Fluorometric Determination of Carbaryl and 1-Naphthol in Honeybees (*Apis mellifera* L.) with Confirmation by Gas Chromatography

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Carbaryl and 1-naphthol were extracted from fortified samples of honeybees, pollen, alfalfa, and honey, and determined quantitatively by the strong fluorescence associated with the sodium salt of 1-naphthol. Recoveries averaged 95% when both methanol and methylene chloride were used for extraction. A Florisil column removed many fluorescent interferences associated with plant extracts. The colorless aqueous phase that resulted when the yellow methylene chloride eluant was extracted with 0.25*N* sodium hydroxide was found suitable for measurement of the fluorescence of any free 1-naphthol present in the sample. For determining carbaryl, the methylene chloride phase was evaporated to near dryness, the carbaryl hydrolyzed by the addition of 0.25*N* sodium hydroxide, the yellow color removed by the addition of petroleum ether, and the fluorescence of the colorless aqueous phase compared with standards. The aqueous phases were shaken with chloroacetic anhydride in benzene to form the chloroacetylated 1-naphthyl derivative for confirmation of the results by gas chromatography.

The toxic effect of carbaryl (1-naphthyl methylcarbamate) on honeybees, *Apis mellifera* L., has been well documented by Morse (1961) and others, and bee poisoning has been reported as far as 3 mi from an apiary (Morse and Gunnison, 1967). Also, Georghiou and Metcalf (1962) showed that the LD_{50} for carbaryl was 0.23 μg per bee by applying 1-μl drops of a solution of the insecticide to the notum. Despite the large number of methods for carbaryl, no method could be found that was amenable to a rapid and independent confirmation of the presence of carbaryl.

The many extraction and workup procedures used for the colorimetric determination of carbaryl and 1-naphthol with *p*-nitrobenzenediazonium fluoroborate were summarized by Johnson and Stansbury (1965a). In basic solutions, carbaryl undergoes rapid hydrolysis to 1-naphthol. The intrinsic fluorescence of 1-naphthol was used by Hercules and Rogers (1958) to quantitatively distinguish 1-naphthol from 2-naphthol in their mixtures. Many methods for the determination of acid phosphatase are based on the fluorescence exhibited by 1-naphthol (White and Argauer, 1970). Carbaryl in fortified milk samples has been determined by Bowman and Beroza (1967) in tetramethylammonium hydroxide-methanol solution by fluorescence.

This present paper describes a quick and efficient method for the analysis of both carbaryl and 1-naphthol since only sodium hydroxide need be added, the use of a color-forming reagent is eliminated, there is greater sensitivity than in the colorimetric procedure, and the results can be confirmed readily by electron-capture gas chromatography. Further advantages are that a mineral oil keeper required in other methods need not be used and the bothersome precipitation step of other procedures is eliminated.

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EXPERIMENTAL

Extraction of Bees, Pollen, and Alfalfa. Twenty-five gram samples of bees, pollen, or alfalfa were macerated in a Waring Blendor with 150 ml of methylene chloride for 1 min. Then 100 g of anhydrous sodium sulfate were added to the blender, and the sample was macerated for an additional 2 min. After the contents of the blender were filtered through a Buchner funnel, the filter cake was returned to the blender and reextracted with 100 ml of fresh methylene chloride (or preferably of 100 ml of methyl alcohol). The reextracted filter cake was washed with an additional 50 ml of methylene chloride, and the combined methylene chloride filtrates were concentrated to a volume of 50 ml in a rotary evaporator under a water-aspirator vacuum. (The combined methylene chloride-methyl alcohol filtrates were evaporated to near dryness under a water-aspirator vacuum and taken up in 50 ml of methylene chloride.)

Column Chromatography. Florisil, as received from the Floridin Co., lost 4.4% of its weight when it was heated to 500° C. Prior to use for column chromatography, an additional 20% water was added to the as received Florisil and allowed to equilibrate overnight. The sample extract in 50 ml of methylene chloride was percolated through 50 g of Florisil (about 24 cm deep) in a Shell-type 20-mm o.d. glass column previously washed with 75 ml of water-equilibrated methylene chloride, and the column was eluted with 100 ml of water-equilibrated methylene chloride. The yellow eluant (about 200 ml) was collected in a 250-ml separatory funnel. Much of the fluorescent material present in the bees remained adsorbed near the top of the column.

