On the basis of these findings, the pathway shown in Figure 4 is proposed for the degradation of azinphos-methyl in soil and by soil bacteria.

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Registry No. I, 86-50-0; II, 30245-38-6; III, 29519-79-7; IV, 87729-12-2; V, 87729-13-3; VI, 28527-10-8; VII, 22305-44-8; VIII, 87729-14-4; IX, 24310-40-5; X, 90-16-4; XI, 85850-18-6; XII, 87729-15-5; XIII, 33953-31-0; XIV, 87729-16-6; XV, 55-21-0; XVI, 69-72-7; XVII, 118-92-3.

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Evaluation of Analytical Methods for the Determination of Residues of the Bee Repellent, Phenol, in Honey and Beeswax

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Reverse-phase high-performance liquid chromatographic (HPLC), fluorometric, and colorimetric methods (based on either the Gibbs or aminoantipyrine reaction) were evaluated for detection of phenol residues in honey and beeswax. All methods required an initial steam distillation prior to analysis. The most suitable method for phenol analysis in honey was found to be the reverse-phase HPLC method using 4-chlorophenol as an internal standard. Both colorimetric methods could also be used for phenol analysis in honey and were the methods of choice for analysis of phenol in beeswax.

The use of phenol, or carbolic acid, as a bee repellent began in the 1930s (*Bee World*, 1968). A concentrated solution (up to 90%) of pure crystalline phenol in water is applied to the adsorbent cloth on the underside of a special hive cover called a phenol board. On the upper surface of the phenol board is a black sheet of metal, which is in contact with the cloth.

The phenol is placed on the top of the hive and the heat from the sun vaporizes the phenol, which then has a repelling effect on the bees, therefore enabling the beekeeper to collect honey and/or beeswax with a minimum of interference. It has been reported that phenol and other bee repellents (*Bee World*, 1968) have been widely used, especially by commercial beekeepers, since honey can be rapidly collected with the assistance of relatively unskilled labor.

Careless use of phenol in collecting honey results in contamination with significantly high residues of phenol that impart a disagreeable medicinal taste to the honey. Since the application of phenol as a bee repellent causes contamination not only in the honey but also in the surrounding wax cells, it may be necessary to monitor phenol residues in beeswax. High levels of phenol in wax frames can be passed on to honey being collected. The potential risk of accumulation of phenol residues is acute because of the normal procedure for beekeepers to reuse empty wax frames. Many countries are considering greater regulation of chemicals, such as phenol, used in beekeeping. Increased regulation will require reliable analytical techniques for the detection of phenol residues in honey.

Sporns (1981) first introduced a procedure using high-performance liquid chromatography (HPLC) for the analysis of phenol in honey. In this paper, we will discuss and compare an alternate HPLC procedure based on reverse-phase separation, colorimetric (Gibbs, aminoantipyrine), and fluorometric techniques for the analysis of phenol in honey and beeswax. These methods are all capable of determining phenol at parts per million levels.

MATERIALS AND METHODS

Honey for recovery studies was obtained from a local beekeeper, who collected the honey without the use of a phenol board. The honey was checked by all methods presented in this paper and an earlier work (Sporns, 1981) and found to be free of detectable phenol.

Beeswax (laboratory grade) was obtained from Fisher Scientific Co. All water used in the analysis was purified by a Millipore Milli-Q system.

Steam Distillation. A honey or wax sample weighing 10 g was transferred to a Kjeldahl flask with 30 mL of water; for the HPLC procedure, 25 mL of distilled water and 5 mL of internal standard solution were used. The contents of the flask were steam distilled by using a Buchi Model 320 Kjeldahl distillation apparatus and the first 10 mL of the steam distillate was collected for analysis.

Cross contamination due to residual phenol in the distillation apparatus was prevented by steam distilling 40 mL of water in a clean Kjeldahl flask, until about 100 mL

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of the distillate was collected.

HPLC Procedure. Two internal standards were used for the HPLC procedure and were added before the steam distillation, 5 mL of either 2,6-dimethylphenol (10 μg/mL) or 4-chlorophenol (40 μg/mL).

The 2,6-dimethylphenol was recrystallized and the solution prepared every 4 days, refrigerated, and protected from light. The 4-chlorophenol solution was freshly prepared every 4 weeks.

