed for pH adjustment. This saves both time and reagents.

Color development is rapid enough so that if color is either too intense or too faint, a more suitable aliquot can be pipetted at once and be evaluated along with the other samples. Color is stable, permitting the analysis of many samples in a single run.

Volume of aliquot needed is small, so that one ashing may provide a volume sufficient for determining Fe, and also Zn, Cu, Mn, and Mo. No interference by Cu or Al has been detected.

The method is sensitive to 1 ppm. Its accuracy compares favorably with results by the *o*-phenanthroline method in the 9th edition of *Official Methods of Analysis*. Through a fortunate circumstance, a referee sample was discovered in storage here that had been submitted for a collaborative study of the *o*-phenanthroline method, reported in 1942 (22) and 1943 (23). This sample (an extract of spinach ash) was analyzed by this 2,2′-bipyridine procedure, and results agreed well with those in the original report. Other comparative studies are in progress.

To become familiar with the present method for determining Al, **6.006**, a number of samples were analyzed in the laboratory. No work has yet been done on new methods for determining Al.

### Recommendation

It is recommended that work be continued on development of more accurate and more efficient methods for determining Al and Fe.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee A and was accepted by the Association. See *This Journal*, **47**, 119 (1964).

This report of the Associate Referee was presented at the Seventy-seventh Annual Meeting of the Association of Official Agricultural Chemists, Oct. 14–17, 1963, at Washington, D.C.

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## DRUGS IN FEEDS

# Polarographic Method for Nitrofurazone and/or Furazolidone in Medicated Feeds

By FRED L. FRICKE, GEORGE E. KEPPEL, and SAMUEL M. HART (Food and Drug Administration, 1141 Central Parkway, Cincinnati, Ohio 45202)

**A polarographic method has been devised by which nitrofurazone and furazone can be determined in combination or alone in feeds. Either drug may be used as standard. In assay of mixtures, an aliquot of extract is polarographed to obtain the diffusion current of both drugs; a second aliquot containing a known amount of standard gives the diffusion current increase due to added standard. A third aliquot is treated with alkali to selectively destroy furazolidone. The pH is adjusted and a polarogram obtained to show the amount of furazolidone from the decrease in diffusion current. The supporting electrolyte is polarographed alone to determine net diffusion currents. Recoveries for the combined drugs in feed ranged from 93 to 108% for nitrofurazone and 87 to 99% for furazolidone.**

Colorimetric methods for nitrofurazone and furazolidone in medicated feeds (1) have given some difficulties in this laboratory. Results are often erratic and difficult to check.

It was suggested that a polarographic method be developed for use as a supplementary procedure. This seemed feasible, since nitrofurazone and furazolidone both contain a nitro group that should be readily reducible in the proper supporting electrolyte. Daftsios and Schall (2) reported results obtained polarographically on a number of nitro drugs commonly used in medicated feeds, including nitrofurazone and furazolidone, with a polarograph modified to give AC derivative curves. The drugs were dissolved in dimethylformamide (DMF) and the supporting electrolyte was an aqueous solution of tetramethylammonium bromide. They worked with the pure compounds, not with feed mixtures.

A polarographic method devised for nitrofurazone in medicated feeds (3) gave good results for some feed mixtures, but interfering substances were found to be present in others. The method has now been revised to eliminate the interferences and extended to include furazolidone and combinations of

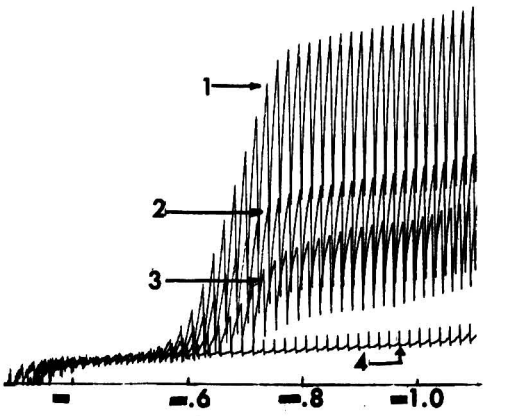


*Fig. 1*—Polarograms of: 1, sample plus added nitrofurazone standard; 2, sample containing nitrofurazone and furazolidone; 3, sample containing nitrofurazone after destruction of furazolidone; 4, supporting electrolyte (dimethylformamide saturated with ammonium chloride). Dropping mercury electrode versus saturated calomel electrode. Sensitivity: 0.015 microamps/mm.

nitrofurazone and furazolidone in medicated feeds. A preliminary extraction with diethyl ether removes interfering substances. The drugs are then extracted with dimethylformamide, and the extract is saturated with $NH_4Cl$ and polarographed. The preliminary ether extract removes some furazolidone, but it can be recovered by a shakeout technique.

Both nitrofurazone and furazolidone solutions, when polarographed, exhibit waves with very nearly the same half-wave potentials and identical diffusion currents for the

same concentrations. Thus, either drug may be used as a standard.

In assaying a mixture, an aliquot of the extract is polarographed to obtain the diffusion current of both nitrofurazone and furazolidone. A second aliquot containing a known amount of standard gives the diffusion current increase from the added standard. A third aliquot is treated with alkali to destroy the furazolidone selectively. After the pH is re-adjusted, the polarogram obtained shows the amount of furazolidone from the decrease in diffusion current. A polarogram of the supporting electrolyte alone is obtained to determine net diffusion currents. Figure 1 illustrates the various waves obtained.