Separation of Carbaryl from 1-Naphthol. Exactly 25 ml of 0.25*N* NaOH were added to the methylene chloride eluant, and the separatory funnel was shaken for 3 min. The clear colorless sodium hydroxide phase was transferred to a 125-ml Erlenmeyer flask for later transfer to a 1-cm cuvette for measurement of the fluorescence of 1-naphthol. The yellow-

colored methylene chloride phase was transferred to a 250-ml Erlenmeyer flask and evaporated to near dryness under a water-aspirator vacuum. Then exactly 25 ml of 0.25*N* NaOH were added to the flask, hydrolysis was allowed to proceed for 10 min, and about 3 to 5 ml of petroleum ether (30° to 60° C) were added to dissolve the oily residue adhering to the sides and bottom of the flask. After another 25 min, during which the standards were prepared, an additional 25 ml of petroleum ether were added, the contents of the flask were poured into a 125-ml separatory funnel, and the lower, clear colorless aqueous phase was transferred to a 125-ml Erlenmeyer flask for later transfer to a 1-cm cuvette for measurement of the fluorescence of carbaryl.

Total Carbaryl and 1-Naphthol. Carbaryl and 1-naphthol were also determined together by simply eliminating the extraction of 1-naphthol from the methylene chloride eluant prior to evaporation and hydrolysis.

Honey Samples. A 100-g sample of honey was weighed in a 500-ml separatory funnel, 150 ml water were added, and the mixture was extracted twice with two portions of 25 ml methylene chloride. The extracts were combined for percolation through Florisil.

Aqueous Sugar Feeding Solutions. The samples were diluted with 0.25*N* NaOH to a concentration of about 1 μg per ml of carbaryl, and the fluorescence was compared with standards.

Standards. Stock solutions of carbaryl and 1-naphthol were prepared in concentrations of 500, 250, and 125 μg per ml in benzene solution and stored in polyethylene-capped amber-colored glass bottles. Hamilton μl syringes were used to transfer aliquots of these stock solutions to 125-ml Erlenmeyer flasks. Because the volumes of the standards used were low, the benzene solvent evaporated quickly in the flask. Exactly 25 ml of 0.25*N* NaOH were then added to each of the flasks. A small stream of nitrogen facilitated evaporation of any residual benzene. Hydrolysis of carbaryl proceeded fast, as shown by the rapid increase of the solutions to maximum intensity of fluorescence. Standards were always run side by side with samples and compared spectrofluorometrically within an hour after the addition of alkali to minimize any effect of oxidation. We have observed a 25% decrease in fluorescence intensity for standards made alkaline and allowed to stand at room conditions overnight. One method for acid phosphatase determination gives a 2-mo period for stability of 1-naphthol standard in alkaline solution when stored in a refrigerator.

Confirmation by Gas Chromatography. The solution in the 1-cm cuvette used to measure fluorescence was returned to the bulk of the solution in the parent Erlenmeyer flask, and 7.5 g of anhydrous sodium sulfate were added. After the salt had dissolved, 10 ml of a freshly prepared 0.5% solution of chloroacetic anhydride in benzene were added, and the flask was shaken mechanically for 3 min. A portion of the benzene layer was decanted into a 15-ml test tube, some anhydrous sodium sulfate was added, and 5 μl of the benzene phase were injected into the gas chromatograph and compared with the standards. For quantitative confirmation, it was occasionally necessary to dilute the fluorescent solution before reaction with chloroacetic anhydride reagent to keep the response in the linear range of the electron capture detector. An example will clarify this point. A fluorescence reading on the 0.1 scale of 98 was obtained that corresponded to a concentration of 2 μg per ml of carbaryl or 50 μg in the total sample. Since linearity for gas chromatography was expected below 8 μg carbaryl per 10 ml reagent when a 5-μl injection was used, a 3- or 4-ml aliquot of the fluorescent solution was diluted to 25 ml with sodium hydroxide solution, and chloroacetylation was conducted as before.

