eral, the Tedion content in the peel increases during the first 2 to 3 weeks after application and then remains relatively constant for at least 8 weeks. [This is in accord with the findings of Cassil and Fullmer that surface residues of Tedion "diminish largely as a result of fruit growth and not by decomposition" (*1*)]. Penetration into the pulp and juice in measurable amounts occurred in only two varieties and in these cases the amounts found were about 1% of the peel content.

Acknowledgment

The author is indebted to R. B. Johnson for supervising the application of the sprays.

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Table II. Tedion Residues[a], P.P.M., in Citrus Peel, Pulp-Juice, and Whole Fruits

(Whole fruit basis calculated from peel content and peel to whole fruit weight ratio)

	Date Sprayed								
	9-17-57	9-17-57	11-19-57	9-23-57	9-25-57	11-12-57	1-27-58	11-14-57	1-27-58
Days after Application	Pineapple Oranges	Hamlin Oranges	Valencia Oranges[a]	Temple Oranges	Marsh Grapefruit	Tangerines	Tangelos	Limes[b]	Lemons
				Peel					
0	0.3	0.4	0.0	0.3	0.1	0.3	0.3	0.1	0.3
3	0.5	0.5	0.2[c]	0.4	0.2	0.4[c]	...	...	...
7	0.8	0.9	0.3	0.7	0.4	1.2	0.6	0.4	0.6
14	1.0	1.4	0.4	1.0	0.6	1.9	...	...	...
21	1.5	1.3	0.5	1.0	0.4	1.7	0.8	0.8	0.9
28	1.0	1.3	0.4	0.7	...	1.8	...	0.9	...
35	1.0	1.3	0.4[d]	0.8	0.4	2.1	0.7	...	0.7
62	1.0	1.1	...	1.0	0.5	1.5[e]	1.1[e]	...	1.2[e]
90	...	...	0.4	1.1	0.5	1.1	1.2	...	1.0
112	1.0	1.0	0.4	0.8[f]	0.5[g]	0.7	...	...	...
				Pulp-Juices					
0	0.01	[h]	[h]	[h]	0.02	[h]	...	...	...
3	0.02	[h]	[h]	[h]	[h]	[h]	...	...	...
7	0.02	[h]	[h]	[h]	0.1	[h]	[h]	...	[h]
14	0.02	[h]	[h]	[h]	[h]	[h]	...	...	...
21	[h]	[h]	[h]	[h]	[h]	[h]	...	...	...
28	[h]	[h]	...	[h]	...	[h]	...	...	...
36	[h]	[h]	...	[h]	...		[h]	...	[h]
				Fruits					
0	0.05	0.1	0.0	0.05	0.03	0.08	0.05	0.02	0.1
3	0.1	0.1	0.05	0.1	0.05	0.1	...	...	...
7	0.2	0.2	0.1	0.1	0.1	0.3	0.1	0.1	0.2
14	0.2	0.3	0.1	0.2	0.2	0.5	...	...	...
21	0.3	0.3	0.1	0.1	0.2	0.4	0.1	0.2	0.4
28	0.2	0.3	0.2	0.1	0.1	0.4	...	0.2	...
35	0.2	0.3	0.1	0.2	0.1	0.5	0.1	...	0.3

[a] Valencias sprayed at the rate of 1/2 lb. Tedion 25% W.P./100 gal.; all others at 1 lb./100 gal. [b] Pulp-juice residues not determined; calculated on assumption of no Tedion penetration through peel. [c] 2 days. [d] 42 days. [e] 49 days. [f] 135 days. [g] 127 days. [h] Nil.

INSECTICIDE RESIDUES

Photofluorometric Method for Determination of Co-Ral Residues in Animal Tissues

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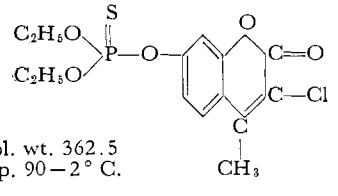
This work was carried out to develop a highly sensitive method for the determination of traces of Co-Ral and/or its oxygen analog in a wide variety of animal tissues. The method has a sensitivity of 0.02 p.p.m. based on a sample of 50 grams and a final solution volume of 5 ml. Cleanup procedures for meat and fat have been developed.

