

acids other than methionine, cystine, and serine. An increase or decrease in the concentration of many amino acids in the white was accompanied by the opposite in the yolk, which indicates that proteins rich or poor in one amino acid were selectively transferred from the white to the yolk.

Acknowledgment

"Hot pressed cottonseed oil" containing 0.17% free fatty acid and about 0.1% gossypol was kindly furnished by the late W. E. Sewell, Chemical Division, The Procter and Gamble Co., Cincinnati, Ohio.

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ACARICIDE DETERMINATION

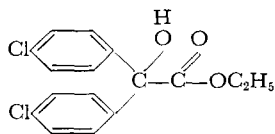
Microdetermination of the Acaricide Ethyl *p,p'*-Dichlorobenzilate (Chlorobenzilate)

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Two analytical methods are presented for the microdetermination of the acaricide ethyl *p,p'*-dichlorobenzilate (Chlorobenzilate) in the presence of citrus extractives. Both methods are based upon the hydrolysis of Chlorobenzilate in the extractive mixture to *p,p'*-dichlorobenzilic acid, which is then selectively oxidized to *p,p'*-dichlorobenzophenone. This ketone is determined either by its absorption at 264 $m\mu$ or by the absorption of its 2,4-dinitrophenylhydrazone in alcoholic alkali at 510 $m\mu$. Both methods are reproducibly sensitive to about 15 γ of Chlorobenzilate in admixture with 3 grams of citrus extractives. These procedures involve a two-stage cleanup of accessory extractives and are, therefore, very specific.

THE COMPOUND ethyl *p,p'*-dichlorobenzilate [2-hydroxy-2,2-bis(4-chlorophenyl)ethyl acetate, Chlorobenzilate, also known as Compound 338, Geigy 338, or G-23992] is a general acaricide against citrus mites and is proving useful in the control of the citrus bud mite, *Aceria sheldoni* (Ewing) (6). Carefully purified Chlorobenzilate possesses the following physical properties:



Molecular weight, 325.2
Boiling point, 141° at 0.1 mm.
Refractive index, n_D^{20} 1.5727.

A_s (95% ethyl alcohol). 661 at 266 $m\mu$, 17,940 at 225 $m\mu$.

A_s (2,2,4-trimethylpentane). 741 at 266 $m\mu$, 19,620 at 230 $m\mu$.

(See Figure 1 for detailed ultraviolet absorption characteristics.)

As with all new insecticides and acaricides, the magnitudes and locales of existing residues of Chlorobenzilate on and in citrus fruits must be established before commercial usage on citrus will be permitted. Two chemical methods are available for the microdetermination of Chlorobenzilate residues on and in foodstuffs: a nitration procedure proposed by Harris (5), and an adaptation of a total chlorine method suggested by Gunther and Blinn (3).

Extensive isolative procedures are required for successful use of the nitration

method in the presence of citrus extractives, while the total chlorine method is nonspecific for Chlorobenzilate.

Chlorobenzilate may be hydrolyzed in a homogeneous system to afford *p,p'*-dichlorobenzilic acid, which is easily oxidized quantitatively to *p,p'*-dichlorobenzophenone by chromic anhydride in glacial acetic acid. In this manner, the hydrolysis step eliminates all interfering substances not soluble in alkali; the oxidation of the alkali-soluble material provides further cleanup, in that only the resultant water-insoluble ketones persist through the remainder of the determination. The final desired ketone can be selectively determined by its absorption at 264 $m\mu$ or by the absorption of its 2,4-dinitrophenylhydrazone in alcoholic alkali (λ_{max} , 510 $m\mu$) if addi-

tional specificity is required by persisting substrate interference.

Reagents

All chemicals are analytical reagent grade, except where specifically indicated.

Potassium Hydroxide, 10% ethyl alcohol solution. Dissolve 10 grams of potassium hydroxide in 100 ml. of 95% ethyl alcohol, filter, and store in a brown bottle.

Petroleum Ether, boiling point 60° to 80°.

