Previous preparations of the titled compounds did not have the structures represented and were inactive as lures for the medfly *Ceratitis capitata* (Weidemann). The compounds now prepared have the proper structures and are very attractive to the medfly.

Table II. Number of Flies Attracted by *tert*-Butyl and *tert*-Pentyl Esters of 6-Methyl-3-cyclohexene-1-carboxylic Acid, Siglure, and Trimedlure

	Olfactometer Data (1)							
		Wick test[c]			Field Tests[a]			
Attractant	Trap test[b]	Fresh	1 Day	4 Days	7 Days	1st Wk.	2nd Wk.	3rd Wk.	Total
Siglure	1535	370	300	30	5	...	...	...	...
tert-Butyl ester	2362	480	430	5	0	3417	971	84	4472
tert-Pentyl ester	1820	425	330	150	100	2730	1472	2785	6987
Trimedlure	...	530	470	250	500	2986	2281	4462	9729

[a] Seven replicates of Steiner traps with 3 ml. of attractant on wick per trap. [b] Glass trap containing 50 ml. of 0.1% emulsion. [c] Wicks with 0.5 ml. of attractant.

The authors' attention was recently directed to a large difference between the boiling point of the *tert*-butyl ester of the series and that of the other butyl isomers (*4*). Furthermore, the refractive index of the *tert*-butyl ester appeared to be incorrect. Large differences were also noted between the physical constants of the *tert*-pentyl ester and its isomers (boiling point and refractive index). An infrared spectrum of these two preparations, which were still available, disclosed that they were not the compounds supposed. Neither of the preparations was attractive.

The compounds were synthesized by reacting the acid chloride with the appropriate alcohol in the presence of pyridine. The physical and chemical data are given in Table I.

The new constants agree with isomeric data, and the infrared spectra are consistent with the proper structures. Furthermore, the new compounds are highly attractive; they exceed the attractiveness of siglure in olfactometer trap and wick tests (*1*), the *tert*-pentyl ester being the more attractive of the two. In field tests, however, the *tert*-pentyl ester was not as attractive as trimedlure [*tert*-butyl *trans*-4(or 5)-chloro-2-methylcyclohexanecarboxylate] (*2*), the hydrochlorinated analog of the *tert*-butyl ester of the siglure series. Trimedlure is the best lure found thus far for the Mediterranean fruit fly.

The most attractive esters of the *trans*-4(or 5)-chloro-2-methylcyclohexanecarboxylic acid (trimedlure series) possess an alcohol moiety with branching in the 1 position (*2*)—i.e., isopropyl, *tert*-pentyl, *sec*-butyl, *tert*-butyl. Now that the two new esters have been shown to be attractive, this statement may be broadened to include the esters of 6-methyl-3-cyclohexene-1-carboxylic acid (siglure series).

Biological data are summarized in Table II. Field tests were run in coffee-growing areas on the southwest coast of Hawaii at an altitude of approximately 1500 feet where the temperature range was 52° to 82° F.

Literature Cited

(1) Beroza, M., Green, N., "Materials Tested as Insect Attractants," U.S.D.A. Handbook No. 239, Agricultural Research Service, pp. 3–4, June 1963.
(2) Beroza, M., Green, N., Steiner, L. F., Miyashita, D. H., J. AGR. FOOD CHEM. **9,** 361 (1961).
(3) Gertler, S. I., Steiner, L. F., Mitchell, W. C., Barthel, W. F., *Ibid.*, **6,** 592 (1958).
(4) Wright, R. H., British Columbia Research Council, Vancouver, Canada, personal communication.

Received for review September 13, 1963. Accepted November 21, 1963.

