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Direct Gas Chromatographic Determination of Carbaryl Residues in Honeybees (*Apis mellifera* L.) Using a Nitrogen-Phosphorus Detector with Confirmation by Formation of a Chemical Derivative

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Carbaryl is the International Organisation for Standardisation's common name for 1-naphthyl methylcarbamate. It is used in the United Kingdom as an insecticide to control capsids, earwigs, and caterpillars of codling, tortrix and winter moths on apple trees; it is also used as a fruit thinner. Carbaryl affects honeybees by attack on the impulse transmitting capability of the nervous system due to inhibition of cholinesterase (Stevenson *et al.* 1977; Westlake *et al.* 1985). Therefore its effects are similar to those of organophosphorus compounds (Aldridge 1981); although in general, the carbamates are less toxic to mammals.

Bees are attracted to flowering crops to collect nectar for honey. If apple trees are sprayed with carbaryl while still in flower, there is a possibility that many bees will be killed and those that survive will carry insecticide back to the hive and affect the whole colony. In order to determine whether carbaryl is involved in the deaths of honeybees in suspected poisoning incidents, a reliable and sensitive method for detecting carbaryl in honeybees is necessary.

Several methods have been described for the determination of N-methylcarbamates. Those of Cohen *et al.* (1970), Holden *et al.* (1969), Holden (1976) and Maitlen and McDonough (1980), all rely on the formation of derivatives from the hydrolysis product. In the case of carbaryl this is 1-naphthol, the major metabolite. This permits the determination of total carbaryl-derived residue in one analysis but precludes the determintion of carbaryl separately from its hydrolysis product. None of these methods had been developed for the analysis of bees.

Lord *et al.* (1980) investigated methods for the analysis of carbaryl in honeybees. They dismissed the possibility of direct gas chromatography of carbaryl and examined derivative formation. The N-acetyl derivative of unhydrolysed carbaryl was produced quantitatively in cleaned bee extracts but on gas chromatography (GC) with an electron capture detector,

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it was subject to major interference and there was some interference when a nitrogen selective detector was used. Conversion of carbaryl to its N-trifluoroacetyl derivative by the method of Lau and Marxmiller (1970) was not quantitative at microgram levels. Four hydrolysis and derivatisation reactions were also found by Lord *et al.* not to be quantitative at the microgram level. For these reasons Lord *et al.* used liquid chromatography in preference to gas chromatography. Our own experience of the determination of carbaryl in honeybees by liquid chromatography with an ultraviolet-absorbance detector showed the technique to lack the sensitivity required for reliable measurement of the small amounts of carbaryl to be found in poisoned bees.

We now report a gas chromatographic analytical method for carbaryl in bee extracts using an extraction technique which allows the sample to be analysed for a number of different pesticide residues. Experience shows that it is necessary to avoid direct injections of uncleaned bee extracts onto the GC column. Due to the high wax content in some of the samples, a two stage clean up procedure is necessary. The first stage involves an adaptation of a pentane/methanol partition (Blaicher *et al.* 1980) followed by a chromatographic clean up using a silica "Sep-Pak" cartridge.

Following this clean up, carbaryl residues could be determined by direct gas chromatography without significant interference. Analysis of "spiked bees" using the N-trifluoroacetyl derivatisation method of Bose (1977) was found to give approximate quantitative agreement with results obtained by direct gas chromatography. This method was more convenient than that of Lau and Marxmiller (1970).