Light Petroleum Ether, boiling point 30° to 60°. Filter through a column of silica gel to eliminate aromatics.

Acetic Acid, glacial.

Chromic Anhydride.

Sodium Sulfate, anhydrous.

Cotton. Any good grade is satisfactory.

Ethyl Alcohol, 95%. Use a spectrophotometrically pure grade.

Methanol, aldehyde- and ketone-free. Reflux 500 ml. of methanol with 3 grams of 2,4-dinitrophenylhydrazine and 1 ml. of concentrated hydrochloric acid for 3 hours, then distill 450 ml. for use.

Methanol-Hydrogen Chloride Solution. Pass dry hydrogen chloride gas into aldehyde- and ketone-free methanol until it has absorbed 9 to 11 grams per 100 ml.

2,4-Dinitrophenylhydrazine Reagent. Prepare daily by dissolving 10 mg. of 2,4-dinitrophenylhydrazine per ml. in methanol-hydrogen chloride solution.

1,2-Dichloroethane (ethylene chloride, ethylene dichloride). Wash with concentrated sulfuric acid until the washings are colorless, then distill the first 90% for use.

Sodium Hydroxide, 1*N* solution.

Hydrochloric Acid, concentrated.

Potassium Hydroxide, 4% ethyl alcohol solution. Dissolve 4 grams of potassium hydroxide in 100 ml. of 95% ethyl alcohol, filter, and store in a brown bottle. Prepare at weekly intervals, so that a colorless solution is available.

Chlorobenzilate Standard. A standard sample of Chlorobenzilate was furnished by the Geigy Co., Inc., Bayonne, N. J., and was reported to be 100% pure by hydroxy and acetyl values. n_D^{20} 1.5727. It has not been possible to obtain crystalline Chlorobenzilate by the usual methods.

***p,p'*-Dichlorobenzophenone**, melting point 146.3–147.1°.

***p,p'*-Dichlorobenzophenone-2,4-dinitrophenylhydrazone**, melting point 242–244°.

Special Apparatus

Spectrophotometer. Any type that responds satisfactorily at 264 and 510 μ may be used. A Beckman Model DU was used for the ultraviolet method, and a Beckman Model B was used for the colorimetric method; absorption characteristics were determined on a Beckman Model DR recording attachment for the Model DU.

Kuderna-Danish Evaporative Concentrator (3, 4).

Stirred Oil Bath, heated to 65° \pm 1°.

Procedure

Hydrolysis of Chlorobenzilate. Place a measured volume of filtered petroleum ether (boiling point 60° to 80°) stripping solution in a 500-ml. § 24/40 Erlenmeyer flask. Add 5 ml. of 10% alcoholic potassium hydroxide solution and evaporatively concentrate on a steam bath through a three-ball Snyder column to about 5 ml. Remove the Snyder column and continue the evaporation on the steam bath with the aid of a jet of air until the residue has a gellike consistency. Add 2.0 ml. of water and 50 ml. of light petroleum ether and swirl gently to mix. Carefully remove most of the petroleum ether through a suction stick. Repeat this extraction procedure twice more with 50-ml. portions of petroleum ether, then remove the remaining petroleum ether from the aqueous extract by warming on the steam bath.

Oxidation of *p,p'*-Dichlorobenzilic Acid. Add 25 ml. of glacial acetic acid and 3 grams of chromic anhydride to the above aqueous residue in the flask and reflux for exactly 10 minutes. It is important that the oxidizing agent be present in excess. This can be determined by the lack of a green cast to the solution. Should the solution be green at this point, add 2 grams more of chromic anhydride and reflux an additional 10 minutes. After allowing the solution to cool to room temperature, transfer it into a 500-ml. separatory funnel, *A*, with 100 ml. of water followed by 75 ml. of light petroleum ether, then shake vigorously for 30 seconds. Draw the separated lower aqueous phase into a second 500-ml. separatory funnel, *B*, containing 25 ml. of light petroleum ether, then shake for 30 seconds. Discard the aqueous phase. Wash the petroleum ether extract in *A* with three 75-ml. portions of water, and use each aqueous phase to wash the petroleum ether extract in *B*. Then filter the washed petroleum ether solution in *A* through cotton and 1 gram of anhydrous sodium sulfate in a Gooch crucible holder into a Kuderna-Danish evaporative concentrator. Use the petroleum ether solution in *B* to rinse *A* and then filter as above into the same evaporative concentrator.