The gas chromatograph used (Varian Aerograph Model 200) was equipped with a 5 ft × 1/8 in. i.d. stainless steel column containing 3% OV-225 on 80/100 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa. 16823) and was maintained at 195° C. The tritium detector was held at 205° C, the injection heater at 210° C, and the nitrogen flow at 30 ml per min at the column outlet.

RESULTS AND DISCUSSION

The fluorescence excitation and emission spectra of hydrolyzed carbaryl in 0.25*N* NaOH (1 μg per ml) recorded on an Aminco-Bowman spectrophotofluorometer are given in Figure 1. The observed maxima of λ_{ex} 340 nm and λ_{em} 465 nm may differ somewhat from instrument to instrument since the spectra have not been corrected for either the quantum distribution of the light source or the response of the emission-monochromator-1P21 electron-multiplier phototube unit (Argauer and White, 1964). The relative intensities of fluorescence *vs.* concentration of carbaryl and the 1-naphthol standards in 0.25*N* NaOH are compared in Figure 2 with quinine sulfate in 0.1*N* sulfuric acid. Since quinine sulfate is widely used as a fluorescence standard for determination of quantum yields, it was chosen as a reference. The fluorescence of hydrolyzed carbaryl and 1-naphthol was strong and linear over at least three decades of concentration. The decrease in response at very high concentrations was expected since the exciting light is absorbed almost totally by the first few millimeters of the concentrated solution where the fluorescence, because of the geometry of the instrument, fails to enter the exit slit for measurement. Solutions may be diluted in half with 0.25*N* sodium hydroxide to be certain that the concentration lies in the linear portion of the standard curve. The bars in the lower left corner of Figure 2 give the values for the fluorescence background obtained when bee samples were used as controls, and was equivalent to about 1 μg per 25 g sample (250 bees).

Figure 3 gives typical chromatograms obtained when the presence of carbaryl was confirmed by gas chromatography.

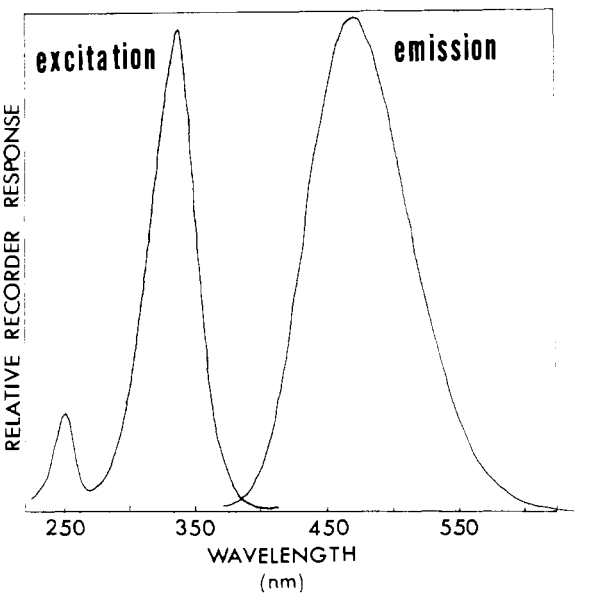

Figure 1. Fluorescence excitation and emission spectra of carbaryl in 0.1*N* NaOH (1 μg/ml)

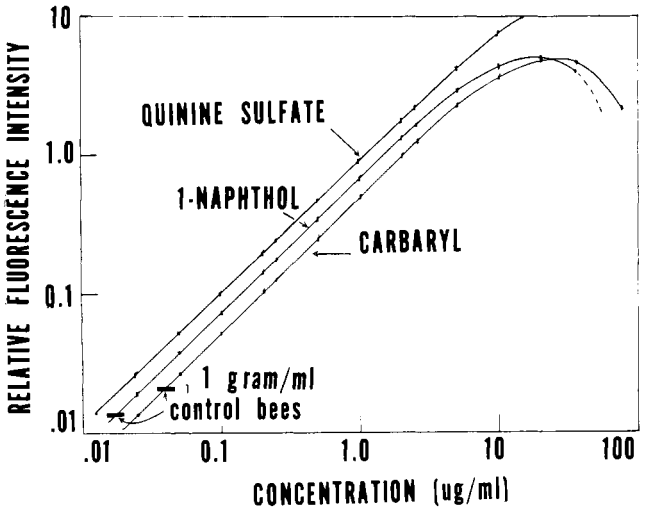

Figure 2. Relationship between intensity of fluorescence and concentration

Quinine sulfate in 0.1*N* H_2SO_4 (λ_{ex} 360 nm; λ_{em} 455 nm); Carbaryl and 1-naphthol in 0.25*N* NaOH (λ_{ex} 340 nm; λ_{em} 465 nm)

Even with a contaminated electron capture detector (evidenced by the low recorder response at high electrometer output), one can readily determine the presence or absence of 1 μg carbaryl per 25 g of bees.

