We chose for our studies an insect that is a very abundant producer of pheromone in comparison with most other insects that have been studied. Many more insects would be required to produce sufficient pheromone for study with a less prolific species. Improvement in the percentage of incorporation of a precursor into the pheromone is considered essential. The 1-h incubation time was chosen because previous work using [^{14}C]acetate (Jones and Berger, 1978) indicated that the maximum level of incorporation was obtained at this time. A much higher percentage of incorporation of ^{14}C was obtained with isolated glands incubated in vitro, but the yield of pheromone was much less (Jones, 1979). Regardless of the techniques employed it is our estimate that approximately 2–3 mg of pure (*Z*)-7-dodecenyl acetate containing 10–12% ^{13}C would be required to readily detect a pathway of synthesis such as that catalyzed by the fatty acid synthetase complex.

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Determination of Carbofuran's Phenolic Metabolites in Hops

The phenolic transformation products of carbofuran were isolated from hop cone extracts by high-pressure liquid chromatography (HPLC) using a reverse-phase, octadecylsilane column. HPLC column fractions were treated with fluorodinitrobenzene, and the dinitrophenyl ether derivatives were determined quantitatively by electron capture gas chromatography. Detection limits were 0.30 mg/kg for 2,3-dihydro-2,2-dimethyl-7-benzofuranol and 2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol and 1.0 mg/kg for 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol in fortified hop samples. At the limit of detection the average through-the-method recovery for these phenolic residues was 81%.

Soil application of the *N*-methylcarbamate pesticide carbofuran results in systemic activity in plants against insects and nematodes. Oxidative metabolism of carbofuran introduces hydroxyl and carbonyl substituents in the 3 position, and these primary metabolites may be hydrolyzed and/or conjugated. In alfalfa, for example, residues are primarily in the form of glucosides of 2,3-dihydro-2,2-dimethyl-3-oxo-7-benzofuranol (II), 2,3-dihydro-2,2-dimethyl-3,7-benzofurandiol (III), and 3-hydroxycarbofuran, and small amounts of the free phenols (Figure 1) are present as well (Knaak et al., 1970). This diversity of chemical forms complicates the analysis of carbofuran residues in crops.

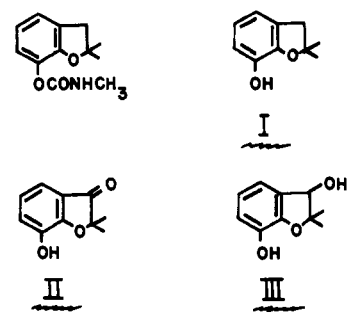

Figure 1. Carbofuran and its phenolic metabolites.

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Whereas the carbamate metabolites may be determined selectively by using element-specific gas chromatograph detectors (microcoulometric, thermionic, or "N–P" detectors), the phenolic products of carbofuran require derivatization for low-level detection; dinitrophenyl ether (Cook et al., 1977), pentafluorobenzyl ether (Archer et al., 1977), and perfluoroacetate ester derivatives are commonly used. Carboxylic acids and phenols, ubiquitous natural products in plants, are derivatized as well, often creating major interferences (Holmstead et al., 1979). Gas chromatography coupled mass spectrometry has been used in the determination of carbofuran's phenols in complex matrices, but this instrumentation is frequently unavailable for routine residue analysis.

We describe here the application of high-pressure liquid chromatography (HPLC) to the isolation of carbofuran's phenolic metabolites from hops. All attempts to determine the benzofuranol residues in this crop were unsuccessful when using conventional sample cleanup procedures including adsorption and reverse-phase column chromatography. The HPLC cleanup procedure described may be useful in the analysis of other complex samples matrices where interferences are encountered.

EXPERIMENTAL SECTION

Chemicals. Analytical reference standards of 2,3-dihydro-2,2-dimethyl-7-benzofuranol (I), II, and III were provided by FMC Corp. (Middleport, NY). 2,3-Dihydro-2,2-dimethyl-3-ethoxy-7-benzofuranol (IV) was prepared by ethoxylation of III by the method of Cook et al. (1977). Reference standards of the corresponding dinitrophenyl ether derivatives, I-DNPE, II-DNPE, III-DNPE, and IV-DNPE, were obtained from the U.S. Environmental Protection Agency (Research Triangle Park, NC). 1-Fluoro-2,4-dinitrobenzene (98%, FDNB) was obtained commercially and stored sealed at −5 °C. Other chemicals were reagent grade; solvents were commerically available pesticide analytical grade, and water for the preparation of solutions and chromatography was distilled.

Apparatus. HPLC was performed on a Varian Model 5021 instrument fitted with a 10-μm, reverse-phase, octadecylsilane column (25 cm) and an absorbance detector monitering at 254 nm. A pellicular, reverse-phase precolumn was used and the column was operated at 35 °C. The mobile phase was pH 2.2, 0.02 M phosphate buffer–acetonitrile (4:1 v/v), and the total flow rate was 2.0 mL/min. At the completion of each isocratic separation the solvent composition was changed to 100% acetonitrile in a 10-min linear gradient.