DIGESTION IN INSECTS

Colorimetric Analysis of Chromic Oxide Used to Study Food Utilization by Phytophagous Insects

A. J. McGINNIS and ROBERT KASTING

Entomology and Chemistry Sections, Canada Agriculture Research Station, Lethbridge, Alberta, Canada

THE PERCENTAGE utilization of diets by animals can be calculated from measurements of food consumed and excreta passed. This procedure has been used with insects (*8, 13*), but is too cumbersome for routine measurement of food utilization. Another method that has been used extensively with higher animals employs an indigestible index compound that is incorporated into the diet. The percentage utilization is calculated from the concentrations of the index compound in the food and the excreta. Although iron oxide, barium sulfate, and lignin have been used as index compounds with higher animals, chromic oxide (Cr_2O_3) is used most extensively (*4, 6, 7, 12*). The index method using Cr_2O_3 would be of value for measuring food utilization by insects. However, the methods for analysis of Cr_2O_3 used with laboratory and farm animals are not sensitive enough for the small quantities that would be present in either food or excreta of individual insects. Therefore, to use Cr_2O_3 as the index compound for studies of food utilization by insects it was necessary to develop a suitable method of analysis. This paper describes a procedure which was satisfactory for the determination of Cr_2O_3 in food and excreta of phytophagous insects.

Materials and Methods

The method developed consists of a wet oxidation of Cr_2O_3 to $Cr_2O_7^{-2}$ (*2*), followed by colorimetric determination of the dichromate ion with diphenylcarbazide (*15*). Other methods, with and without modification, were

A method satisfactory for measuring 150 to 1200 μg. of chromic oxide—quantities which would be present in 5 to 20 mg. of either food or excreta when insects were fed diets containing about 4% chromic oxide—is described. The samples were digested with a perchloric acid–sulfuric acid–sodium molybdate mixture for 30 minutes. The cooled digest was diluted, an aliquot was treated with diphenylcarbazide, and absorbance was measured at 540 mμ. The method can also be used to measure other chromium compounds.

considered but did not appear promising for the authors' purposes (*3*, *14*). The procedure finally adopted is outlined below.

Reagents. Digestion Mixture (*2*). Dissolve 10 grams of sodium molybdate ($Na_2MoO_4.2H_2O$) in 150 ml. of distilled water; slowly add 150 ml. of concentrated sulfuric acid and cool the solution; slowly add with stirring 200 ml. of 70% perchloric acid.

Color-Developing Reagent (*15*). Diphenylcarbazide, 0.25% (w./v.), in 50% aqueous acetone prepared daily.

Procedure. Weigh a sample containing from 150 to 1200 μg. of Cr_2O_3 into a 100-ml. Kjeldahl flask. Add 10 ml. of the digestion mixture and heat for 30 minutes on a six-unit, electric micro-Kjeldahl digestion rack drawing 630 watts. (Within the first 15 minutes, the digest changes color from green to yellow or orange.) Remove from heat and cool to room temperature. Dilute the digest to 500 ml. with distilled water. Transfer 5.0 ml. of the diluted digest to a test tube (18 × 150 mm.) and add 4.5 ml. of 0.25*N* sulfuric acid. Add 0.5 ml. of diphenylcarbazide reagent with a hypodermic syringe (1-ml. capacity) so that the color-developing reagent is forcibly injected into the sample to provide immediate and thorough mixing. Allow at least 3 minutes for color development, and measure the absorbance at 540 mμ against a blank consisting of 9.5 ml. of 0.25*N* sulfuric acid and 0.5 ml. of diphenylcarbazide reagent.

Standard Curve. The sensitivity of the method requires that samples for a standard curve contain between 150 and 1200 μg. of Cr_2O_3. Because such small samples could not be weighed without difficulty, a 4% mixture of Cr_2O_3 in cellulose powder was prepared by weighing both components, after oven-drying at 120° C. for 2 hours, and mixing in a tumble mixer for 48 hours. Five- to 30-mg. quantities of this mixture were analyzed for Cr_2O_3 as outlined.

Materials Analyzed. Thatcher wheat sprouts (*10*) and pith from Golden Ball wheat stems (*9*) were lyophilized and ground to pass a 40-mesh screen, and each was tumble-mixed with appropriate quantities of Cr_2O_3. These mixtures were similar to two of the diets used in subsequent digestibility studies with insects. Feces from cutworms (*Agrotis orthogonia* Morr.) fed a diet containing equal parts by weight of wheat sprouts and cellulose powder were bulked, dried at 120° C. for 2 hours, ground to pass a 40-mesh screen, and tumble-mixed with Cr_2O_3. The mixture of cellulose powder (Whatman Standard Grade) and Cr_2O_3 was prepared by tumble-mixing for 24 to 48 hours. The Cr_2O_3 used in all mixtures was ground in a mortar to pass a 60-mesh screen.