If only one of the drugs is present, the procedure is the same, except that the alkali decomposition step is omitted (see Figs. 2 and 3).

### Nitrofurazone and Furazolidone

#### *Reagents*

(a) *Ethyl ether.*—Reagent grade.
(b) *Potassium hydroxide.*—Pellets.
(c) *Acetonitrile.*—Redistilled.
(d) *Nitrofurazone Reference Standard.*—NF.
(e) *Furazolidone Reference Standard.*—NF.

#### *Apparatus*

(a) *Polarograph.*—Sargent Model XV or equivalent; with "H" type cell, saturated calomel reference electrode.

(b) *Suction filtration apparatus.*—Such as Fisher Filtrator and funnel.

(c) *Shaking machine.*—International Bottle Shaker or equivalent.

#### *Determination*

Into a 250 ml centrifuge bottle, weigh 20.0 g well-mixed ground sample. If product is in pellet form, grind and reduce to 20-mesh or smaller before weighing sample.

To remove interfering substances, add about 100 ml ethyl ether to bottle, stopper, and shake for 3 minutes. Remove stopper carefully and brush adhering feed particles back into bottle. Centrifuge for 1 min.; decant ether carefully through 12.5 cm S&S No. 589 Blue Ribbon filter paper on a filtration apparatus, keeping ether level low in filter and retaining most of feed in bottle. Collect and save ether filtrate in an Erlenmeyer flask. Repeat ether extraction two or more times

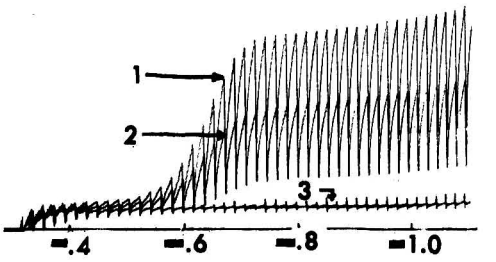


*Fig. 2*—Polarographs of: 1, sample plus added furazolidone standard; 2, sample containing furazolidone; 3, supporting electrolyte (dimethylformamide saturated with ammonium chloride). Dropping mercury electrode versus saturated calomel electrode. Sensitivity: 0.03 microamps/mm.

with 50 ml portions of ether, combining all filtered extracts. Final extract should be nearly colorless. Wash paper with ether and dry by suction. Remove ether remaining in centrifuge bottle and sample by evaporating and drying with stream of air over top of bottle. Transfer the dried filter paper to bottle and hold for dimethylformamide extraction.

To recover the furazolidone that has dissolved in the ethyl ether extract, partition between acetonitrile-petroleum ether as follows:

Evaporate combined ether extracts on steam bath just to dryness, using air current. Take up residue in 5 ml acetonitrile and transfer to 125 ml separatory funnel. Wash flask with five 5 ml portions of acetonitrile, transferring each to same funnel. Add 40 ml petroleum ether and shake. Drain lower layer into a second separatory funnel. If emulsion forms, drain it into second funnel and continue with the petroleum ether extraction, repeating five times with 40 ml portions of petroleum ether. Drain lower acetonitrile layer into beaker, and evaporate just to dryness on steam bath with current of air. Discard petroleum ether extracts.

Dissolve residue in dimethylformamide. Transfer to 100 ml volumetric flask, rinsing beaker and making to volume with dimethylformamide. Mix. Label as *Solution A*. This solution contains the furazolidone removed by the preliminary ether extract.

Pipet 100 ml dimethylformamide into the centrifuge bottle containing the ether-extracted feed and the filter paper. Stopper, and shake 20 min. on shaking machine. Filter (S&S No. 560 HF) and store filtrate in glass-stoppered flask. Label as *Solution B*.

Pipet 25 ml Solution A and 25 ml Solution B into glass-stoppered flask and mix. Label as *Solution X*. This mixture contains all of the nitrofurazone and furazolidone extracted from

the medicated feeds with interfering substances eliminated.

Pipet 15 ml Solution X into glass-stoppered flask. Add 0.1–0.2 mg nitrofurazone standard accurately weighed on a Cahn Electrobalance. Calculate standard concentration as mg/ml. Label as *Solution Y*.

Transfer about 20 ml Solution X to 100 ml beaker and determine its pH with pH meter. Add dropwise, with stirring, saturated alcoholic KOH, freshly prepared, until pH of 14 is obtained. Transfer to glass-stoppered graduated cylinder and shake 1 min. Solution should be a deep violet or purple. Let stand for 20 min., protected from light. Return to beaker and add dropwise a solution of acetic acid in dimethylformamide (45 + 5) until solution has returned to original pH. Solution should regain its original color. Transfer to glass-stoppered flask and label as *Solution Z*. This solution contains nitrofurazone in the same concentration as in Solution X, but the furazolidone has been destroyed by the alkali.