THE COMPOUND *O*-(3-chloro-4-methylumbelliferone) *O,O*-diethyl phosphorothioate (Bayer 21/199, more recently Co-Ral) has been registered recently in the United States and Canada for the control of cattle grubs, screwworms, horn flies, lice, and ticks. The compound possesses the following properties:



Mol. wt. 362.5
M.p. 90–2° C.

A survey of possible approaches to the determination of Co-Ral residues in animal tissues revealed several colorimetric methods for the estimation of low concentrations of coumarin derivatives in various types of biological material. In two cases, procedures of this type have been applied to Co-Ral (*1, 2*). However, in no case could the desired degree of sensitivity (0.01 to 0.02 p.p.m.) be obtained without the use of large samples of tissue and involved extraction and concentration procedures.

Fluorescence has been used extensively for the determination of trace amounts of various types of organic compounds—e.g., thiamine, riboflavin—in biological material (*11*). Although this method has not been used for the determination of pesticide residues, it has been used extensively for naturally occurring coumarins (*3, 4, 10*). Hornstein (*5*) has reported on the fluorescence of several pesticides.

Co-Ral is a highly fluorescent compound; however, its fluorescence is much less than that of 3-chloro-4-methyl-7-hydroxycoumarin. As this latter compound or a related coumarin derivative can be formed from Co-Ral by hydrolysis, the possibility of obtaining a very sensitive residue method presented itself.

A satisfactory pesticide residue method should determine residues of toxic metabolites as well as residues of the parent compound. In the case of Co-Ral, extensive metabolic studies (*8*) have shown that the only toxic metabolite present in animal tissues is the oxygen analog. The compound formed by opening the lactone ring has been identified in animal urine, but not in tissues (*8*). In developing the method described, provision has been made for inclusion in the results of any of the oxygen analog which may be present and the exclusion of hydrolysis products such as 3-chloro-4-methyl-7-hydroxycoumarin.

Co-Ral, its oxygen analog, and 3-chloro-4-methyl-7-hydroxycoumarin produce equivalent amounts of fluorescence on hydrolysis with 1*N* potassium hydroxide. The fluorescence spectra of the hydrolyzates and the infrared absorption spectra of the phosphate-free hydrolyzates were identical for each of the three compounds.

The fluorescent compound which has an acid equivalent corresponding to that of 3-methyl-7-hydroxycoumarilic acid was purified by repeated recrystallization from water. Other workers in this laboratory (*6*) have shown that the chlorine is removed from Co-Ral on alkaline hydrolysis. Work on the further identification of the fluorescent material is continuing.

By using the fluorescence principle, a sensitive method for determination of Co-Ral residues in animal tissues has been developed. Meat samples are extracted by grinding successively with acetone and benzene. On combination of the extracts, an aqueous phase separates, which is discarded. On addition of chloroform to the resulting solution, additional water and insoluble material separate and are removed by filtration. After evaporation to dryness, fat is separated from the desired constituents by extraction of a Skellysolve B solution of the residue with acetonitrile. 3-Chloro-4-methyl-7-hydroxycoumarin is removed by passage of a chloroform solution of the extractives through acid-washed alumina. Co-Ral and its oxygen analog are not retained by the column. After evaporation of the column effluent, the residue is hydrolyzed with aqueous potassium hydroxide and after extraction with amyl alcohol to remove interfering fluorescent materials, fluorescence is determined.

Procedure

Reagents. All chemicals are reagent grade unless otherwise specified.

Alumina, chromatographic grade, acid-washed (Merck). Co-Ral, standard solution (chloroform solution of Co-Ral containing 2.5 γ per ml.).

All solvents should be redistilled in an all-glass still.