Use a 50-ml. portion of petroleum ether to rinse *B*, then *A*, and then as above into the evaporative concentrator. Completely remove the solvent from the ketone residue in the usual manner; removal of the last traces of solvent requires the aid of a gentle jet of dry air.

Ultraviolet Determination of *p,p'*-Dichlorobenzophenone. To the dry oxidation residue in the Kuderna-Danish receiving tube add 4 ml. of spectrophotometrically pure ethyl alcohol, and boil the solution gently until the volume is reduced to about 1 to 2 ml. to remove persisting traces of volatile aromatic substances by codistillation. Adjust the final volume to exactly 10 ml., and determine the transmittancy at 264 μ , setting the instrument with a control solution prepared from untreated fruit and subjected to the same analytical procedures as the sample. A standard calibration curve can be prepared from pure *p,p'*-dichlorobenzophenone (melting point 146.3–147.1°) but until interferences from new substrates have been evaluated, it is recommended that it be prepared from control extractives fortified with purified Chlorobenzilate.

Colorimetric Determination of *p,p'*-Dichlorobenzophenone. To the dry oxidation residue in the Kuderna-Danish receiving tube add 1.5 ml. of aldehyde- and ketone-free methanol and 1 ml. of 2,4-dinitrophenylhydrazine reagent, and place the tube in a stirred oil bath heated at 65° \pm 1°. After 5 minutes fit the tube with a generously greased glass stopper and leave in the oil bath for 3 hours. Remove the tube from the oil bath, add exactly 4 ml. of 1,2-dichloroethane, mix gently, then add 10 ml. of 1*N* sodium hydroxide solution and shake vigorously for 30 seconds. Remove the upper aqueous phase by means of a suction stick, add 5 ml. of concentrated hydrochloric acid, and shake vigorously for 15 seconds. This removes excess 2,4-dinitrophenylhydrazine. Again remove the upper aqueous phase, add 5 ml. of water, and mix thoroughly. Remove the upper aqueous phase by means of a suction stick as completely as possible, then pipet 2 ml. of the remaining 1,2-dichloroethane solution into a 25-ml. volumetric flask.

Table I. Solubility of Chlorobenzilate and Pertinent Derivatives in Various Solvents at 26°

	Compound, γ /Ml.				
	Petroleum Ether		95% ethyl alcohol	Methanol	1,2-Dichloroethane
	B.p. 30–60°	B.p. 60–80°			
Chlorobenzilate ^a	v.s. ^b	v.s.	v.s.	v.s.	v.s.
<i>p,p'</i> -Dichlorobenzilic acid	530	940
<i>p,p'</i> -Dichlorobenzophenone	3700	4980	4900	5400	...
2,4-Dinitrophenylhydrazone	Ca. 50	Ca. 50	1530

^a Ethyl *p,p'*-dichlorobenzilate.

^b Very soluble.