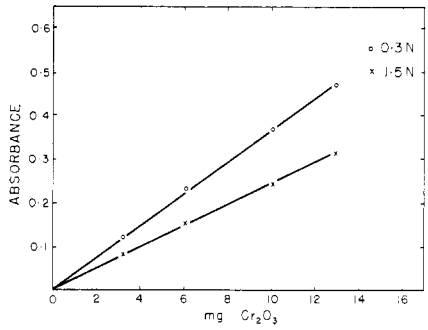

Figure 1. Effect of acid concentration on absorbance when diphenylcarbazide is added to diluted chromic oxide digests

Color developed with 0.5 ml. diluted digest + 9.0 ml. reagent blank + 0.5 ml. diphenylcarbazide; X—X reagent blank diluted to 100 ml. before use (1.5N), O—O same reagent blank diluted to 500 ml. before use (0.3N)

Results and Discussion

The chemical determination of Cr_2O_3 described here involves two steps, oxidation of the chromium to dichromate and development of color with diphenylcarbazide. Blundy (*1*) summarized some of the methods that have been used for oxidation of chromium and concluded that incomplete oxidation was the main cause of erratic and unreliable results. Completeness of oxidation may be affected by time and temperature. Studies here showed that varying the oxidation period from 20 to 40 minutes had little effect on the final value, and for convenience, a 30-minute oxidation period was adopted. When the reflux rate during digestion was too slow, oxidation as judged by color change did not occur; if the reflux rate was too rapid, oxidation occurred very quickly, but results were erratic and unreliable. The optimum rate permitted oxidation, as judged by the change in color, to occur in 10 to 15 minutes with only a small decrease in volume of digest.

Several factors that might affect development of the color with diphenylcarbazide were investigated. To obtain reliable results, the diphenylcarbazide reagent must be mixed rapidly with the acid digest. This was achieved by using a hypodermic syringe preset so that 0.5 ml. could be forcibly injected into the sample. Color intensity was not affected by varying the concentration of diphenylcarbazide from 0.20 to 0.30%. Likewise color intensity was not affected when the diphenylcarbazide reagent was prepared in either 40 or 60% aqueous acetone. Urone and Anders (*15*) reported that the diphenylcarbazide solution darkens on standing. The present authors found that standing for as long as 6 hours did not affect color development with dichromate.

The color developed with diphenylcarbazide was more intense in a diluted digest that had stood at room temperature for several hours than in the same digest immediately after dilution. Color intensity increased about 1% as a result of the digest standing for 2 hours after dilution. In the authors' laboratory, 36 samples were analyzed on the same day by one operator, and it took less than 2 hours after digestion to dilute and complete the analyses. Therefore, any effects of standing after dilution were negligible.

The intensity of color produced by the diphenylcarbazide was also affected by the concentration of acid in the solution to be analyzed (Figure 1). The intensity of color development was materially increased by decreasing the acid concentration to 0.3*N*. Czarnocki *et al.* (*5*) found that higher acid concentrations also decreased the absorbance of the dichromate ion.

In colorimetric analyses, it is not uncommon for the slopes of standard curves to vary slightly from day to day. Initially, in the Cr_2O_3 analyses, major variations were noted in the slopes of these curves. Nonetheless, quantitative recoveries of Cr_2O_3 were obtained. At that time, reagent blanks consisting of only the oxidation mixture, which was treated like other digests and diluted to 500 ml., were added instead of 0.25*N* sulfuric acid prior to color development. Figure 2 shows the variability of standard curves obtained with digests when diluted with different reagent blanks. Initially, 0.5 ml. of diluted standard digest and 9.0 ml. of diluted reagent blank constituted the sample in which color was

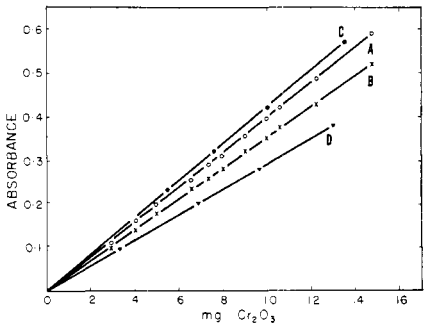

Figure 2. Effect of reagent blanks on diphenylcarbazide color of digests containing known quantities of chromic oxide