Sample Preparation. Muscle and Animal Organs. A 50-gram sample of tissue is placed in a blender (Waring type) with 200 ml. of acetone and 5 grams of Super-Cel. After blending for 5 minutes, the mixture is filtered with suction through a Whatman No. 3 paper. The residue is placed in the blender and extracted with 200 ml. of benzene for 5 minutes. After filtering, the benzene and aqueous acetone solutions are combined in a separatory funnel and shaken vigorously. On separation, the lower (aqueous) phase is discarded. Five grams of Super-Cel and 200 ml. of chloroform are added to the benzene-acetone solution. After mixing and filtration, the solvent is evaporated on the steam bath under an air jet. The residue is dissolved in 300 ml. of Skellysolve B and extracted three times with 50-ml. portions of acetonitrile. Each acetonitrile fraction is washed in turn with a 200-ml. portion of Skellysolve B. The acetonitrile extracts are combined and the solvent is evaporated on the steam bath. The residue is dissolved in 10 ml. of chloroform.

Fat. A 20-gram sample of fat is placed in a blender with 20 grams of anhydrous sodium sulfate and 200 ml. of Skellysolve B. After blending for 5 minutes, the mixture is filtered through a pledget of glass wool. After the filter is washed, the resulting Skellysolve B solution is extracted with acetonitrile as for other tissues.

Chromatography. A column is prepared by packing 5 grams of dry Super-Cel into a 20 × 400 mm. coarse-porosity chromatographic tube. A slurry of 30 grams of acid-washed alumina in chloroform is poured into the column. The column is washed with 100 ml. of chloroform. The samples are chromatographed using a total volume of 100 ml. of chloroform to elute the Co-Ral. The effluent is evaporated to dryness.

Hydrolysis. The residue from the chromatographic separation is dissolved in exactly 25 ml. of chloroform. A 5-ml. aliquot is pipetted into each of two screw-cap tubes labeled *A* and *B*. A 1-ml. aliquot of the standard Co-Ral solution (2.5 γ) is added to tube *B*. The solvent in the tubes is removed on the steam bath under a jet of air. A 5-ml. portion of 1.0*N* aqueous potassium hydroxide is added to each tube. The tubes are capped and placed in an oven at 92° C. for 2.5 hours or in an autoclave at 15 p.s.i.g. for 30 minutes. After cooling, the solutions are extracted with an equal volume of *n*-amyl alcohol. The mixture is centrifuged and the amyl alcohol is discarded.

Measurement of Fluorescence. Slit arrangement 3 of the Aminco Bowman spectrophotofluorometer is used for all measurements. The fluorescence of each solution is determined, using an activating wave length of 330 mμ, and measuring fluorescence at 410 mμ.

Calculation of Co-Ral Concentration.

Let A = fluorescence due to Co-Ral in $^1/_5$ of original sample.
B = fluorescence due to Co-Ral in $^1/_5$ of original sample plus fluorescence of added Co-Ral.
$B - A$ = fluorescence due to 2.5 γ of added Co-Ral.

$$\text{Then, Co-Ral in original sample (p. p. m.)} = \frac{A\,(2.5)(5)}{(B - A)(\text{weight of sample in grams})}$$

Discussion

Hydrolysis Conditions. An investigation of the time required to hydrolyze Co-Ral by heating with 1.0*N* potassium hydroxide was conducted. The effect of the hydrolysis time on the fluorescence produced is shown in Figure 1. It is apparent that the fluorescence reaches a maximum after 60 minutes of heating. Additional heating up to 3.5 hours has no effect on the fluorescence.