After steam distillation, a 20-μL sample of distillate was injected into a Whatman Partisil 5 ODS-3 RAC column protected by a Whatman Pellosil ODS guard column. The solvent used was 50:50 acetonitrile (HPLC grade)–water and was maintained at a flow rate of 2 mL/min by a Beckman Model 110A pump. Peak heights were measured at an absorbance of 195 nm by using a UV variable-wavelength spectrophotometer (Laboratory Data Control Spectromonitor III) connected to a Hewlett-Packard 3388A integrator.

Sample injections were alternated with standard injections of mixtures of phenol (1 μg/mL) and 2,6-dimethylphenol (2 μg/mL) or 4-chlorophenol (3.2 μg/mL).

Gibbs Procedure. Gibbs (1927) first introduced the reagent 2,6-dichloroquinone-4-chloroimide, which reacts with phenols to give a blue indophenol.

To the 10 mL of steam distillate were added 1 mL of buffer (pH 9.8, 60 g of sodium carbonate monohydrate and 40 g of sodium bicarbonate in 1 L of water) and 1 mL of 0.04% (w/v) 2,6-dichloroquinone-4-chloroimide in 95% ethanol (prepared fresh daily and refrigerated). The mixture was allowed to stand for 5 min and then placed for 30 min in a water bath, maintained at 40 °C, before the absorbance was measured at 610 nm with a Beckman DU-8 spectrophotometer. A reagent blank containing 10 mL of distilled water in place of the steam distillate was prepared with each set of samples.

Aminoantipyrine Procedure. This procedure was adapted from methods used to examine waste water ("Standard Methods for the Examination of Water and Wastewater", 1971). To the 10 mL of steam distillate were added 1 mL of ammonium chloride solution (50 g of ammonium chloride in 1 L of water adjusted to pH 10.2 with concentrated ammonium hydroxide), 1 mL of aminoantipyrine solution (2 g of 4-aminoantipyrine in 100 mL of water), and 1 mL of potassium ferricyanide (8 g of potassium ferricyanide in 100 mL of water). The last two solutions were prepared fresh daily. The absorbance was read at 510 nm and measured against a blank prepared with 10 mL of distilled water in place of the steam distillate.

Fluorometric Procedure. The steam distillate was measured directly by using a Perkin-Elmer 650-10LC fluorescence spectrophotometer. The excitation wavelength was set at 270 nm and the emission wavelength monitored at 300 nm. Both excitation and emission slit widths were 5 nm. A water blank was measured with each set of samples.

RESULTS AND DISCUSSION

The HPLC procedure used in this paper has been modified considerably from the original analysis of phenol in honey (Sporns, 1981). In the original HPLC procedure, separation of phenol and the internal standard, 2-phenylethanol, was achieved by using a silica column. We found that there can be substantial variations in the separation of these two compounds when the silica column is obtained from a different manufacturer. Determination of the analysis on a Whatman Partisil PXS 5/25 analytical column, rather than the original Spectral Physics Sphersorb 5-μm silica column, resulted in phenol being almost unretained, while 2-phenylethanol had a markedly longer retention time when the same solvent, water, was used. Although in the earlier study (Sporns, 1981), using a silica column, there is never a noticeable problem with interference with substituted phenols, we felt that it was prudent to use a system where better resolution of phenol and substituted phenols was obtained. We, therefore, decided to use a reverse-phase system, similar to the procedure of Chao and Suatoni (1982), which gave excellent separation of phenol from other substituted phenols. The change, however, required the use of a solvent of the composition 50:50 acetonitrile–water.

