Colorimetric Determination of Small Quantities of Methylenedioxyphenyl-Containing Pyrethrum Synergists

MURRAY S. BLUM
Department of Entomology, University of Illinois, Urbana, Ill.

The ability of methylenedioxyphenyl compounds to form colored complexes in the presence of gallic and sulfuric acids has been utilized in devising a quantitative test for pyrethrum synergists containing this group. The test provides a convenient method for studying the complex mode of action of such pyrethrum synergists as sesamin, *n*-propyl Isome, piperonyl butoxide, and piperonyl cyclonene.

PYRETHRUM SYNERGISTS, substances which activate pyrethrum, although not very toxic in themselves, have found wide use in the past several years. One of the first of these to be discovered was sesame oil (*2*), the active component of which was shown by Haller and coworkers (*6*) to be sesamin. These workers further showed that the methylenedioxy groups attached to the aromatic portions of the molecule were associated with synergistic activity (*5*). With this fundamental piece of information at their disposal, various workers began to synthesize new methylenedioxy compounds in a search for better pyrethrum synergists. This search has produced such excellent synergists as piperonyl cyclonene (*11*), *n*-propyl Isome (dipropyl ester of 5,6,7,8-tetrahydro-7-methylnaphtho[2,3]-1,3-dioxole-5,6-dicarboxylic acid) (*9*), piperonyl butoxide (*11*), and sulfoxide (*10*).

Research on the mechanism of action of these synergists has been extremely difficult because of the lack of sensitive and specific methods of assay. Jones and coworkers (*7*) described a method of assay for piperonyl butoxide, but this method is specific for this and closely related compounds and for no other methylenedioxy-containing synergists. Labat (*8*) described a qualitative color test for methylenedioxyphenyl compounds which is simple to perform and very sensitive. In the presence of concentrated gallic and sulfuric acids, methylenedioxyphenyl compounds produce a green color which immediately changes to blue when the solution is heated. This reaction was found to be applicable to all pyrethrum synergists containing methylenedioxyphenyl groups. By modifying Labat's procedure, it has been possible to obtain a green complex which is stable for several hours and which follows Beer's law. The method is sufficiently sensitive to make possible studies on the metabolism of pyrethrum synergists in insects.

Method

Apparatus A 25- or 50-ml. buret. A photoelectric colorimeter or Beckman DU spectrophotometer.

Analytical Procedure

Reagents. Gallic Acid. Several samples of gallic acid were tried, all of which gave good results. Different grades of gallic acid (pure, reagent, or U.S.P.) which were recrystallized three times from hot water were sufficiently pure for the test.

Acetonitrile, redistilled.

Sulfuric acid, concentrated, C.P.

Procedure. Place 10 mg. of gallic acid in a dry 13 × 100 mm. culture tube and add 0.1 ml. of acetonitrile containing between 5 and 80 γ of the methylenedioxyphenyl-containing synergist, delivered by means of a blowout pipet. By means of a buret, add 3 ml. of sulfuric acid down the side of the tube, so that it layers. After 1 minute, thoroughly mix the acid and acetonitrile. Prepare a blank containing all the reagents except the synergist and treat as above. After 2 hours, read the unknown against the blank in the colorimeter or spectrophotometer at 660 mμ.

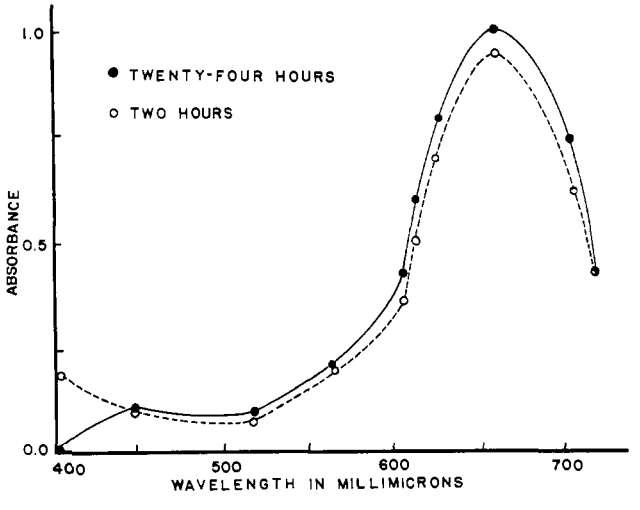
Preparation of Standard Curve Prepare solutions of the synergist dissolved in acetonitrile in concentrations of 5 to 80 mg. per 100 ml. Standard curves should be prepared whenever samples are analyzed.