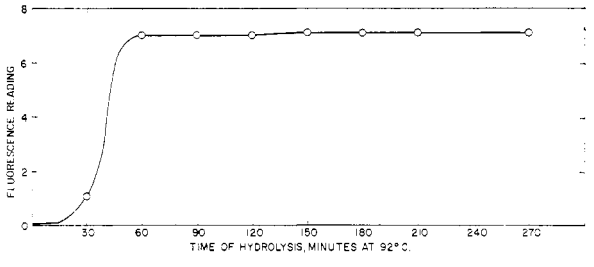


Figure 1. Effect of hydrolysis time on the fluorescence of Co-Ral

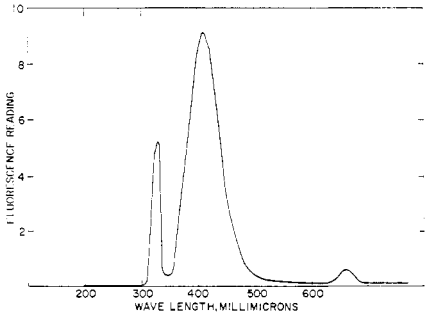


Figure 2. Fluorescence spectrum of hydrolyzed Co-Ral in 1*N* potassium hydroxide

Heating in an autoclave at 15 p.s.i.g. for 30 minutes was as effective as the longer period at 92° C.

Both the oxygen analog of Co-Ral and 3-chloro-4-methyl-7-hydroxy coumarin yield the same fluorescent product as Co-Ral itself on treatment with alkali. This is substantiated by identical infrared spectra of the fluorescent derivatives from all the compounds.

Fluorescence Characteristics. The fluorescence spectrum of hydrolyzed Co-Ral is shown in Figure 2. The maximum fluorescence is obtained with ultraviolet radiation of wave length 330 mμ. The fluorescence maximum is at 410 mμ. The activation and fluorescence spectra of hydrolyzed 3-chloro-4-methyl-7-hydroxycoumarin and the oxygen analog of Co-Ral are identical to those obtained with hydrolyzed Co-Ral.

In the concentration range up to 5 γ per ml. of the final solution, there is a linear relationship between fluorescence intensity and concentration.

The fluorescent compound is relatively unstable to irradiation with ultraviolet light. In spite of this, no detectable decrease in fluorescence is obtained in the length of time required to make satisfactory readings.

The use of the internal standard increment method eliminates the need for a separate fluorescent material for instrument calibration. Errors due to fluorescence quenching are also avoided by this technique.

Extraction Methods. The extraction of pesticide residues from biological materials is the step in all procedures which is most difficult to check properly. The addition of the compound itself to a sample of the material in question, followed by its subsequent recovery, affords an excellent check on all steps following the extraction. However, recovery experiments of this type do not prove that the substance in question is being quantitatively extracted from the original tissue.

To test the efficiency of various extraction systems, a study was carried out on goat liver taken from an animal which had been sprayed 6 days previously with Co-Ral containing radioactive phosphorus. The liver was cut up into small pieces and these were thoroughly mixed before weighing out 10-gram samples. The samples were ground in a blender with 100 ml. of an appropriate solvent. After filtration, the residue was reground with 100 ml. of a second solvent. The two extracts were combined and evaporated to dryness and the residue was dissolved in 100 ml. of Skellysolve B. The Skellysolve B was extracted successively with equal volumes of both water and acetonitrile. The acetonitrile extract was evaporated to a small volume and the entire sample was plated for radioassay. In view of the small amount of residue from the acetonitrile extract, no corrections for self-absorption were made. The results are shown in Table I.

Table I. Efficiency of Various Solvent Systems for Extraction of Radioactivity from Liver

First Solvent	Second Solvent	Counts per Minute in Acetonitrile
Ethyl alcohol, 95%	Skellysolve B	60
	Benzene	70
Acetone	Benzene	230
Chloroform	Benzene	100

On the basis of this experiment, it was concluded that of the solvent pairs examined, the acetone-benzene system is by far the most efficient for the extraction of radioactive organophosphorus compounds from liver. It has been reported in a similar experiment (*8*) using the identical solvent extraction system, that the radioactivity in the acetonitrile fraction can be entirely chromatographed as either Co-Ral or its oxygen analog. Ideally, one would prefer a solvent system which would extract all the radioactivity. However, this is not possible, because of the size and complexity of the phosphorus metabolic pool. In the case of the liver used in our study, the acetone-benzene system removed approximately 20% of the total radioactivity in the sample. A modification of the acetone-benzene system was adopted for routine extraction of tissue samples.