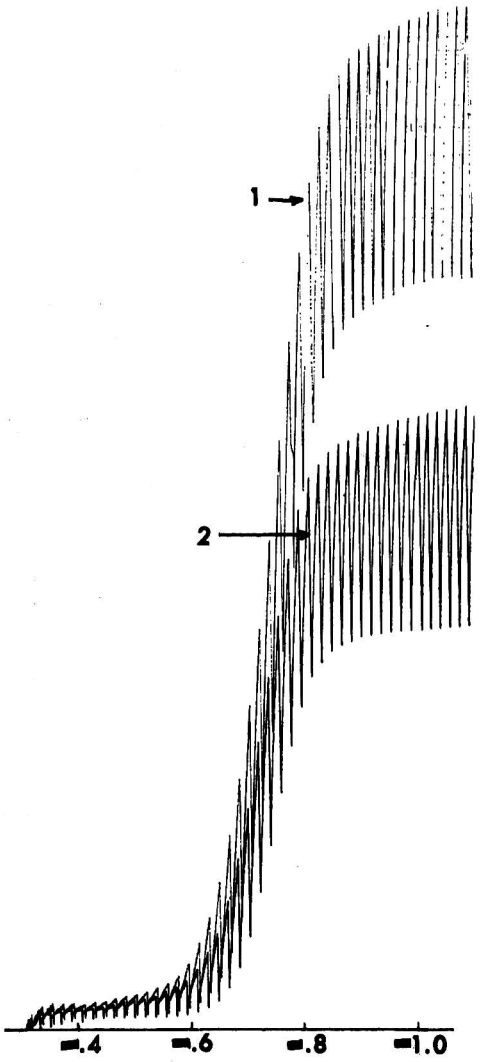


*Fig. 3*—Polarographs of: 1, sample plus nitrofurazone standard; 2, sample containing nitrofurazone. Supporting electrolyte: dimethylformamide saturated with ammonium chloride. Dropping mercury electrode versus saturated calomel electrode. Sensitivity: 0.03 microamps/mm.

Add about 0.5 g $NH_4Cl$ to Solutions X, Y, and Z, and shake for 1 min. Decant solution into polarograph cell, rinsing cell with solution. Deaerate 10 min. with nitrogen passed through DMF as a scrubbing solution. If other solutions have been previously used in scrubber, clean nitrogen tubing to remove any contaminants.

Obtain polarograms for: 1, Solution X (containing NFZ + FZ); 2, Solution Y (NFZ + FZ + standard NFZ); 3, Solution Z (NFZ only); 4, supporting electrolyte (DMF saturated with $NH_4Cl$). Range: 0 to −1.5 volts. To obtain diffusion currents, measure wave heights at −0.9 volts and multiply by sensitivity factor.

***Calculations***

% Furazolidone = concn NFZ std in mg per ml × Id(X − Z) × diln factor × 100/g sample × 1000 × Id(Y − X),

where Id(X − Z) = diffusion current of furazolidone in microamps = Wave heights in mm of Solution X − Solution Z × sensitivity factor;

Id(Y − X) = diffusion current of added standard nitrofurazone; and dilution factor = 200. (Sample was extracted with 100 ml solvent. Furazolidone recovered from ether extract was diluted to 100 ml. Solutions polarographed consisted of equal volumes from each of these two solutions, so that they represent the total amount of the drugs in 200 ml.)

% Nitrofurazone = concn NFZ std in mg per ml × Id(Z − SE) × diln factor × 100/g sample × 1000 × Id(Y − X),

where Id(Z − SE) = diffusion current of nitrofurazone present in sample, and Id(Y − X) = diffusion current of added standard nitrofurazone; dilution factor = 200

**Furazolidone Alone**

Follow the procedure for combination of furazolidone and nitrofurazone, with following changes:

1. Use 10.0 g sample,

2. Pipet 50 ml dimethylformamide, instead of 100 ml to make Solution A.
3. Make Solution B to 50 ml.
4. Mix 15 ml each of Solutions A and B for Solution X.
5. Omit destruction of furazolidone by alkali.
6. Use Furazolidone Reference standard to make Solution Y.

Obtain polarograms for: 1, Solution X; 2, Solution Y; 3, supporting electrolyte. Range: 0 to −1.5 volts. To obtain diffusion currents, measure wave heights at −0.9 volts and multiply by sensitivity factor.

***Calculations***

% Furazolidone = concn FZ std in mg per ml × Id(X − SE) × diln factor × 100/g sample × 1000 × Id(Y − X),

where Id(X − SE) = furazolidone diffusion current, and Id(Y − X) = diffusion current of added standard furazolidone; dilution factor = 100.

### Nitrofurazone Alone

Follow the procedure for the combination of furazolidone and nitrofurazone, with following changes:

1. Use 10.0 g sample.
2. Extract with 50 ml DMF Solution X.
3. Discard ether extract.
4. Take 15 ml aliquot from Solution X and add 0.1–0.2 mg NFZ standard, accurately weighed to make Solution Y.
5. Omit furazolidone destruction by alkali.

Obtain polarograms for: 1, Solution X; 2, Solution Y; 3, supporting electrolyte. Range: 0 to 1.5 volts. Measure wave heights at − 0.9 volts and multiply by sensitivity factor to obtain diffusion currents.

***Calculations***

% Nitrofurazone = concn NFZ std in mg per ml × Id(X − SE) × diln factor × 100/g sample × 1000 × Id(Y − X),

where Id(X − SE) = diffusion current of nitrofurazone present; Id(Y − X) = diffusion current of added NFZ standard; and dilution factor = 50.

### Discussion

Dimethylformamide, the solvent used in this method, is polar and has an alkaline reaction. It will contain small amounts of water extracted from the feed material so that small amounts of $NH_4Cl$ can be dissolved. If supporting electrolyte in the form of various aqueous solutions is added, the filtrate becomes cloudy and cannot be clarified by filtration or by immiscible solvent extraction. When solid $NH_4Cl$ is added, the filtrate remains clear. This method for adding supporting electrolyte by saturating the extract with $NH_4Cl$, although somewhat unconventional, appears to give the best results.