Two modifications of the chloroacetylation procedure (Argauer, 1969) have been introduced. Aizawa and Pincus (1964) showed for benzoylation of estrogens that the addition of sodium carbonate to the mixture facilitated the formation of that derivative. We found that this addition also increased chloroacetylation in our procedure. However, sodium sulfate proved as effective and kept the foaming action of sodium carbonate during the shaking step to a minimum. By using OV-225 in place of XE-60 for the column substrate, an interference peak reported earlier by Argauer (1969) as being bothersome was resolved.

RECOVERIES

Each of the recovery steps can be monitored easily because of the strong fluorescence of the compounds. As an example,

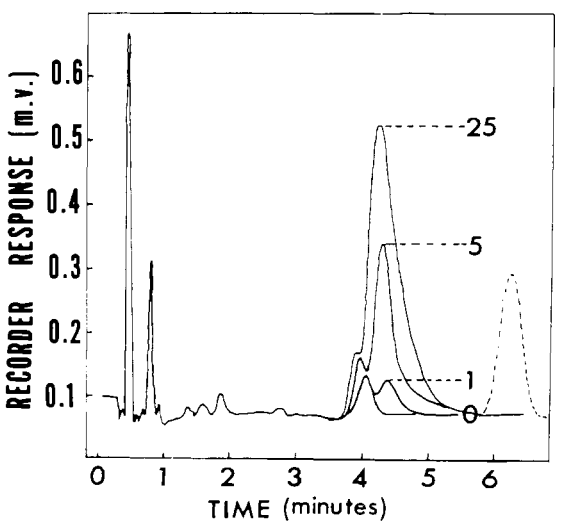

Figure 3. Gas chromatograms for carbaryl after hydrolysis and and chloroacetylation

Solid line: 0, 1, 5, 25 μg carbaryl in 25 ml of 0.25*N* NaOH. Ten ml reagent added. 5-μl aliquot of 10-ml benzene phase injected. Dotted line: Relative response for 0.5 ng heptachlor epoxide

for determining the recovery from the column, 50 μl of the 500-μg per ml stock standard solution were added to a 250-ml Erlenmeyer flask. Then, after the solvent evaporated, 50 ml of methylene chloride were added for percolation through the Florisil. The eluant was evaporated under vacuum, and 25 ml of 0.25*N* NaOH were added. The same amount of standard and NaOH solution were added to another flask as a control. The intensity of fluorescence of the two solutions were compared (λ_{ex} 340 nm; λ_{em} 465 nm).

Likewise the fluorescence of aqueous sodium hydroxide solutions obtained by extraction of petroleum ether and methylene chloride that contained carbaryl or 1-naphthol served to indicate the degree of separation of the two solutes (Table I). Table II gives the recoveries when bees, pollen, and alfalfa were first fortified with the compound and then extracted with methylene chloride or with methylene chloride followed by methanol. The recoveries when methylene chloride was used as the sole extracting solvent appeared near those found by Johnson and Stansbury (1965b) for bees and by Johnson (1964) for apples and lettuce. When methanol was used, the recoveries increased significantly. Porter *et al.* (1969) also showed improved recoveries of carbaryl from fruits and vegetables by acetonitrile.

Table I. Extraction of Carbaryl and 1-Naphthol from the Two Solvents with Alkali[a]

Compound	Solvent	Extraction Time	Percent Extracted
50 μg carbaryl	50 ml petroleum ether	1 min	79
50 μg carbaryl	50 ml petroleum ether	2 min	95
50 μg carbaryl	50 ml petroleum ether	3 min	100
50 μg carbaryl	150 ml CH_2Cl_2	3 min	1
250 μg carbaryl	150 ml CH_2Cl_2	5 min	3
25 μg 1-naphthol	150 ml CH_2Cl_2	3 min	98

[a] 25 ml of 0.25*N* NaOH used for extraction in a separatory funnel.