The possibility that the unextractable radioactive fractions might contain some Co-Ral or its oxygen analog was considered. It was thought that the most probable reason for failure to extract these substances from tissue might be due to protein binding. Accordingly, a study of the protein-binding properties of both Co-Ral and its oxygen analog was carried out. In this experiment, 50 ml. of homogenized milk, blood plasma, or a 7% solution of egg albumin in water was placed in a blender jar (Waring type). To each of these was added 0.5 ml. of an acetone solution containing 5 mg. or 0.5 mg. of Co-Ral or its oxygen analog. The samples were incubated at room temperature for 4 hours and then extracted by the acetone-benzene technique. The purpose of adding the compounds in acetone was to ensure intimate contact of the material with the protein. The concentrations of the acetone used will not denature the protein. In each case, more than 90% of the added material was recovered. From this experiment it can be concluded that under the conditions used, neither Co-Ral nor its oxygen analog was protein-bound in a form which prevented its extraction by the solvent system used. This type of experiment does not prove that these compounds are not bound by other proteins or under other conditions, but it furnishes strong support for the contention that this is not a serious problem in the extraction of these compounds from animal tissues.

Table II. Recovery of Co-Ral and Its Oxygen Analog from Various Tissues

Chemical	Tissue	Compound Added, P.P.M.	Compound Found, P.P.M.	Mean Recovery, %
Co-Ral	Fat	0.05	0.05	100
		0.11	0.10	91
		0.21	0.21	100
	Muscle	0.20	0.19	95
	Liver	0.20	0.16	80
		0.40	0.36	90
	Brain	0.20	0.20	100
Oxygen analog	Muscle	0.04	0.04	100
		0.08	0.08	100
		0.12	0.12	100
		0.20	0.22	110

Table III. Precision of Method for Various Animal Tissues

Compound	Tissue	P.P.M.	
Co-Ral	Fat	0.022 ± 0.001	(2)[a]
	Kidney	0.021 ± 0.002	(3)
	Fat	0.050 ± 0.006	(4)
		0.210 ± 0.013	(4)
	Muscle	0.190 ± 0.016	(12)
	Liver	0.360 ± 0.014	(2)
Oxygen analog	Muscle	0.220 ± 0.018	(3)

[a] Values are followed by average deviation from the mean and, in parentheses, by number of determinations carried out.

The extraction of fat samples is much simpler than that of muscle or other tissues. Samples of fat are dissolved in Skellysolve B. Extraction with acetonitrile (*7*, *9*) separates the pesticide residues from the bulk of the fat. The same basic technique is used for the removal of fat from extracts of meat and other tissues. In the latter cases, the initial extraction solvents must be evaporated before the residue can be dissolved in Skellysolve B.

Removal of Interfering Fluorescence. A main source of interfering fluorescence could be 3-chloro-4-methyl-7-hydroxycoumarin which may be present in tissues as a result of the hydrolytic decomposition of Co-Ral or its oxygen analog. A study of adsorption characteristics revealed that this compound is retained quantitatively on acid-washed alumina when chloroform is used as the eluting solvent. Under the same conditions both Co-Ral and its oxygen analog are recovered quantitatively. Accordingly, this technique is included in the routine method described.

In spite of the elaborate cleanup system developed, traces of animal extractives which produced interfering fluorescence on hydrolysis with potassium hydroxide, were still present in the final solution. Examination of a large number of solvents showed that *n*-amyl

alcohol would remove the interfering fluorescence from the hydrolysis mixture without affecting the fluorescence due to the Co-Ral hydrolysis products.

Recovery Experiments. To test the efficiency of the cleanup procedures described, known amounts of Co-Ral were added to samples which were subsequently analyzed. In the case of fat samples, the Co-Ral was dissolved in Skellysolve B. With the muscle and other tissues, the Co-Ral was dissolved in acetone. In both cases, the appropriate amounts of solution were added to the blender in the initial extraction steps. The results of these experiments are shown in Table II.