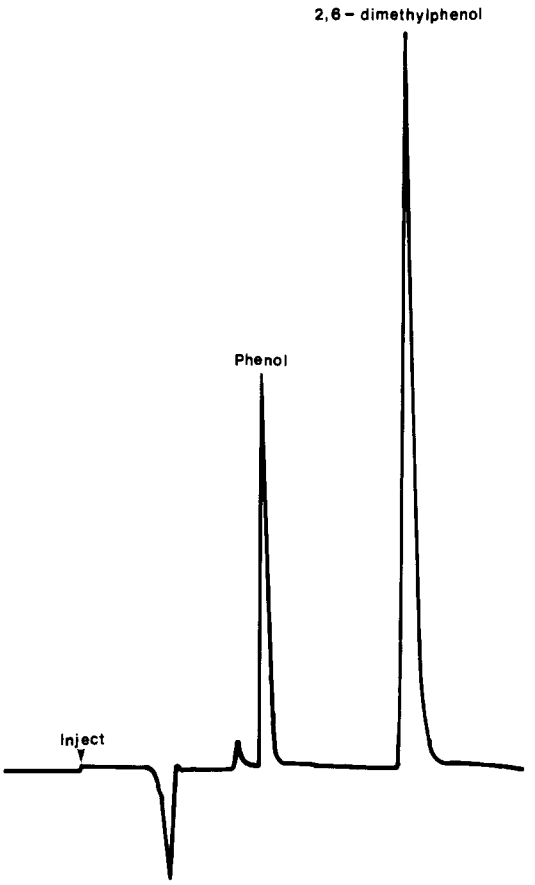

Figure 1. HPLC chromatogram of the steam distillate of a honey containing 5.7 ppm of phenol with 2,6-dimethylphenol as the internal standard and monitored at 195 nm.

We have also discovered that not all variable-wavelength detectors are capable of accurate analysis at 195 nm. The high noise level experienced at 195 nm when using a Tracor 970 variable-wavelength detector required that phenol be monitored at an absorbance of 269 nm. The Laboratory Data Control Spectromonitor III could be used successfully at 195 nm, allowing approximately a 25-fold increase in sensitivity as compared to that at 269 nm.

The change to reverse-phase separation resulted in the discontinuation of the use of 2-phenylethanol as an internal standard. It was found that both 2,6-dimethylphenol and especially 4-chlorophenol worked quite well as internal standards. Both are crystalline solids, although 2,6-dimethylphenol is not a stable in solution as 4-chlorophenol.

Quantitation of phenol for the HPLC method involved the use of the equation for the internal standard method based on peak height measurements as described in an earlier paper (Sporns, 1981). Calculation of phenol using the internal standard 2,6-dimethylphenol or 4-chlorophenol required the multiplication of the equation by a factor to account for the difference in volatility of phenol and the

Table I. Parameters for Phenol Analytical Methods

method of analysis	material analyzed	no. of samples	linear region	systematic error	correlation coefficient
Gibbs	honey	9	0 to 30 ppm	+0.4 ppm	0.999
Gibbs	wax	6	0 to 47 ppm	+0.3 ppm	0.997
aminoantipyrine	honey	6	0 to at least 70 ppm	negligible	0.998
aminoantipyrine	wax	5	0 to at least 107 ppm	+2.1 ppm	0.999
fluorometric	honey	5	0 to 17 ppm	+0.2 ppm	0.999
fluorometric	wax	6	0 to 27 ppm	+0.4 ppm	0.995

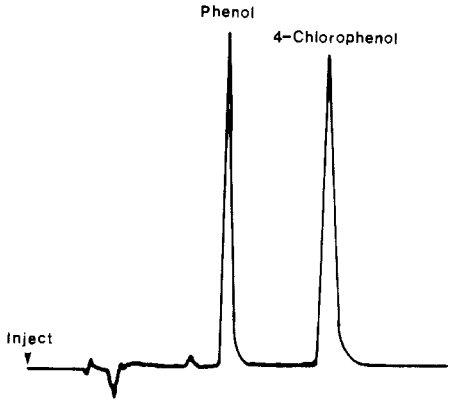

Figure 2. HPLC chromatogram of the steam distillate of a honey containing 7.6 ppm of phenol with 4-chlorophenol as the internal standard and monitored at 195 nm.

internal standard used. The order of volatility of the compounds is as shown: 2,6-dimethylphenol > phenol > 4-chlorophenol. Figures 1 and 2 show typical chromatograms of separation of phenol and each internal standard used (retention times were as follows: for phenol, 4.0 min; for 2,6-dimethylphenol, 7.2 min; for 4-chlorophenol, 6.1 min). The factors calculated for 2,6-dimethylphenol and 4-chlorophenol were 3.305 and 0.6215, respectively. The factors were determined by adding phenol to honey (free of phenol) at levels ranging from 0.5 to 30 ppm in the presence of the internal standard and quantitating the phenol recovered by using the HPLC procedure. Any variation in the procedure with respect to changes in amounts or volumes used in the steam distillation would affect the correction factor. Slight variation would have a negligible affect.