Table II. Recoveries of Carbaryl and 1-Naphthol from Fortified Samples

Sample	Amount of Compound Added, ppm	Percentage Compounds Recovered by Extraction with CH_2Cl_2	Percentage Compounds Recovered by Extraction with CH_2Cl_2 followed by CH_3OH
	Carbaryl		
Honeybees (25 g sample)	3.0	81	89, 93, 95
Honeybees (25 g sample)	1.0	85	93
Honeybees (25 g sample)	0.5	83	96
Honeybees (25 g sample)	0.25	80	91
Honey (100 g sample)	0.25	90, 96, 94	...
Pollen (10 g sample)	5.0	...	98
Pollen (10 g sample)	1.0	...	94
Alfalfa (50 g sample)	0.5	80, 84	91, 94
Alfalfa (25 g sample)	1.0	78, 84	99, 93
	1-Naphthol		
Honeybees (25 g sample)	1.0	82, 84	81, 85
Honey (100 g sample)	0.25	83, 86	87
	Carbaryl	**No solvent extraction**	
Aqueous carbaryl feeding solution	50.0	95	
Aqueous carbaryl feeding solution	100.0	100	
Aqueous carbaryl feeding solution	200.0	97	
Untreated Controls			**(ppm)**
Honeybees (25 g sample)	0.0	...	0.04
Honey (100 g sample)	0.0	...	0.01
Pollen (25 g sample)	0.0	...	0.04
Alfalfa (50 g sample)	0.0	...	0.03

Table III. Carbaryl and 1-Naphthol Recovered from Bees Fed Carbaryl

A. Each Bee Fed 0.4 μg Carbaryl in a Sugar Solution from a Dutky-Fest Applicator. 100 Bees Analyzed ≈ 12-g Sample

Hours at Room Temperature	Percentage Recovered		
	1-Naphthol	Carbaryl	Total
0	...	...	99[a]
0	...	...	96[a]
24	18.5	40.5	59
24	...	...	66[a]
60	...	...	63[a]
88	...	...	50[a]
96	12.0	27.5	39.6

B. Mass Feeding on Sugar Solutions Containing Carbaryl. Fifty-gram Sample of Bees Analyzed (≈ 400 bees)

Sugar Solution (μg Carbaryl/ml)	Mortality at 48 hr	μg Found	
		1-Naphthol	Carbaryl
225	100%	46	80 (77)[b]
80	97%	21	17 (21)[b]
30	75%	21	6.7 (8)[b]

[a] 1-Naphthol and carbaryl not separated prior to determination. [b] ( ) indicates values by GC confirmation.

Dead bees suspected of dying from carbaryl poisoning are usually submitted for evaluation some time after exposure to the weather. Consideration was therefore given to the possibility that enzymatic and microbial action in dead bees may produce both positive and negative interferences. Live bees that had foraged on wild flowers in the Beltsville, Md., area were fed sugar solutions containing known amounts of carbaryl from a Dutky-Fest micro-applicator. The bees were immediately quick frozen in a test tube immersed in dry ice-alcohol to prevent regurgitation of the carbaryl-contained sugar solutions, and then kept at room temperature for a specified period (Table III). Even after 96 hr, carbaryl still could be detected in the dead bees. Table IIIB gives the mortalities and the residues of carbaryl and 1-naphthol when the bees were kept in a controlled environment and forced to feed on an aqueous sugar solution containing carbaryl. (The solutions were prepared by diluting an ethanol solution of carbaryl with 50% aqueous sugar syrup.) The values obtained for the carbaryl residues by fluorescence agreed with those obtained by gas chromatography.

We believe that the fluorometric procedure given here can be used with little modification to determine carbaryl in other food and agricultural products. Since the fluorescence of 25 μg carbaryl in 25 ml of sodium hydroxide solution (25 g sample at 1 ppm) appears strong when the solution is examined visually under ultraviolet light, it may be possible to estimate carbaryl visually prior to spectrofluorometric measurement for products that have a tolerance set between 1.0 and 100 ppm.

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