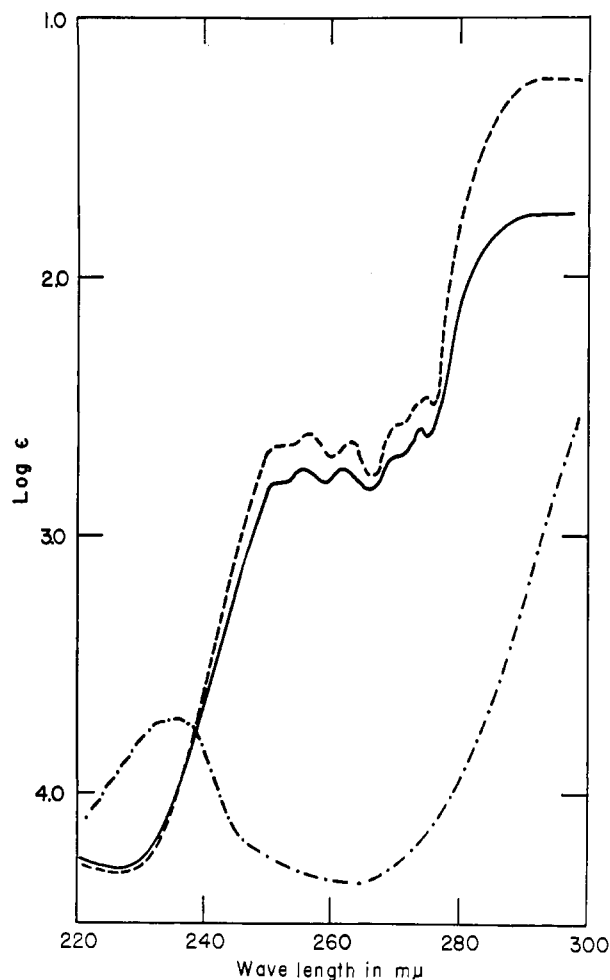


Figure 1. Ultraviolet absorption characteristics
 --- *p,p'*-Dichlorobenzilic acid
 - · - *p,p'*-Dichlorobenzophenone
 — Chlorobenzilate

Add exactly 1 ml. of 4% alcoholic potassium hydroxide solution and dilute to volume with 95% ethyl alcohol. Determine the transmittancy of the resulting red solution, which is stable for 24 hours, at 510 $m\mu$, setting the instrument with a control solution prepared from untreated fruit and subjected to the same analytical procedures as the sample. A standard curve can be prepared from purified *p,p'*-dichlorobenzophenone (melting point 146.3–147.1°) or directly from purified *p,p'*-dichlorobenzophenone-2,4-dinitrophenylhydrazone (melting point 242–244°), but until interferences from new substrates have been evaluated, it is recommended that it be prepared from control extractives fortified with purified Chlorobenzilate.

Comments on Procedures

Effects of Solvents. In Table I are presented the solubilities of Chlorobenzilate and certain pertinent derivatives in various solvents. Petroleum ether (boiling point 60° to 80°) was selected as the stripping solvent because of its nonpolar character, low boiling point and heat of vaporization, lack of interference, low

order of toxicity, and formation of quick-breaking emulsions during processing operations.

Hydrolysis Step.

The hydrolysis of Chlorobenzilate by 10% alcoholic potassium hydroxide solution was complete within the time required to remove the alcohol by evaporation. Thus, the alcoholic potassium hydroxide solution may be added directly to the stripping solution just before evaporating to dryness. For samples consisting solely of citrus oil, a 5- to 10-minute reflux period is suggested.

Excess ethyl alcohol must be largely removed from the hydrolysis solution prior to the oxidation. This was accomplished most advantageously immediately following the refluxing operation by use of an air jet. Although citrus extractives prevent the quantitative

removal of alcohol, there was not enough left to consume excess amounts of the oxidizing agent.

The amount of water used to dissolve the resulting potassium *p,p'*-dichlorobenzilate was critical. Less water resulted in an increased possibility of losses, whereas more water resulted in losses and emulsion trouble during the following oxidation step.

Oxidation Step. The two oxidizing agents evaluated were alkaline permanganate and chromic anhydride in glacial acetic acid. Alkaline permanganate resulted in interference, low recoveries, emulsions, and difficulties derived from manganese dioxide formation. Chromic anhydride, in contrast, proved ideally suited for this procedure.

The oxidation step resulted in the oxidation of the benzoic acid to the ketone and the oxidation of interfering material into easily removed products. With citrus oils, a 10-minute reflux period was required to achieve adequate oxidation. In most cases, 3 grams of chromic anhydride proved to be in excess of the amount required for complete oxidation. When the oxidizing agent was not in excess, serious interferences occurred

unless more oxidizing agent was added and reacted for an additional reflux period.