"Spiking" an aliquot of the extract with the standard is a variation of the well-known method of "standard addition." It will give best results because it compensates for variable factors that may affect results. Pure nitrofurazone and furazolidone exhibit waves with maxima. However, in the feed extracts, no maxima are evident; apparently there is an inherent maximum suppressor in the feed.

Interfering substances that were present in the feeds examined had half-wave potentials about the same as that for nitrofurazone. It was found, after much experimentation with various solvents and columns, that extraction with ethyl ether removed the interferences effectively. Identity of interferences is not known.

Time required for an analysis (not including grinding and preparing the samples) is approximately 2 hours.

Table 1 presents the results of determina-

**Table 1. Results for drugs containing nitrofurazone and/or furazolidone by polarographic method**

| Drug | Added, mg | Found, mg | Recovery, % |
|---|---|---|---|
| Nitrofurazone | 3.208 | 3.190 | 99.4 |
| | 0.760 | 0.758 | 99.7 |
| | 0.667 | 0.642 | 96.2 |
| Furazolidone | 0.804 | 0.769 | 95.6 |
| | 0.996 | 1.030 | 103.4 |
| | 1.285 | 1.265 | 98.4 |
| | 1.965 | 2.000 | 101.8 |
| | 1.488 | 1.510 | 101.5 |
| | 0.899 | 0.901 | 100.2 |
| Combination | | | |
| Nitrofurazone | 1.343 | 1.251 | 93.1 |
| Furazolidone | 0.528 | 0.521 | 98.7 |
| Nitrofurazone | 1.306 | 1.410 | 107.9 |
| Furazolidone[a] | 0.476 | 0.416 | 87.3 |

[a] The total decomposition of furazolidone takes 20 minutes. In this case the decomposition was stopped after 15 minutes, which may be the cause of the high recovery of nitrofurazone and the low recovery of furazolidone.

tions by analysts in this laboratory, using the above methods. Control samples of feeds without medication were obtained and ground to 20-mesh, varying amounts of drug were added, and these samples were then given to the analysts as unknowns for determination. The results obtained indicated that the per cent recoveries should be in the range of 95–100% for nitrofurazone, 95–102% for furazolidone; for the combination of both drugs, recoveries should be in the range of 93–108% for nitrofurazone and 87–99% for furazolidone.

Attempts will be made to apply the method to medicated feeds containing other nitro-derivative drugs.


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## BOOK REVIEWS

**Farm Chemicals Handbook.** Meister Publishing Company, 37841 Euclid Avenue, Willoughby, Ohio, 1964. 502 pp. Price $10.

The *Farm Chemicals Handbook* is published annually to keep up with the latest information on pesticides and fertilizers, and this year's edition is 130 pages larger than the previous edition.

It now has eight principal divisions, which include a directory of companies and plants; lists of organizations, associations, and State Control Officials; dictionaries of plant foods and pesticides; buyer's guide for farm chemicals; and an alphabetical list of farm chemicals manufacturers. Two comprehensive charts on spray compatibility and spray safety are special features and are easy to use. Advertisements by manufacturers and distributors provide additional product information. Advertisements and subject content are well indexed for quick reference.

This complete directory is very useful to manufacturers, buyers, distributors, and users of farm chemicals as well as to control and research groups. Production cost is high, and the price has been raised to $10 this year. It will be $12 for the 1965 edition. However, its utility appears well worth the price.

LUTHER G. ENSMINGER

**Organic Reactions.** Volume 13. Arthur C. Cope, Editor-in-Chief, John Wiley and Sons, Inc., New York, 1963. vii + 382 pp. Price $12.50.

This is the thirteenth volume in this series devoted to organic reactions. The chapters are comprehensive and critical reviews of important synthetic transformations of organic compounds, and each is authored by chemists who have had extensive working experience with the reaction surveyed. Each chapter includes sections on the history, mechanism, scope, and limitations of the reaction as well as an exhaustive tabular survey of applications which have been reported in the literature.

The chapters are as follows:

1. Hydration of Olefins, Dienes, and Acetylenes via Hydroboration. By George Zweifel and Herbert C. Brown; 54 pages, 70 references, 18 pages of tables.

2. Halocyclopropanes from Halocarbenes. By William E. Parham and Edward E. Schweizer; 36 pages, 140 references, 13 pages of tables.

3. Free Radical Additions to Olefins to Form Carbon-Carbon Bonds. By Cheves Walling and Earl S. Huyser; 59 pages, 160 references, 25 pages of tables.

4. Formation of Carbon-Hetero Atom Bonds by Free Radical Chain Additions to

Carbon-Carbon Multiple Bonds. By F. W. Stacey and J. F. Harris, Jr.; 227 pages, 631 references, 133 pages of tables.

The book also contains a cumulative index for Volumes 1 through 13 of authors and chapters. It is concisely and clearly written, and the printing, paper, and binding measure up to the usual high standards of the publisher.

LLEWELLYN H. WELSH

**Analytical Methods for Pesticides, Plant Growth Regulators, and Food Additives.** Vol. 1. Edited by Gunter Zweig. Academic Press, New York and London, 1963. 637 pp. Price $24.00.