MATERIAL AND METHODS

Carbaryl (≥ 99%) was obtained from the Murphy Chemical Company Ltd. Solvents, distilled in glass, were obtained from Rathburn Chemicals Ltd., Walkerburn, Borders, Scotland. Pyridine and anhydrous sodium sulphate were "analytical" grade from BDH Ltd., Dagenham, Essex, England; the sodium sulphate was heated to 400°C for 24 hr before use to remove traces of organic impurities. Sand was acid purified, 40-100 mesh size, from BDH Ltd. and trifluoroacetic acid (> 99%) came from the Sigma Chemical Company Ltd. of Poole, Dorset, England. All-glass interchangeable plunger 10mL glass syringes, obtained from BDH Ltd. were used in conjunction with silica "Sep-Pak" cartridges obtained from Millipore (UK) Ltd., Harrow, Middlsex, England, for sample clean up. Centrifuge tubes graduated to 10mL with ground-glass stoppers (Exelo T6/15S) and Soxhlet extraction equipment (Quickfit FR 150/3S, EX 5/53 and CX 5/25) were manufactured by J. Bibby Ltd. of Stone, Staffordshire, England. Cellulose Soxhlet thimbles (28 mm i.d. x 80 mm long) were obtained from Whatman Labsales Ltd. of Maidstone, Kent, England. Glass pestles and mortars (95 mm internal diameter) and Pasteur pipets were

supplied by Gallenkamp Ltd., Loughborough, Leicestershire, England. The vortex mixer ('Whirlimix') was from Fisons Scientific Equipment, also of Loughborough. 5 μL syringes used for both manual and automatic injection onto a gas chromatograph were type 85-RNE manufactured by Hamilton A.G., Bonaduz, Switzerland.

The gas-chromatograph was a Hewlett-Packard HP5880A instrument with packed-column injection port, nitrogen-phosphorus ionisation detector and "level 4" integration and data processing. A Hewlett-Packard automatic injector (HP7672A) was used routinely once the method had been developed. The initial development work used a 0.6 m x 2 mm i.d. glass column packed with 100-120 mesh Chromosorb W-HP coated with 2% m/m OV101 stationary phase. The carrier gas was nitrogen (oxygen free) at a flow rate of 30 ml min^{-1}. The injection, column oven, and detector temperatures were 210, 150, and 300°C, respectively. The detector gases (hydrogen and air) and the detector element current were adjusted as described in the instrument instruction manual. Under these conditions, carbaryl had a nominal retention time of 3.25 min. To obtain higher resolution a 10 m x 0.53 mm i.d. fused-silica capillary column coated internally with a dimethyl silicone phase (e.g., BP1 from Scientific Glass Engineering (UK) Ltd., Milton Keynes,England) has been used. This required an adaptor at the GC injection port and the addition of carrier gas to make up the required flow at the base of the detector. Suitable operating conditions were carrier flow 8 mL min^{-1}, make-up gas flow 20 mL min^{-1}, injection temperature 200°C, detector temperature 300°C, column oven 100° for 1 min then 8° per min to 140° then 5° per min to 180°, held for at least 5 min. Under these conditions, the retention time of carbaryl was 12 min. With this column, the maximum injection volume used was 2 μL.

Reagents and apparatus were individually checked for interferents during method development and "reagent-blank" analyses (without sample) were performed at frequent intervals when the method was in use.

Weigh accurately up to 4 g of whole dead honeybees (*Apis mellifera*. L.) into a mortar with up to 20 g of anhydrous sodium sulphate and 4 g of sand (acid purified). Grind with a pestle until a fine homogeneous powder has been produced. Quantitatively transfer this powder to an extraction thimble, and extract with 100 mL of diethyl ether for 12-16 h using a Soxhlet apparatus. Allow the extract to cool before transferring it to a 100-mL volumetric flask and adjust to the volumetric mark by either concentration or dilution.

A methanol/pentane partition is used to remove the non-polar co-extractives from the sample before analysis. Whilst carbaryl is soluble in both pentane and methanol, it is preferentially soluble in methanol, and whilst the co-extractives are soluble in both pentane and methanol, most of them appear to be

preferentially soluble in pentane.