After construction of the standard curve, the readings from the unknown solutions can be read directly off the curve.

Discussion

Reagent. Sulfuric Acid. The reaction rate varies with different samples of sulfuric acid. The sensitivity of the reaction appears to be unaffected by small amounts of atmospheric moisture, but large amounts of water inhibit the formation of the complex. One sample of acid produced a charring, but all other samples were found to be suitable. As Fairing and Warrington (*3*) point out, it is difficult to prepare sulfuric acid of an exact concentration and even more difficult to keep such acid at a constant concentration for any length of time. Therefore, standard curves should be run with each group of unknowns to compensate for any changes in the strength of the acid.

Figure 1. Absorption of complex after 2 and 24 hours



The color was fully developed in 5 minutes with some samples of acid, while others required up to 60 minutes. The color was found to be fully developed in 2 hours in all cases, and was fairly stable for 24 hours.

Acetonitrile. Acetonitrile was se-

lected as a solvent for several reasons. Labat used ethyl alcohol as a solvent for methylenedioxyphenyl compounds. This solvent reacted extremely vigorously with sulfuric acid, sometimes charring the methylenedioxyphenyl compound. Labat heated the reactants on a water bath, another procedure which often results in charring. Under the conditions cited in the paper, the heat that is produced on mixing the solvent and acid is rapidly dissipated through the solution and charring does not occur.

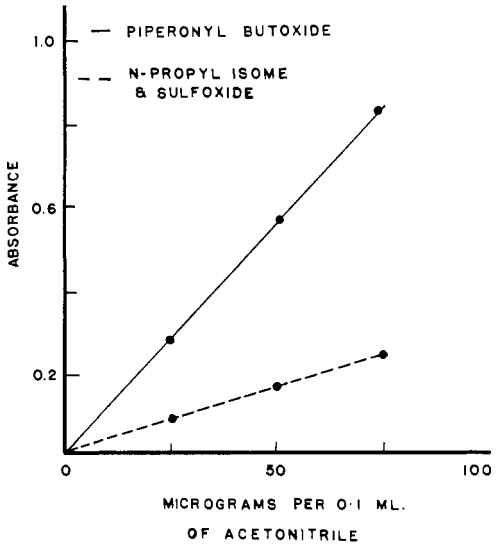


Figure 2. Calibration curves for piperonyl butoxide, *n*-propyl Isome, and sulfoxide (660 mμ)

Gallic Acid. As gallic acid is insoluble in acetonitrile, it is added in the dry form; 10 mg. was selected because of the convenience of weighing out this amount. An excess of the gallic acid does not interfere with this reaction. Tannic acid can also be used, but gives a less intense color.

Preparations of Solutions for Testing. The synergist is extracted from the material under consideration with a low boiling solvent such as hexane or low boiling petroleum ether. After the solvent has been removed, the residue is taken up in acetonitrile. If the fat or oil content is not too great, clear solutions can be obtained which yield quantitative results when measured against a blank prepared from an extract of the material from which the synergist was extracted. When the oil content is high, saponification is necessary. The method of Fairing and Warrington has been found suitable. Jones and coworkers describe several methods for removing interfering materials from extracts containing piperonyl butoxide.

Pyrethrum causes no interference when present at concentrations of 5 or 10 to 1, the standard ratios of synergist to pyrethrum.

Measurement of Complex. The absorption spectrum of the complex (piperonyl butoxide) was scanned in the Beckman DU spectrophotometer (Figure 1) 2 hours after its formation. Two peaks were found, a small one at 400 mμ and one at 660 mμ. When the complex was examined 24 hours later (Figure 1), the peak at 400 mμ had disappeared, while the peak at 660 mμ was slightly greater. As the complex had changed from green to blue, it is believed that the two initial peaks correspond to the yellow (400 mμ) and the blue (660 mμ) components of the spectrum of the green complex. The transition from green to blue probably involves extinction of the yellow component of the spectrum.

Standard curves for three methylenedioxyphenyl pyrethrum synergists were prepared. *n*-Propyl Isome, piperonyl butoxide, and sulfoxide were dissolved in acetonitrile and made up to different concentrations. Figure 2 presents the results of this experiment. When examined in the Beckman DU spectrophotometer at 660 mμ, all compounds produced complexes which obeyed Beer's law over a range from 5 to 80 mg. per 100 ml. At higher concentrations, a straight-line function is not followed.