This volume covers the principles, methods, and general applications of analytical methods for pesticides, plant growth regulators, and food additives. Twenty-three chapters have been prepared by 27 contributors.

The first seven chapters are largely devoted to principles of formulation and residue analysis, analysis of intentional and incidental food additives, extraction and cleanup procedures, and toxicological testing methods. Chapters 8 through 19 present general methods of analysis with examples selected from the field of pesticides and food additives. Various charts and tables are included. Chapters 20 through 23 give a general survey of residue analysis in the food, dairy, and meat industries, and analysis of both formulations and residues in government laboratories.

In all, this book presents a general picture of analytical methods for pesticide residues, formulations, and food additives. The principles of these analyses are covered together with the requirements of both industrial and government laboratories.

Because of the advance of methodology in this field, the chapters on general methods may require revision, for example, the chapter on gas chromatography. It is not current mainly because of the rapid advance of the electron capture detection technique in 1962 and 1963. Extraction and cleanup techniques have now advanced beyond those described in this book.

Each chapter includes a bibliography covering the literature prior to early 1962.

Succeeding volumes of this treatise will include specific methods for many materials: Insecticides (Volume II), Fungicides, Nematocides, Soil Fumigants, Rodenticides, and Food and Feed Additives (Volume III), and Herbicides (Volume IV).

JERRY A. BURKE

**Coffee Processing Technology.** Volume 1, Fruit-Green, Roast, and Soluble Coffee. By Michael Sivetz and H. Elliott Foote. xv + 588 pages. Price $17.25. Volume 2, Aromatization, Properties, Brewing, Decaffeination, Plant Design. By Michael Sivetz. xii + 379 pages. Price $12.50. Avi Publishing Co., Westport, Conn., 1963.

The authors are chemical engineers with a background in industrial food manufacture. The text reflects their professional status and experience, and deals to the greater extent with the techniques of preparing coffee and its products for the market and for consumption. Practices in coffee product preparation, with special emphasis on soluble coffee ("instant coffee"), are brought up-to-date. A present day concept of what coffee is and does, is reflected in the frontispiece, *A Sense of Value.* "Coffee is not bought or consumed for nutrition. It may be purchased by bag, pound or cup, but weight (the quantity measure) has value only insofar as it has acceptable flavor (the intensity measure). Coffee has only one value: to give the consumer pleasure and satisfaction through flavor, aroma, and desirable physiological and psychological effects." This book was written for coffee growers, roasters, and manufacturers; those with financial interest in the industry; manufacturers of coffee machinery; and consultants to the food and coffee industries.

The opening chapter, "History of Coffee," which is devoted principally to the past decade, explains the present status of coffee. The expansion of the production of Robusta coffee (the lowest quality commercial coffee)

and its place in the industry are discussed. In the 19th century, *Coffea arabia* was the variety grown on the island of Java to produce the then world-famous "Java coffee." Coffee tree diseases, especially coffee blight, depleted the trees on the island. When the culture of rubber trees was developed at the close of the century on Java, it was found necessary to shade the emergent tree. The Robusta coffee tree seed was planted with the rubber seed to afford the required shade. Because of its rapid growth, prolific yields, and hardiness, the Robusta tree was chosen to replace the fast-disappearing arabica trees. In the early 20th century Robusta coffee, grown for its coffee value alone in the favorable environment, became a commodity of relative importance and furnished competition to the lower-priced coffees from other localities. There were those who claimed it was not entitled to be recognized as coffee and others who wanted to trade in it as "Java coffee." The situation posed a problem in the enforcement of the Federal Food, Drug and Cosmetic Act. As a result of an investigation (Viehoever, A., and Lepper, H. A., *This Journal,* **5**, 274 (1921)), it was formally decided that Robusta was coffee but not of the identity known as "Java coffee." To this day Robusta has not taken its place as one of the more desirable coffees. In some markets of the world, price is its inducement.

Horticulture problems are discussed in a separate chapter.

Various facets of green coffee processing are presented and evaluated. A chapter is devoted to the details of removing the pulp from the fruit by the "natural" (dry) or "washed" (wet) processes. With either process the coffee must be dried; chapters give detailed discussions of sun-drying and machine drying. Discussion of green coffee preparation for the market is concluded in a chapter on "Hulling, Classification, Storage, Transportation, and Grading of Green Coffee." In "Green Coffee Processing at the Roasting Plant" the handling practices complete the story for coffee in the state customarily purchased by the consumer who brews his own.

The emphasis on soluble coffee begins with chapters on "Percolation: I. Equipment and II. Theory and Practice," and one each on "Spray Drying Equipment" and "Spray Drying Process." Volume 1 concludes with "Packaging of Roast and Soluble Coffee."

Those interested in the scientific aspects of coffee as a food product will find Volume 2 more rewarding. In the chapter on "Aromatizing Soluble Coffee" the recovery, retention, and storage without change of aroma and flavor are discussed. In the chapter on "Physiological and Psychological Effects of Coffee," cup testing for taste effect is described, and flavor factors in coffee evaluation are listed and defined. The volatile constituents that produce the aroma (aldehydes, ketones, heterocyclic and sulfur compounds, esters, nitriles, alcohols, and hydrocarbons) and the nonvolatiles (caffeine, chlorogenic and other acids, and phenols) are discussed. Factors that might be broadly classified as psychological are covered in a few paragraphs and no principles specific or inherent to coffee tasting are presented, other than those of recognized application to food tasting in general.