Unfortunately, the determination of phenol in wax could not be adapted to analysis by the HPLC procedure. The major problem arising was the steam distillation of components from the wax, which interfered with the detection of phenol. Also, some of these compounds had very long retention times for our solvent conditions, causing increased problems after repeated injections.

The colorimetric tests and the fluorometric technique could be used for the analysis of both honey and wax. Some parameters for each method are given in Table I. These parameters are obtained from calibration curves and recovery studies, which involved the addition of known concentrations of phenol to honey and wax to check for interferences. The linear regions for each analysis were determined directly from calibration curves by using standard phenol solutions. Differences in the linear region between honey and wax occur because of the increased volatility of phenol in the honey analysis compared to wax analysis. After experiments were carried out by adding known amounts of phenol to honey and wax, the volatility of phenol in honey analyses was found to be approximately 55% greater than the phenol volatility in wax analyses. Concentration of phenol in μg/mL determined in analyses was converted directly to ppm of phenol (for the conditions given under Materials and Methods) by multiplication of a factor of 0.4267 for honey analyses and 0.6693 for wax analyses.

Table II. Precision of Phenol Analytical Methods

method	average value of phenol, ppm	no. of replicates	SD
HPLC (4-chlorophenol internal standard)	7.90	5	0.10
aminoantipyrine (honey)	11.74	5	0.17
aminoantipyrine (wax)	2.17	3	0.12
Gibbs (honey)	3.66	5	0.09
Gibbs (wax)	3.02	5	0.19
fluorescence (honey)	9.70	7	0.27
fluorescence (wax)	6.15	4	0.49

Possible interfering compounds from honey and wax were evaluated by spiking a honey and wax at various levels and analyzing the resulting data by using linear regression analysis (correlation coefficient given in Table I). The systematic error (line intercept, since, with no phenol added, there should be none detected) represents interferences. In all cases, interferences are in a positive direction and are less than 0.5 ppm, except for the wax determination using the aminoantipyrine method.

Samples of honey and wax that contained levels of phenol higher than those given in Table I (for the linear region) could, of course, still be analyzed after appropriate dilution. In fact, the linear region for the Gibbs reaction is limited by the concentration of the Gibbs reagent. A more concentrated Gibbs reagent, however, would give a significant absorbance in the blank at the wavelength measured (610 nm), increasing the error, especially for samples containing smaller amounts of phenol. Possible interferences in the color reactions due to the presence of steam volatile phenolic compounds have been suggested (Dacre, 1971; "Standard Methods for the Examination of Water and Wastewater", 1971). For fluorometric detection, interferences could be due to other steam volatile fluorescent compounds. It should be noted that only one honey and wax were spiked with phenol at various levels and used for the systematic error determination. One might expect, with the variety of honeys produced, that some samples could contain more interfering compounds than others. This, again, points out the great advantage of HPLC where quantitation is coupled with a separation and identification of the compound analyzed.

A comparison of the precision of the various methods of analysis is given in Table II. These results and data using these methods of phenol analysis for a variety of honey and wax samples (P. Daharu and P. Sporns, unpublished results) indicate the precision of the methods to be comparable, except for the fluorescence method.

In conclusion, the method of choice for phenol analysis in honey is the HPLC method using 4-chlorophenol as an internal standard. For laboratories without HPLC equipment, either colorimetric test (aminoantipyrine or Gibbs) may be used, with the added advantage that beeswax can also be analyzed. The aminoantipyrine method is quicker than the Gibbs test with a greater linear

region; however, it seems affected by interferences from beeswax to a greater extent.

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Insecticidal Activity of Tralomethrin: Electrophysiological Assay Reveals That It Acts as a Propesticide

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The activity of the pyrethroid tralomethrin was assessed by using an electrophysiological assay (monitoring the release of miniature excitatory postsynaptic potentials) on the larvae of *Heliothis virescens*, *Chilo partellus*, and *Plutella xylostella*. Small quantities of deltamethrin in the tralomethrin sample were sufficient to explain the observed activity. Tralomethrin is shown to be unstable and debromination of tralomethrin to deltamethrin occurred rapidly in simulated sunlight. There is therefore evidence that tralomethrin is not intrinsically active.