It will be noted in Figure 2 that piperonyl butoxide forms a more intensely colored complex than either of the other two synergists. This is due to the fact that the butyl Carbitol side chain of the butoxide molecule contributes to the color of the complex. Castagnou and Quilichini (*1*) have shown that ethylenedioxy groups give the Labat reaction. Thus butoxide has a methylenedioxy group and two ethylenedioxy groups contributing to the colored complex. The other pyrethrum synergists produce a colored complex solely because of their methylenedioxy groups.

Concentration Range and Precision. As shown in Figure 2, the relationship between color intensity and concentration obeys Beer's law in the range from about 5 to 80 mg. of pure synergist per 100 ml. A deviation from a straight-line function occurs above 80 mg. per 100 ml.

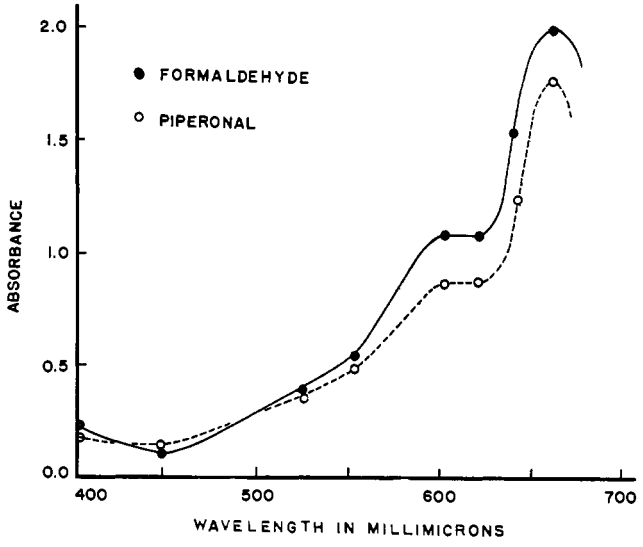
Working in the range from 15 to 80 mg. of synergist per 100 ml., the standard deviation of the results obtained with any group of samples was about ±4%. The precision of the method can be increased by using larger aliquots; however, charring occurs more frequently with larger aliquots when small amounts of tissue extract are present along with the synergist.

Specificity. Compounds containing methylenedioxy or ethylenedioxy groups give the test. Methylenedioxybenzene, piperonal, safrole, piperonyl butoxide, sulfoxide, sesamin, piperonyl cyclonene, *n*-propyl Isome, 3,4-methylenedioxycinnamic acid, and butyl Carbitol all form green complexes.

An analysis of technical samples of sulfoxide (88% pure) and piperonyl butoxide gave results similar to those obtained with the pure synergists. This must mean that the impurities present in the technical mixtures consist of closely related compounds forming the same colored complex. If the percentage purity of the technical material is known, the results can be corrected with reasonable accuracy.

Reaction Mechanism. The colored complex which is formed in this reaction is the product of the reaction of formaldehyde, produced by acid hydrolysis of the methylenedioxy ring, and the polyhydric phenol (gallic acid) which is present. This qualitative test, first described by Weber and Tollens (*12*), is the basis of the Gaebel test for methylenedioxy groups (*4*). Although phenols will react with formaldehyde in acid solution, a green colored complex is not formed by many phenols other than gallic acid. When the spectrum of the formaldehyde complex was examined in the Beckman DU spectrophotometer (Figure 3), it was found that the peak was the same as that in the complex formed by a methylenedioxy compound (Figure 3, piperonal), demonstrating the validity of the proposed reaction mechanism. The absence of a plateau in the spectrum formed with piperonyl butoxide (Figure 1) is possibly due to the fact that the butyl Carbitol side chain contributes to the color.

Figure 3. Absorption of complexes formed by formaldehyde and piperonal



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Literature Cited

(1) Castagnou, R., and Quilichini, R., *Bull. soc. pharm. Bordeaux*, **90,** 118 (1952).
(2) Eagleson, C., U. S. Patent **2,202,145** (May 28, 1940).
(3) Fairing, J. D., and Warrington, H. P., Jr., *Advances in Chem. Ser.*, No. **1,** 260 (1950).
(4) Gaebel, G. O., *Arch. Pharm.*, **248,** 225 (1910).
(5) Haller, H. L., LaForge, F. B., and Sullivan, W. N., *J. Org. Chem.*, **7,** 185 (1942)
(6) Haller, H. L., McGovran, E. R., Goodhue, L. D., and Sullivan, W. N., *Ibid.*, **7,** 183 (1942).
(7) Jones, H. A., Ackermann, H. J., Webster, M. E., *J. Assoc. Offic. Agr. Chemists*, **35,** 771 (1952).
(8) Labat, J. A., *Bull. soc. chim. biol.*, **15,** 1344 (1933).
(9) Synerholm, M. E., and Hartzell, A., *Contrib. Boyce Thompson Inst.*, **14,** 79 (1945).
(10) Synerholm, M. E., Hartzell, A., and Cullmann, V., *Ibid.*, **15,** 35 (1947).
(11) Wachs, H., *Science*, **105,** 530 (1947).
(12) Weber, K., and Tollens, B., *Ann.*, **299,** 316 (1898).