Densities, color, particle size, solubilities, and thermal properties are discussed under "Physical Properties of Coffee." The chapter also deals with rapid moisture measurement and the important factors affecting a physically clean instant coffee brew. "Chemical Properties of Coffee" is a limited review of composition studies. The identified constituents of essence of coffee aroma are listed. Coffee oil is given more extended coverage as to constants, fatty acids, and its preparation from the bean. Uniform quality is an important requirement for successful marketing of instant coffee. Process control rests principally on taste tests, a subject included in the chapter on "Control of Soluble Coffee Processing and Product Quality." The chapter on "Special Aspects of Coffee Processing," describes and discusses decaffeination and brew preparation in the home, restaurant, or by vending machine. The espresso method, coffee flavor, its substitutes and imitations for food and beverage use, and creme de café are additional subjects dealt with.

The concluding chapter, "Soluble Coffee Plant Design," confirms the impression given by the work, as a whole, that soluble coffee

is a product of vital concern to the future of the industry.

To those who have followed soluble coffee from its inception, this work explains some facets of present-day acceptance and popularity of the product. "Instant coffee is a convenience food compared with brewed roast coffee. It minimizes consumer attention to preparation, eliminates cleaning after preparation, reduces product waste, and does away with investment in and maintenance of a brewing device. Some instant coffees do not become stale as do roasted and ground coffee. The fact that a cup of instant coffee is about half the price of brewed coffee is also an important factor of public acceptance." Under a discussion of economic considerations it is stated that "the cost ratio of brewed to instant coffee is 1.7 to 1.1 cents per cup." Since soluble coffee is the dried soluble solids of coffee brew, it is difficult to appreciate how the additional cost of manufacture involved could result in lower cost with cups of comparable quality.

The authors point out that definite improvements in techniques of production have resulted in better quality of present-day soluble coffee. Price does not appear to be the reason for increased popularity. They regard the present-day product as leaving much to be desired in that characteristic for which the discriminating purchaser buys coffee, inseparable from quality, namely aroma. Potentialities of improvement are proposed, the most important of which is aromatization, i.e., "preserving or restoring the characteristic aroma of roast coffee in the preparation of the water-soluble powder." The complexity of the coffee aroma problem suggests the development of a synthetic coffee flavor.

The authors state that an increasing number of plants for making soluble coffee are being constructed in the coffee-growing countries. Marginal quality green coffees can be purchased locally and inexpensively. When this marginal quality green coffee is processed, none of the poor appearance and little of the bad flavor remains. A very pleasant-flavored instant coffee can be produced in a properly designed, equipped, and operated plant.

The authors do not debate the use of otherwise unsalable coffee for the production of instant coffee, and what effect this practice may have on the development of a quality product. Neither do they appear to share the more important concern of the law enforcement officials for the legal problems that might arise from such practices.

This work brings the knowledge available on coffee up to date. The discussions are extensively supported by bibliographic and patent references including many as recent as 1963. It has numerous illustrations and is a substantial contribution to the literature on a specific food.

HENRY A. LEPPER

# ANNOUNCEMENTS

***Standing Committees:***

L. G. Ensminger has been appointed as a member of the Committee on Foreign Visitors.

W. V. Eisenberg has been appointed as co-chairman of the Joint Committee with the American Association of Cereal Chemists. K. L. Harris is a member of the Committee.

***Chlorinated Insecticides and Miticides:***

Jerry A. Burke, Food and Drug Administration, Washington 25, D.C. has been appointed as General Referee on Chlorinated Insecticides and Miticides to replace Paul A. Mills, of the same address.

***Maple Products:***

John C. Kissinger, Eastern Utilization Research and Development Division, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia 18, Pa., has been appointed as Associate Referee on Microbiological Methods to replace Aaron Wasserman, of the same address.

***Microbiological Methods:***

Bernard F. Surkiewicz, Food and Drug Administration, Washington 25, D.C., has been appointed as Associate Referee on Frozen Food Analysis.

Ezra P. Casman, Food and Drug Administration, Washington 25, D.C., has been appointed as Associate Referee on Staphylococcus Toxin in Food.

Edward F. Baer, Food and Drug Administration, Washington 25, D.C., has been appointed as Associate Referee on Staphylococcus in Food.

Paul L. Poelma, Food and Drug Administration, Washington 25, D.C., has been appointed as Associate Referee on Salmonella in Food.

Donald A. Kautter, Food and Drug Administration, Washington 25, D.C., has been appointed as Associate Referee on Anaerobes in Food.

Harry Korab, American Bottlers of Carbonated Beverages, 1128 Sixteenth Street, N.W., Washington, D.C., has been appointed as Associate Referee on Microbiological Methods for Carbonated Beverages.

Arnold C. Salinger, Maryland Department of Health, Baltimore 18, Md., has been appointed as Associate Referee on Bacteriological Methods for Crab Meat.

John Lanier, Food and Drug Administration, Atlanta 9, Ga., has been appointed as Associate Referee on Microbiological Methods for Nut Meats.

***Food Additives:***

David M. Takahashi, Food and Drug Administration, San Francisco, Calif., has been appointed as Associate Referee on Antioxidants in Foods, and resigned as Associate Referee on Antioxidants in Cereal Foods.