Curves *A* and *B* represent the same digests diluted with different reagent blanks; curves *C* and *D* are standard curves prepared with different digests and different reagent blanks; color developed with 0.5 ml. diluted digest + 9.0 ml. reagent blank + 0.5 ml. diphenylcarbozide; reagent blanks were diluted to 500 ml. before use

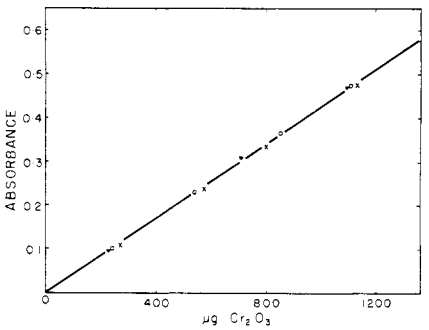

Figure 3. Three standard curves (X, O, Δ) obtained by the procedure finally adopted for chromic oxide analysis

Color developed with 5.0 ml. diluted digest + 4.5 ml. 0.25*N* sulfuric acid + 0.5 ml. diphenylcarbazide

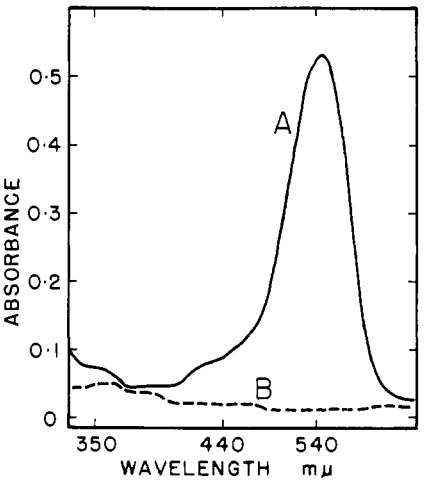

Figure 4. Absorption spectra of dichromate solution equivalent to 24 μg. per ml. of chromic oxide (Cr_2O_3) with (*A*) and without (*B*) addition of diphenylcarbazide and sulfuric acid

Table I. Quantitative Analysis of Different Chromium Compounds

Substance	Sample Weight, mg.	Recovery Weight, mg.	Recovery %	Mean
Cr metal	6.50	6.14	94.5	
	8.18	7.87	96.2	
	10.30	10.19	98.9	
				96.5
$CrCl_2$	10.52	10.36	98.5	
	21.22	21.44	101.0	
				99.8
$CrCl_3.6H_2O$	23.70	23.10	97.5	
	32.23	32.48	100.8	
	40.97	40.25	98.2	
				98.8
CrO_3	9.40	9.34	99.4	
	12.00	12.11	100.9	
	15.95	16.32	102.3	
				100.9
$Na_2Cr_2O_7 \cdot 2H_2O$	6.08	6.04	99.3	
	12.85	12.55	97.7	
	19.40	19.61	101.1	
	24.98	25.49	102.0	
				100.0

Table II. Quantitative Recovery of Chromic Oxide from Different Mixtures

Mixture	Sample Weight, mg.	Cr_2O_3 Added, μg.	Cr_2O_3 Recovered, μg.	Recovery, %	Mean
Cellulose powder: Cr_2O_3	7.1	284	284	100	
	11.2	448	460	103	
	15.6	623	636	102	
	29.2	1170	1184	101	
					102
Wheat sprouts: Cr_2O_3	5.3	265	264	100	
	9.5	475	464	98	
	10.0	500	485	97	
	13.8	690	672	97	
					98
Pith: Cr_2O_3	4.6	230	232	101	
	9.3	465	460	99	
	14.7	733	724	99	
	18.6	930	930	100	
					100
Excreta: Cr_2O_3	31.3	626	616	98	
	40.9	818	784	96	
	50.7	1014	1020	101	
					98

developed. For unknown samples appropriate volumes of diluted digest up to 9.5 ml. were used with correspondingly less of the diluted reagent blank. To avoid the variability caused by reagent blanks, 0.25*N* sulfuric acid was used to make the solution to the required 9.5-ml. volume. Moreover, it proved advisable to use the same volume of diluted digest from all standard and unknown samples for color development. These modifications allowed preparation of standard curves with reduced variability (Figure 3).