Evaporate 40 mL of the diethyl ether extract to dryness in 10mL aliquots in a 10mL graduated test tube under a gentle stream of nitrogen. Re-dissolve the residue in 1 mL of pentane using a vortex mixer ('Whirlimixer'). After this add a further 3 mL of pentane followed by 2 mL of methanol. Mix the pentane and methanol using a vortex mixer. Stand the test tube in an ice bath to allow the two phases to separate completely. Using a Pasteur pipet transfer the top (pentane) layer to a second tube. Evaporate the contents of this second tube to dryness. Add a further 5 mL of pentane to the first test tube and vortex mix, then stand the test tube in an ice bath until both phases have completely separated. Transfer the pentane layer to the second test tube and then add 2 mL of methanol to this test tube. This is to back extract any carbaryl which may be in the pentane. Vortex mix the contents of this tube and allow the two phases to separate completely by standing the tube in an ice bath.

Discard the pentane layer and then combine the two methanol layers in a test tube. Evaporate the methanol under a stream of nitrogen with the test tube standing in warm water (30-50°C). Add 1 mL of hexane and then evaporate this to dryness to remove any residual methanol. Re-dissolve the residue in 1 mL of 5% (v/v) diethyl ether in hexane ready for clean up using a silica 'Sep-Pak' cartridge.

In order to remove any interferents the 'Sep-Pak' should be thoroughly pre-washed with the following : (i) methanol (20 mL), (ii) dichloromethane (20 mL), (iii) diethyl ether (20 mL) and (iv) hexane (20 mL).

Vortex mix the residue dissolved in 5% (v/v) diethyl ether in hexane immediately before introducing it into the 'Sep-Pak' cartridge. Once the residue has soaked into the 'Sep-Pak', elute the 'Sep-Pak' with 10 mL of 10% (v/v) diethyl ether in hexane; this fraction may be discarded. Then elute the 'Sep-Pak' with 10 mL of 65% (v/v) diethyl ether in hexane. This is the carbaryl-containing fraction. Evaporate the diethyl ether/hexane solvent and re-dissolve the residue in 1 mL of hexane. Analyse the sample by GC by injecting 5 μL of the hexane solution onto the chromatograph.

If a sensitive GC-mass spectrometer is available, the same analysis conditions may be used to confirm the identification of carbaryl, otherwise the identification of carbaryl may be confirmed by using the following procedure (Bose 1977).

Evaporate the solvent (now 1 mL of hexane) and re-dissolve the residue in 1 mL of a mixture of ethyl acetate and pyridine (9 + 1 v/v) in a graduated test tube. Vortex mix the contents of the tube, add 0.5 mL of trifluoroacetic anhydride, and again vortex mix the reaction mixture. Care must be taken to exclude any moisture from the derivatisation reaction. Stand this mixture in

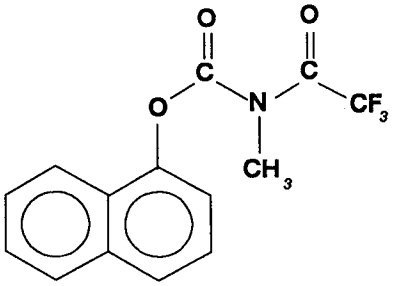

Figure 1. The carbaryl derivative (N-trifluoroacetylcarbaryl)

warm water (30-50°C) for 15-20 min, then evaporate the solution to dryness. Re-dissolve the residue in 2 mL of hexane and vortex mix, then add 8 mL of water and mix again (this removes any trifluoroacetic acid from the hexane).

Using the same conditions as above, inject 5 μL of the derivatised sample. The carbaryl derivative has a retention time of 2.30 min.

RESULTS AND DISCUSSION

Quantitative analysis is performed by comparing the carbaryl or carbaryl derivative peak height and retention time with that of a carbaryl or carbaryl derivative standard (external standard). The reproducibilities of both the derivatised and underivatised carbaryl peak heights and retention times were within 3%. The reproducibility of derivative formation was good, as derivatives formed on different days from the same standard yielded responses on the gas chromatogaph within 5% of each other. The derivative (Fig.1) appears to be stable when stored at room temperature (or below) for more than five days. A linear GC response to carbaryl and its derivative was obtained using standards in the range 0.35-100 μgmL^{-1}.