***Alcoholic Beverages:***

J. V. Spilman, Internal Revenue Service, 1114 Commerce St., Dallas, Tex., has resigned as Associate Referee on Methanol (Refractometric).

Paul A. Reeves, Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Philadelphia, Pa., has been appointed as Associate Referee on Alcohol (Immersion Refractometer).

***Extraneous Materials in Foods and Drugs:***

Frank R. Smith, Food and Drug Administration, Washington 25, D.C., has resigned as Associate Referee on Cacao Products.

John D. Wildman, Food and Drug Administration, Bureau of Scientific Research, Boston, Mass., has been appointed as Associate Referee on Mold Count Methods for Tomato Catsup, to replace V. S. Troy, Continental Can Co., 1350 W. 76th St., Chicago 20, Ill.

Alberto W. Vazquez, Food and Drug Administration, Washington 25, D.C., has been appointed as Associate Referee on Vegetable Products, to replace Frank R. Smith, of the same address.

***Preservatives and Artificial Sweeteners:***

George Schwartzman, Food and Drug Administration, Cincinnati, Ohio, has been appointed as General Referee on Preservatives and Artificial Sweeteners to replace E. M. Hoshall, Food and Drug Administration, Baltimore 2, Md.

***Feeds:***

W. W. Turner, EFCO Laboratories, Erly-Fat Livestock Feed Co., P.O. Box 3054, Tucson, Ariz., has been appointed as Associate Referee on Crude Fat or Ether Extract.

***Analytical Biology of Foods and Drugs:***

O'Dean L. Kurtz, San-Serv Associates, Baltimore, Md., has resigned as Associate Referee on Breakfast Cereals.

***Dairy Products:***

Sam H. Perlmutter, Food and Drug Administration, Washington 25, D.C., has been appointed as Associate Referee on Phosphatase Tests to replace J. E. Campbell, R. A. Taft Engineering Center, 4676 Columbia Pkwy., Cincinnati 26, Ohio.

***Antibiotics:***

John J. Mayernik, Chemical Division, Merck and Co., Inc., Rahway, N.J., has been appointed as Associate Referee on Penicillin in Animal Feed.

***Meat and Meat Products:***

H. G. Fugate, Meat Inspection Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md., has been appointed as Associate Referee on Identification of Meats by Serological Methods, to replace Ralph D. Barner, of the same address.

***Vitamins and Other Nutrients:***

T. K. Murray, Vitamin and Nutrition Section, Food and Drug Directorate, Ottawa, Ontario, Canada, has resigned as Associate Referee on Vitamin A in Margarine.

***Decomposition:***

James B. Hyndman, Food and Drug Administration, Dallas 4, Texas, has resigned as Associate Referee on Pyoverdine in Eggs.

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# CORRECTIONS

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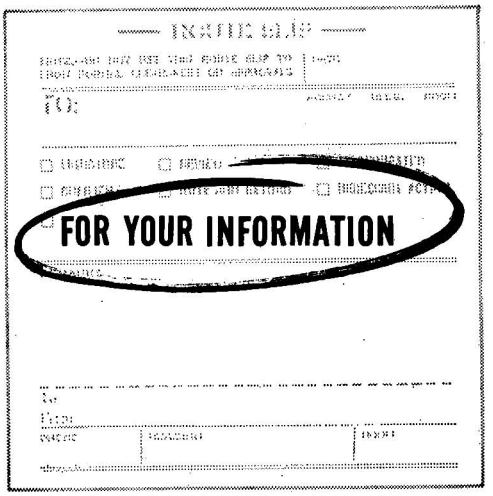
| *This Journal,* **46**, 893 (1963), "A Method for the Analysis of Chlorinated Benzenes in Clams (*Mercenaria mercenaria*) and Oysters (*Crassostrea virginica*)," by N. Schwartz, H. E. Gaffney, M. A. Schmutzer, and F. D. Stefano, p. 893, abstract | Line 6 should read: ". . . with 300 ml of the 1:1 mixture of *n*-hexane and isopropanol." |
| *Ibid.,* **47**, 129 (1964), "Report of Subcommittee A on Recommendations of Referees," under Fertilizers, p. 120 | Delete recommendation (20). |
| *Ibid.,* **47**, 293 (1964), "A Flame Ionization Detector Highly Selective and Sensitive to Phosphorus—A Sodium Thermionic Detector," by Laura Giuffrida, p. 295; p. 298 | Caption for Fig. 3 should read: ". . . *A* and *B* are repeat samples of the first eluant. *C* and *D* are repeat samples of the second eluant."<br>Figs. 5 and 6 should be reversed (the captions remain the same). |




***Toxicity of Household Substances***

The National Academy of Sciences—National Research Council has recently published a 29-page pamphlet entitled *Principles and Procedures for Evaluating the Toxicity of Household Substances,* as Publication 1138. The pamphlet was prepared by the Committee on Toxicology of NAS-NRC, under the chairmanship of Arnold J. Lehman, Food and Drug Administration. The chapters are titled as follows: I, Oral Ingestion; II, Evaluation of the Aspiration Hazards of Liquids; III, Percutaneous Absorption, Eye and Skin Irritation and Sensitization; IV, Inhalation. Price of the pamphlet is $1.50.

***WHO Expert Committees Issue Reports***

The World Health Organization Expert Committee on Addiction-Producing Drugs has issued its thirteenth report as No. 273 in WHO's Technical Report Series. The 20-page report is priced at $0.30.