The absorption spectra for the diphenylcarbazide-dichromate complex (*A*) and for dichromate alone (*B*) are compared in Figure 4. Absorbance by the complex at the maximum (540 mμ) was about 10 times greater than that of the same solution at 350 mμ (*5*) without addition of sulfuric acid and diphenylcarbazide. This increase in sensitivity permits analysis of 5 to 20 mg. of either food or excreta of insects containing as little as 4% Cr_2O_3 on a dry-weight basis.

In some digestibility studies with higher animals (*11*), recoveries of Cr_2O_3 have been less than 100%. Possibly such losses occurred, at least in part, through biological oxidation or reduction to compounds not measured by the analytical methods. Thus, it was important to determine whether chromium in compounds other than Cr_2O_3 could be measured with the present method. Table I shows that the recovery of chromium from different compounds was satisfactory. In these analyses, the outlined procedure was modified to accommodate the larger quantities of chromium. After the standard and unknown digests were diluted, 0.5-ml. aliquots and 9.0 ml. of 0.25*N* sulfuric acid were added before the diphenylcarbazide reagent. Three to 15 mg. of Cr_2O_3 were used for the standard curves.

Typical results obtained by the method outlined when applied to mixtures containing Cr_2O_3 are shown in Table II. When the Cr_2O_3 content of the sample to be digested fell within the limits of the method (150 to 1200 μg.), the quantity of organic matter present did not affect precision. The results also show that the medium into which the Cr_2O_3 was incorporated did not affect either accuracy or precision of the method. For nutritional studies with insects, a con-

centration of 4% Cr_2O_3 in the diet was satisfactory since it was not toxic to feeding insects and did not appear to affect consumption (unpublished data). This concentration was high enough, however, to provide measurable quantities of Cr_2O_3 in insect food and excreta.

Acknowledgment

The technical assistance of A. G. Hewitt is gratefully acknowledged.

Literature Cited

(1) Blundy, P. D., *Analyst* **83,** 555 (1958).
(2) Bolin, D. W., King, R. P., Klosterman, E. W., *Science* **116,** 634 (1952).
(3) Brisson, G. J., *Can. J. Agr. Sci.* **36,** 210 (1956).
(4) Currier, A., Kitts, W. D., Cowan, I. McT., *Can. J. Zool.* **38,** 1109 (1960).
(5) Czarnocki, J., Sibbald, I. R., Evans, E. V., *Can. J. Animal Sci.* **41,** 167 (1961).
(6) Dansky, L. M., Hill, F. W., *J. Nutr.* **47,** 449 (1952).
(7) Hill, F. W., Anderson, D. L., *Ibid.*, **64,** 587 (1958).
(8) Kasting, R., McGinnis, A. J., *Can. J. Zool.* **37,** 713 (1959).
(9) McGinnis, A. J., Kasting, R., *Can. J. Plant Sci.* **41,** 469 (1961).
(10) McGinnis, A. J., Kasting, R., *Entomol. Exptl. Appl.* **5,** 313 (1962).
(11) Moore, J. H., *Brit. J. Nutr.* **11,** 273 (1957).
(12) *Ibid.*, **12,** 24 (1958).
(13) Smith, D. S., *Ann. Entomol. Soc. Am.* **52,** 674 (1959).
(14) Troelson, J. E., M.Sc. thesis, University of Saskatchewan, 1960.
(15) Urone, P. F., Anders, H. K., *Anal. Chem.* **22,** 1317 (1950).

Received for review July 1, 1963. Accepted September 18, 1963.