Light petroleum ether was selected as the extraction solvent at this stage because it minimized interference in the ultraviolet region.

Ultraviolet Determination. The ultraviolet absorption characteristics of Chlorobenzilate, *p,p'*-dichlorobenzilic acid, and *p,p'*-dichlorobenzophenone are reproduced in Figure 1. A calibration curve for the *p,p'*-dichlorobenzophenone prepared by the present procedure conforms to Beer's law at 264 $m\mu$ from 10 to 140 γ . Solutions also follow Beer's law and allow the determination of higher concentrations of Chlorobenzilate. The ultraviolet determination has an over-all efficiency of from 87 to 103% based upon recovery of pure *p,p'*-dichlorobenzophenone in the presence of citrus extractives (see Table II).

Interferences result from traces of aromatic substances in the light petroleum ether used to extract the *p,p'*-dichlorobenzophenone from the oxidation mixture, water not removed from the final ketone residue, and contamination of glassware. This latter consideration requires scrupulous care; it is recommended that glassware be soaked in sulfuric acid-dichromate cleaning solution, rinsed with water, acetone, and then water, and dried above 100°. Ground-glass surfaces should be lightly

Table II. Typical Recoveries of Chlorobenzilate from Citrus Extractives^a by Ultraviolet Method

Added, γ	Recovered ^b , γ	Recovery ^c , %
0	0	..
0	4	..
30	30	95
30	28	87
100	105	103
100	99	97
400	385	96
400	395	98

^a Either 3 grams of commercial oil of lemon or orange or total petroleum ether extractives from 1 pound of lemon or orange peel.

^b Solvent blank used as reference.

^c Corrected for control value (2 γ).

Table III. Typical Recoveries of Chlorobenzilate from Citrus Extractives^a by Colorimetric Method

Added, γ	Recovered ^b , γ	Recovery, %
0	0	..
0	0	..
30	24	80
30	24	80
100	94	94
100	80	80
400	338	85
400	372	93

^a Either 3 grams of commercial oil of lemon or orange or the total petroleum ether extractives from 1 pound of lemon or orange peel.

^b Solvent blank used as reference.

greased with a silicone-type lubricant, as other types may contain aromatics.

Colorimetric Determination. While the basic procedure of Claborn and Patterson (2) for the preparation of the 2,4-dinitrophenylhydrazone was followed some major modifications were required to attain consistent results. These authors were concerned with determining both *p,p'*-DDT and *o,p'*-DDT and recommended a 24-hour heating of the ketones with the 2,4-dinitrophenylhydrazine reagent to ensure reaction with the *o,p'*-ketone. As there is little if any *o,p'*-isomer in technical grade Chlorobenzilate (7), the shorter heating period was adopted. In this study, it was essential to wash the 1,2-dichloroethane solution of the 2,4-dinitrophenylhydrazone with concentrated hydrochloric acid and then with water to remove excess reagent and thus to avoid otherwise serious interferences. The final colored solution was diluted to 25 ml. instead of to 10 ml. as recommended by Claborn and Patterson to ensure the dissipation of any cloudiness due to moisture. The absorption characteristics of the final colored solution are shown in Figure 2; $A_s = 28,500$ at 510 $m\mu$. A calibration curve for the *p,p'*-dichlorobenzophenone-2,4-dinitrophenylhydrazone prepared by the present procedure conforms to Beer's law at 510 $m\mu$ from 15 to 200 γ without dilution. Higher values may be determined by dilution.

The over-all efficiency of the colorimetric determination, based upon the recovery of pure *p,p'*-dichlorobenzophenone-2,4-dinitrophenylhydrazone, and in the initial presence of citrus ex-

Figure 2. Absorption characteristics of *p,p'*-dichlorobenzophenone-2,4-dinitrophenylhydrazone in alkaline solution