It is apparent from the data in Table II that Co-Ral in concentrations down to 0.04 p.p.m. can be recovered quantitatively by the method described.

Precision and Sensitivity. The data in Table III show the precision of results obtainable by this method. In the range up to 0.1 p.p.m., the average deviation from the mean is approximately 10% of the measured value. At higher concentrations, the precision is somewhat better. The precision is considered to be excellent for the low concentrations being measured.

The sensitivity of the procedure is determined, in this case, by the amount of fluorescence obtained when the method is applied to untreated samples. In the case of both fat and meat samples this value is equivalent to about 0.02 p.p.m. of Co-Ral. Thus, to contain a reportable residue, a sample must give a value twice that obtained for an untreated control. On this basis, the minimum sensitivity of the method would be set at 0.02 p.p.m. of Co-Ral, although this concentration is many times greater than the instrumental sensitivity limit.

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INSECT REPELLENT ANALYSIS

Colorimetric Determination of 2-Ethyl-1,3-hexanediol

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2-Ethyl-1,3-hexanediol gives a colored compound upon reaction with concentrated sulfuric acid and *p*-dimethylaminobenzaldehyde which can be estimated colorimetrically. The method can be used for the determination of the polyalcohol on glass, cloth, and human skin.

2-ETHYL-1,3-HEXANEDIOL is an effective repellent against various arthropod pests (*1*). An analytical method for this compound in aqueous or alcoholic solution was required in conjunction with studies on the evaporation and absorption of insect repellents. A method known to function for the higher alcohols in the presence of ethyl alcohol was selected for study.

In the presence of concentrated sulfuric acid, higher alcohols (*2, 3*) are dehydrated to unsaturated compounds, which then react with aromatic aldehydes to produce colors (known as the Komarowsky reaction). Snell (*3*) stated that this reaction is not given by polyalcohols. On the contrary, 2-ethyl-1,3-hexanediol reacts with *p*-dimethylaminobenzaldehyde in the presence of strong sulfuric acid to give a colored product. An analytical method for this repellent has been developed, based upon this reaction.

The colored product has an absorption maximum at 500 mμ (Figure 1). Beer's law is obeyed within the range of 5 to 105 γ when the reaction time, temperature, and the concentrations of sulfuric acid and *p*-dimethylaminobenzaldehyde are controlled.

The method has given satisfactory recoveries of 2-ethyl-1,3-hexanediol applied to glass, cloth, and human skin and functions in either aqueous or alcoholic medium.

Procedure

Chromogenic Reagent. Reagent grade *p* - dimethylaminobenzaldehyde (100 mg.) is mixed with 50 ml. of reagent grade concentrated sulfuric acid and dissolved by shaking. This reagent should be freshly prepared before each analysis.

Preparation of Standard Curve. 2-Ethyl-1,3-hexanediol (105 mg.) was diluted to 200 ml. with distilled water; 20 ml. of this solution was diluted to 200 ml. to give a standard solution containing 52.5 γ per ml.

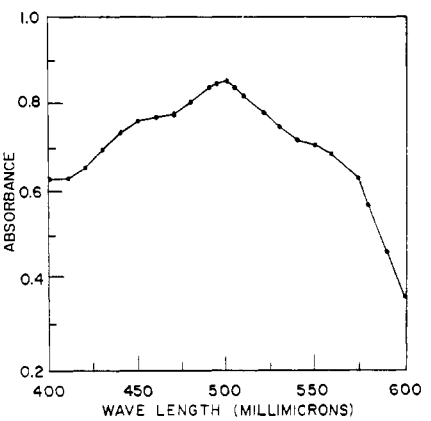

Figure 1. Absorption spectrum of color produced by reaction of 2-ethyl-1,3-hexanediol with p-dimethylaminobenzaldehyde in 66% sulfuric acid

Aliquots of the standard solution con-