The Expert Committee on Biological Standardization has issued its sixteenth report as No. 274 in the Technical Report Series. This report comprises 92 pages and is priced at $1.00. Contents include sections on antibiotics, hormones and enzymes, antigens, antibodies, biological diagnostic reagents, and international requirements for biological substances, plus a short index.

Both reports may be obtained from the World Health Organization, Geneva, Switzerland.

***Early Publication of Reports***

At the last meeting of the Editorial Board of AOAC, held in Washington, April 23, it was decided to expedite publication of Associate Referee reports that were received long in advance of the annual meeting. In the past, a report could not appear earlier than the corresponding reports of the General Referee and the Subcommittee, both of which contained the formal recommendations for action by the Association. To comply with this formality, reports received in the summer preceding the annual meeting could not be published earlier than the following February, and because of limited space in the *Journal,* some reports have not appeared until more than a year after they were submitted, even though publication had been approved much earlier.

Under the new system, as soon as a report is received it will be sent to the General Referee, who will make his recommendation and notify the Subcommittee and the AOAC office. The Subcommittee members may make their recommendations at any subsequent time, and will also notify the AOAC office. The report may be published at the earliest opportunity thereafter, with a footnote stating that recommendations await official action by the Association. Thus a report received in the spring or early summer may be published in October or December of the same year. By this means, the AOAC hopes to encourage early submission of reports.

***Journal of the Association of Public Analysts***

Contents for Volume 2, No. 1, of the Journal of the Association of Public Analysts, are as follows:

The Detection and Determination of Propionic Acid and Sorbic Acid in Bread and Flour Confectionery, by G. H. Walker, M. S. Green, and C. E. Fenn; New Foods and Drugs Legislation during 1963, by G. H. Walker; The Occurrence of Lead in Tea, by H. A. Williams; Radioactivity of Tea Samples; A Study of the Determination of *p*-Hydroxybenzoic Acid Esters in Food, by G. B. Thackray and A. Hewlett; Changes in Fats on Storage, by D. M. Freeland.

### *Dr. Randle Receives Award*

Dr. Stacy B. Randle, of Rutgers University, received the 1964 award of the Central Atlantic States Association of Food and Drug Officials for his service and contributions to the Association. The award consisted of a framed scroll and a $100 bond, presented at a luncheon during the Association's annual meeting in May.

Dr. Randle is a member of the Executive Committee of AOAC and chairman of Subcommittee E, has served on a number of committees, and has been Associate Referee on topics in the fields of feeds and fertilizers.

### *AOAC Cooperates with PAC*

Several AOAC members have been appointed to panels of the Pesticides Analysis Advisory Committee, Ministry of Agriculture, Fisheries and Food, England. The PAC works in conjunction with the Collaborative Pesticides Analytical Committee for adoption of collaborative methods.

Appointments are as follows:

*Dithiocarbamates Panel:* W. R. Bontoyan, U.S. Department of Agriculture, Beltsville, Md.

*Lindane Panel:* R. L. Caswell, U.S. Department of Agriculture, Beltsville, Md.

*Emulsifiability Panel:* E. E. Fleck, U.S. Department of Agriculture, Beltsville, Md.

*Herbicides Subcommittee:* R. F. Thomas, U.S. Department of Agriculture, Beltsville, Md.

*Monuron and Diuron Panel:* H. Hammond, State Laboratories Department, Bismarck, N. Dak.

### *Change in Name of Attapulgus Clay Company*

We have been notified that the Attapulgus Clay Company, listed in *Official Methods of Analysis*, 9th Ed., 1960, sec. **24.106(a)**, as the source of Attaclay, has changed its name to Minerals and Chemicals Philipp Corporation. The Corporation is located at Essex Turnpike, Menlo Park, N.J.

### *John B. Smith Wins Wiley Award*

John B. Smith, Rhode Island Agricultural Experiment Station, Kingston, Rhode Island, has been selected to receive the 1964 Harvey W. Wiley Award of the AOAC. The $500 award, which will be presented at the banquet during the Annual Meeting in October, was given to Professor Smith for his outstanding contributions to methods for fertilizers and other agricultural products.

In notifying Professor Smith of the award, Charles V. Marshall, Canada Department of Agriculture, who is President of the AOAC, said:

"The Award Committee was very impressed by the large number of publications you have made, especially in the development of analytical methods in agricultural chemistry, and by your long, faithful, and productive service to our Association. We are indebted to you for the methods developed for manganese and magnesium for feeds and fertilizers, and for your extended services as an officer and as a member of several committees."

Professor Smith began his professional career as an assistant chemist at the Massachusetts Agricultural Experiment Station in 1916. After serving in the U.S. Army Sanitary Corps during World War I, he returned to the Station in 1919, and then went to the Texas Agricultural Experiment Station in 1920. In 1923 he was appointed Associate Chemist at the Rhode Island Agricultural Experiment Station, was promoted to Chemist in 1927, and in 1941 was made Professor of Agricultural Chemistry and Department Head, positions he held until his recent retirement. At a testimonial dinner given in his honor, he was presented with the University of Rhode Island Award, and received a color TV set.

Professor Smith was born in Williamstown, Vermont, and received his professional education at Tufts College, Rhode Island State College, and Ohio State University. He was president of the AOAC in 1960, was Associate Referee on various topics in the field of fertilizers, and was a member of a number of committees.