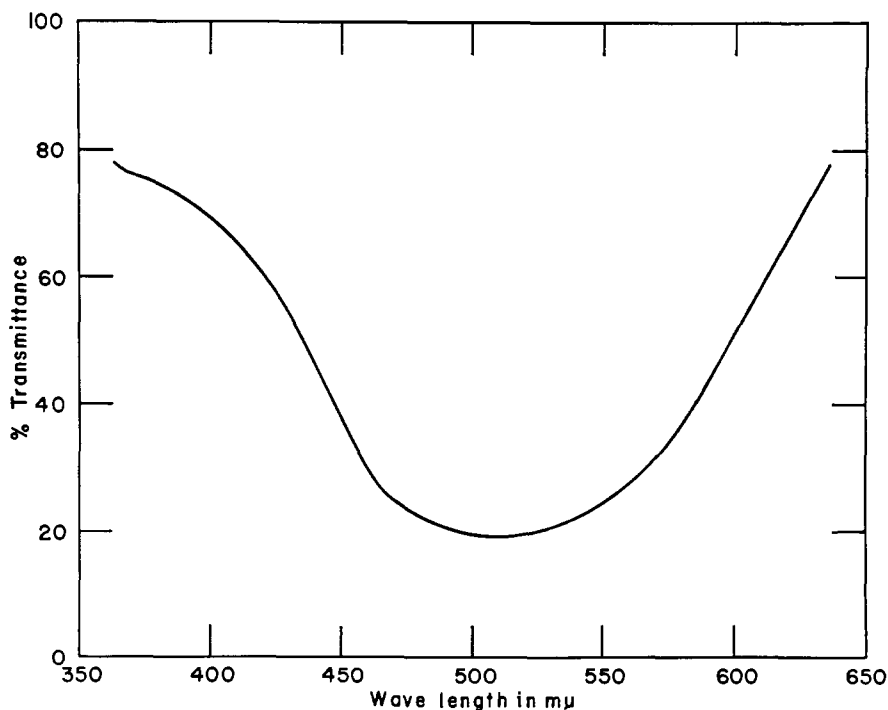


Table IV. Chlorobenzilate Residues on Lemons^a

Days after Treatment	Peel Residue, P.P.M. ^b		Pulp Residue, P.P.M. ^c	
	Ultraviolet	Colorimetric	Ultraviolet	Colorimetric
1	8.6	7.1	0.0	0.0
15	8.2	7.3	0.0	0.0
30	8.2	7.6	0.0	0.0
60	5.7	5.6	0.0	0.0

^a Sprayed with 1 pound of 25% wettable powder per 100 gallons at approximately 20 gallons per tree.

^b Based on fresh weight of part concerned.

^c Pulp refers to portion of fruit, including juice, remaining after peel is removed.

tractives, is from 80 to 94% (see Table III).

It is essential that all glassware used for this determination be free of acetone. Traces of acetone adhering to glassware used in preparing the final colored solution from the washed 1,2-dichloroethane extract and alcoholic potassium hydroxide result in a red color. This acetone can be eliminated by rinsing glassware with water and drying above 100°.

Field Data. To illustrate the magnitudes of residues encountered in current practice, some typical Chlorobenzilate residue determinations on lemons are shown in Table IV. The residues were determined by both the ultraviolet and the colorimetric methods for comparison.

Discussion

The ultraviolet method is simpler and faster, and possesses slightly better recovery and sensitivity. An added advantage in the use of the ultraviolet method is that the full spectrum may be plotted to aid in the characterization of the final product. Either of these pro-

cedures should be successful with most types of substrates encountered.

With the conversion of Chlorobenzilate to *p,p'*-dichlorobenzophenone, there is concurrently a two-stage cleanup of accessory substances. In order to exist and interfere in the final measurement steps, a substance must be made aqueous or alkaline-soluble by the hydrolysis step, and then converted to a water-insoluble substance by oxidation. It must exist as a ketonic substance to interfere with the colorimetric method or as one that absorbs radiation at 264 $m\mu$ to interfere with the ultraviolet method. Few if any of the currently used insecticidal or acaricidal materials will cause appreciable interference. However, many DDT-type compounds are converted in part to diphenylacetic acid derivatives by the hydrolysis procedure, and will therefore interfere in the final measuring steps.

Acknowledgment

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