HERBICIDE RESIDUE IN SEAFOOD

Determination of Butoxyethanol Ester of 2,4-Dichlorophenoxyacetic Acid in Shellfish and Fish

J. E. COAKLEY,[1] J. E. CAMPBELL and E. F. McFARREN

Milk and Food Research, Robert A. Taft Sanitary Engineering Center, Public Health Service, Cincinnati, Ohio

In view of the extensive shellfish industry operating in the Chesapeake Bay, information on the uptake and retention of the butoxyethanol ester of 2,4-dichlorophenoxyacetic acid by commercially important species of shellfish became a prerequisite to the consideration of the widespread use of this herbicide to control Eurasian watermilfoil. A colorimetric method is described which showed that oysters and clams contained between 3.5 and 3.7 p.p.m. of esters when exposed for 3 days at the center of a 1-acre plot treated at a dosage rate of 30 pounds acid equivalent per acre. The method as verified by gas chromatography can be used to determine either the free acid or ester content of shellfish.

Because of the rapid invasion and explosive growth of Eurasian watermilfoil, *Myriophyllum spicatum*, in the Chesapeake Bay Region, joint field studies were conducted by the Natural Resources Institute of the University of Maryland, the Virginia Institute of Marine Science, the Maryland Game and Inland Fish Commission, and the U. S. Fish and Wildlife Service to find a method for controlling this aquatic plant. These studies revealed that esters of 2,4-dichlorophenoxyacetic acid (2,4-D) impregnated on attaclay granules were more effective than other phenoxy compounds for the control of Eurasian watermilfoil (*11*).

In view of the extensive shellfish industry operating in those waters, information concerning the uptake and retention of these esters by commercially important species of shellfish became a prerequisite to a consideration of the widespread use of this herbicide. Accordingly, a plot containing watermilfoil was experimentally treated with the butoxyethanol ester of 2,4-D at a dosage rate of 30 pounds of acid equivalent per acre, and oysters, clams, fish, and crab were held at the center of this plot. Three days after herbicide treatment, samples of each of the above-mentioned species were collected by the Chesapeake Biological Laboratory (*1*), Natural Resources Institute of the University of Maryland, Solomons, Md., and sent to the Robert A. Taft Sanitary Engineering Center for analysis. As a result, it became necessary to develop a method of analysis for 2,4-D and its esters in shellfish and fish.

Freed (*4*) found that a characteristic wine-purple color was produced when 2,4-D was heated in concentrated sulfuric acid with 4,5 dihydroxy-2,7-naphthalenedisulfonic acid (chromotropic acid). Marquardt and Luce (*8*) developed a quantitative colorimetric application of this reaction to the determination of 2,4-D in milk. In part, their method has been applied to the determination of 2,4-D in shellfish and fish.

The method described here for determining the butoxyethanol ester or 2,4-D content of shellfish and fish involves hydrolysis of the ester to 2,4-D by shaking with a dilute base, acidification and extraction of the 2,4-D with benzene, extraction of the benzene with buffer, extraction of the buffer with carbon tetrachloride, chromatography on Florisil (*9, 10*) to remove some of the fats and pigments, elution of the 2,4-D from the Florisil with methanol, evaporation of the methanol, reaction of the 2,4-D with chromotropic acid, and spectrophotometric measurement of the color.

Reagents

2,4-Dichlorophenoxyacetic acid (2,4-D) recrystallized from benzene (Matheson, Coleman and Bell). Dissolve 0.1000 gram of purified 2,4-D in absolute methanol and dilute to volume in a 100-ml. volumetric flask. Store in amber-colored bottle with an aluminum-lined screw cap. Prepare from this stock solution 2,4-D standards containing 5, 10, 20, 40, 60, and 80 μg. per ml.

Butoxyethanol ester of 2,4-D (Technical, Amchem Products, Inc., Ambler, Pa.). Dissolve 0.4000 gram of butoxyethanol ester of 2,4-D in methanol and dilute to volume in a 100-ml. volumetric flask. Prepare from this stock solution 2,4-D ester standards containing 1000, 2000, and 3000 μg. per ml.

Phosphate buffer, pH 6.7. Dissolve 25 grams of reagent-grade dibasic sodium phosphate and 10 grams of monobasic sodium phosphate in 1 liter of distilled water. Adjust to pH 6.7 by adding monobasic sodium phosphate.

4,5 - Dihydroxy - 2,7 - naphthalenedisulfonic acid, practical (chromotropic acid) (Matheson, Coleman and Bell).

[1] Present address: Food and Drug Administration, Washington 25, D. C.