Tralomethrin [HAG 107: (*S*)-α-cyano-3-phenoxybenzyl (1′*RS*)-*cis*-(1*R*,3*S*)-3-(1,2,2,2-tetrabromoethyl)-2,2-dimethylcyclopropanecarboxylate] is known to be unstable, decomposing to deltamethrin [NRDC 161: (*S*)-α-cyano-3-phenoxybenzyl *cis*-(1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate] in ultraviolet light (Ruzo and Casida, 1981), on topical application to houseflies, and on feeding to cabbage looper larvae (Ruzo et al., 1981).

Despite the instability of tralomethrin, it is not known whether its insecticidal activity is due to decomposition to deltamethrin or whether it possesses insecticidal activity in its own right. To find out if tralomethrin is intrinsically active, the pyrethroid must be tested at its site of action in the insect. Only by this method can reliable information be obtained without the complicating factors of penetration and metabolism.

Pyrethroid insecticides kill insects by affecting the nervous system (Gammon et al., 1982). Within the nervous system, motor nerve terminals have been identified as particularly susceptible to disruption by pyrethroids and their disruption correlates with pyrethroid poisoning in vivo (Adams and Miller, 1979; Miller et al., 1983; Miller and Adams, 1982; Narahashi and Lund, 1980; Salgado, 1981; Salgado et al., 1983; Omer et al., 1980). The onset and degree of pyrethroid poisoning can be assayed by monitoring the change in function of a process that is under nerve terminal control. The rate of release of miniature excitatory postsynaptic potentials (mepps) is such a process—indeed, the first sign of pyrethroid poisoning in vitro is a vast increase in the frequency of mepps. By increasing the concentration of pyrethroid until an increase in mepp frequency is observed, a reproducible measure of the intrinsic activity of a pyrethroid can be obtained. Such data, in a series where differences of penetration and metabolism can be ignored, give a good correlation between in vivo and in vitro potency (Miller and Adams, 1982; Miller et al., 1983; Salgado, 1981; Salgado et al., 1983; Irving, 1983). Some previous electrophysiological assays on pyrethroids have yielded a poor correlation between in vivo and in vitro potency [e.g., Narahashi et al. (1977)] probably because the tissue used (nerve cord, in this case of crayfish) is not the primary site of action of pyrethroids, necessitating the use of excessive concentrations.

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MATERIALS AND METHODS

Chemical Analysis. Tralomethrin and deltamethrin were analyzed by high-performance liquid chromatography (HPLC). The system used consisted of a Waters Associates 6000A pump, an Altex Model 210 injection valve, and a Pye Unicam LC–UV detector. Columns used were 100 mm × 4.6 mm i.d., and all solvents were prefiltered and of HPLC or glass distilled grade. Two contrasting separation systems were used, both giving base-line separation of deltamethrin and the two isomers of tralomethrin: system A, Spherisorb S5ODS (Chrompack), mobile phase 85% methanol/15% water, flow rate 1 mL/min, and analytical wavelength 230 nm; system B, Spherisorb S5W silica (Chrompack), mobile phase 60% hexane/40% dichloromethane, flow rate 1 mL/min, and analytical wavelength 240 nm. Concentrations of deltamethrin and tralomethrin were measured by direct comparison with standards of similar concentration.

Photochemistry. Tralomethrin (formulated as a 3.75% emulsifiable concentrate containing a red azo dye (to act as a filter for ultraviolet light) was irradiated as thin films (0.75 μg of active ingredient/cm^2) on glass under simulated sunlight. The lamp array used (6 × 40 W Thorn UV fluorescent tubes, 20 cm above the sample plates) has been shown to be 2–5 times less intense than British summer sunlight in its ability to photodegrade a variety of pesticides (Fraser, 1983).

Electrophysiological Assay. Mepp release was monitored by using the fourth instar larvae of the lepidopterans *Heliothis virescens*, *Chilo partellus*, and *Plutella xylostella*. The larvae were dissected by dorsal incision and the gut/viscera/fat removed to expose the nervous system and musculature. The preparations were flooded with saline

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