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ANTICOAGULANT RODENTICIDE

Toxicity and Antidotal Studies on 2-Pivalyl-1,3-indandione (Pival), an Anticoagulant Rodenticide

J. R. BEAUREGARD, T. W. TUSING, and R. F. HANZAL

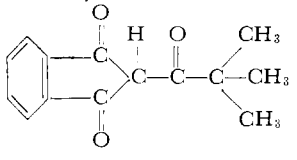
Hazleton Laboratories, Falls Church, Va.

The anticoagulant properties of 2-pivalyl-1,3-indandione and the use of vitamin K preparations as antidotes for poisoning in dogs and secondary toxicity in cats have been investigated. 2-Pivalyl-1,3-indandione is a much more effective poison in small daily dosages than in single large doses. Vitamin K_1 is a more effective intravenous antidote than vitamin K. Under the conditions of this study, secondary poisoning of cats does not appear to be a significant hazard.

RAPID ACTING, single-dose poisons used to eradicate commensal rodents have always presented a serious problem in public health. The discovery of the feasibility of using anticoagulants as rodenticides has eliminated some of these hazards. The early work has been reviewed by several recent authors (*2–4, 14, 17*).

Anticoagulants, as the name implies, elicit changes in the body which reduce or prevent clotting of the blood. The effect of these compounds on the blood clotting mechanism can be measured in many ways, including determination of the prothrombin time or the coagulation time. As the anticoagulant effects become more pronounced, the prothrombin and coagulation times will become longer. If there is no treatment to correct these alterations in the blood, fatal hemorrhages will occur. The use of anticoagulants such as some dicoumarin derivatives or 2-substituted 1,3-indandiones as rodenticides takes advantage of the above facts. To be effective, these materials are incorporated in cereal baits in very low concentrations (0.025%) and made easily accessible to the rodents. After several days of feeding, hemorrhages occur and the animals die a relatively painless death. In such low concentrations, and because death results only after several feedings, anticoagulant rodenticides are advantageous in reducing acute or single-dose hazards to humans or domestic animals.

2-Pivalyl-1,3-indandione (Pival, also referred to as *tert*-butylvalone) is a typical 2-substituted 1,3-indandione prepared by reaction of pinacolone with diethyl phthalate (*8*). This compound, a bright yellow crystalline material with a very slight odor, was first synthesized and patented by Kilgore about 1937 (*7*) as one of a series of 2-acyl-1,3-indandiones. It has the following chemical structure:



2-Acyl-1,3-indandiones are active as insecticides and the optimal insecticidal effectiveness is reached when there are five carbons in the acyl radical, as in Pival (*8*). These insecticidal properties are advantageous in a compound to be incorporated in cereal baits and left open to attack by cereal-destroying insects.

Data presented were obtained from limited toxicity studies, in dogs and cats, of Pival both as the pure material and as a 0.5% mixture in an inert medium.

Methods

Healthy mongrel dogs, male and female, were selected at random. The animals had been acclimated to the laboratory environment for approximately 2 weeks, had received inoculations of rabies vaccine and anticanine distemper and antiinfectious canine hepatitis serum, and had received a vermifuge. The investigations in dogs were separated into two phases: (1) the acute toxicity of Pival, and (2) the subacute toxicity of Pival and the effectiveness of vitamin K preparations in reversing the hemorrhagic tendencies caused by Pival.

Prothrombin and coagulation times were determined before Pival was administered and at frequent intervals thereafter. Prothrombin times were determined by a modification of Quick's method (*15*).

Blood was drawn by venipuncture, mixed in a 9 to 1 ratio with 0.1*M* sodium oxalate, and centrifuged for 10 minutes at approximately 2000 r.p.m. A test tube containing 0.2 ml. of Simplastin (brand of thromboplastin extract, Chilcott Laboratories) suspension and another tube containing plasma were then incubated at 37° C. in a water bath for 6 minutes. Next, 0.1 ml. of the plasma was added to the tube containing the Simplastin, and timing was begun. The mixture was slowly stirred with a small loop of No. 22 Nichrome wire until the