The effectiveness of the analytical method was determined by measuring the recovery of carbaryl added to dead bees before analysis. Ten samples of 'control' bees (each sample consisting of 4 g of dead honeybees that had not been exposed to carbaryl) were treated by topical addition of a solution of carbaryl in hexane and the bees were kept at room temperature for two hours to allow the solvent to evaporate. Each of these samples and an untreated control sample were analysed as described in the Materials and Methods section and the recovery of added carbaryl was found to be in the range 95-100% (Table 1).

The method of analyses was also applied to experimentally poisoned bees. Two hundred live bees were treated with a topical application to each bee of 1 μL containing an average LD_{50} dose of carbaryl in acetone solution. After 24 hr, the surviving bees were separated from the dead bees and sacrificed by freezing at -20°C. 'Dead' and 'surviving' bees were

separately analysed, the results are shown in Table 2.

Table 1. Recovery of carbaryl from 4g samples of 'control' bees fortified with carbaryl prior to analysis

Sample	Carbaryl Added (mg/kg)	Carbaryl Recovered (mg/kg)	% Recovery *
1	None	0.007	-
2	0.09	0.089	99
3	0.10	0.096	96
4	0.10	0.097	97
5	0.10	0.097	97
6	0.10	0.097	97
7	0.10	0.10	100
8	0.10	0.095	95
9	0.18	0.18	100
10	0.30	0.30	100
11	0.30	0.29	98

*figures corrected for background

Table 2. Results of experimental dosing of 200 bees with carbaryl

Nominal dose	Dead/Survived	Analysis results mg/kg	µg/bee	bees/g
1.3 µg bee	D (77)[a]	0.80	0.073	11
	S (123)[a]	0.40	0.049	8

[a] - The figure in brackets indicate the numbers of bees which died (D) or survived (S)

Honeybees thought to have died due to pesticide poisoning have been analysed by this method. Table 3 shows the carbaryl residues measured in bees which died shortly after carbaryl had been sprayed on fruit trees near to their hives.

Recoveries of samples fortified with carbaryl indicate that the analytical method described in this paper is suitable for the direct gas chromatographic analysis of carbaryl residues in dead honeybees together with confirmation of its presence by substitution of a trifluoroacetyl group for the carbamoyl hydrogen. The limit of determination is between 0.03 and 0.10 $mgkg^{-1}$ (0.003 and 0.010 µg per bee, assuming a typical bee weighs 0.1 g).

Experimental data (Table 2) and sample analysis (Table 3) show that the bees exposed to carbaryl contained residue levels of carbaryl well above the limit of determination of this method. Hence this is a convenient method of analysis as it is rapid and reliable. An individual cartridge clean up can be used for each sample and eliminates the possibility of cross

Table 3. Results of the analysis of honeybees where carbaryl was suspected to be the cause of death.

Sample	Weight analysed (grams)	No of bees per gram	Amount of carbaryl found			
			Underivatised		Derivatised	
			mg/kg	µg/bee	mg/kg	µg/bee
A	4.10	12	12.0	0.97	9.1	0.76
B	4.00	11	5.0	0.45	5.3	0.48
C	3.98	10	2.5	0.25	3.1	0.31
D	4.06	13	4.3	0.33	4.2	0.32
E	4.25	12	9.7	0.81	11.0	0.87
F	4.00	14	13.0	0.96	11.0	0.80
G	4.10	12	2.7	0.23	2.4	0.22
H	4.12	14	6.4	0.46	6.2	0.44

contamination. The method of analysis outlined above has been successfully applied to a number of field samples where carbaryl was suspected to be the cause of death of bees.

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