A Tracor Model MT 220 gas chromatograph fitted with a ^{63}Ni electron capture detector (D.C. mode) and a 1 m × 4 mm (i.d.) glass column packed with 60–80-mesh Gas-Chrom Q coated with 5% OV-210 was used. Operating conditions were as follows: carrier gas flow rate, 60 mL of N_2/min; column oven temperature, 210 °C; inlet temperature, 230 °C; detector temperature, 325 °C.

Extraction and Sample Preparation. The procedure of Cook et al. (1977) was used with minor modifications. Green hops (10 g) or dry hops (5 g) were homogenized in 150 mL of 0.25 N hydrochloric acid prior to hydrolyzing the sample under reflux. The hydrolysate was filtered through a large plug of glass wool in a funnel, and the boiling flask was rinsed with 2 × 50 mL of 0.25 N hydrochloric acid. The entire hydrolysate was extracted with 2 × 150 mL of methylene chloride and 2 × 150 mL of methylene chloride–diethyl ether (3:1 v/v). Tenacious emulsions formed, but these were broken slowly upon addition of approximately 5 g of sodium chloride. The combined extract was dried (sodium sulfate) and subjected to ethoxylation and carbamate–phenol separation (Cook et al., 1977).

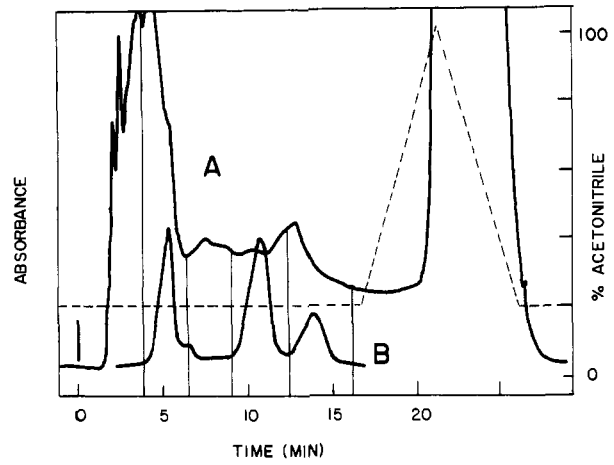

Figure 2. Liquid chromatogram of an ethoxylated hop cone extract (A) and a mixed standard containing II, I, and IV (B). The dashed line indicates the composition of the mobile phase.

The Nuchar column cleanup was replaced with a high-efficiency HPLC separation. The extract, containing I, II, and IV, was concentrated just to dryness on a rotary evaporator fitted with a 35 °C water bath. Considerable loss of I may occur if vacuum evaporation is not done carefully, and Cook et al. (1977) recommend use of a steam table. The flask was cooled in an ice bath, 1.00 mL of acetonitrile was introduced, and the residue was dissolved. The stoppered, round-bottom flask was allowed to sit several minutes, and the sample was transferred to a screw-top vial for storage at 0 °C until analysis.

A small portion of the sample (50 μL) was injected into the chromatograph, and II, I, and IV were collected in the column effluent at 4.5–6.5, 9.5–13.5, and 13.5–17 min, respectively; column fractions were not combined in the analysis of hops. Retention times were determined each day to define optimum collection intervals, but little variation was observed. Fractions were collected in stoppered test tubes and extracted with 3 volumes of dichloromethane. Extracts were combined for derivatization with FDNB.

Derivatization was modified as follows: a pH 8.8, borate buffer was used, the reaction mixture was heated in a 95 °C water bath, and heating was continued 45 min after boiling off the volatile solvent. Acetone was added in place of methylene chloride prior to drying, and the drying agent was rinsed with 10 mL of hexane.

Analysis. Samples were concentrated on a rotary evaporator to approximately 1 mL, transferred quantitatively with hexane to volumetric tubes, reduced under a stream of nitrogen, and adjusted to 4.0 mL with hexane. Injection volumes were 6–8 μL and quantitation was achieved by absolute calibration relative to mixed standards containing 0.2 or 0.5 ng/μL I-DNPE, II-DNPE, and IV-DNPE.

RESULTS AND DISCUSSION

The ethoxylated sample extract dissolved rapidly in a small volume of acetonitrile to give a dark brown solution with a pungent aromatic odor. Only 5% of this mixture was separated with the liquid chromatograph to minimize contamination of the analytical-scale column; the aliquot was equivalent to 0.25–0.50 g of plant material. Only a small fraction of the plant extract was required due to the

Table I. Recovery of Carbofuran Phenols from Hops

treatment, mg/kg	residue found, mg/kg[a]			% recovery[b]		
	I	II	III	I	II	III
control[c]	0.03 ± 0.03	0.04 ± 0.09	0.13 ± 0.12			
0.30	0.21	0.31	nd[d]	60	90	nd
0.30	0.31	0.26	nd	93	73	nd
0.30	0.26	0.23	nd	77	63	nd
1.0	0.93	1.2	0.81	90	116	68
1.0	0.82	0.92	1.1	79	88	97
1.0	1.01	0.96	1.2	98	92	107

[a] Control not subtracted. [b] Control subtracted. [c] Mean and standard deviation of nine determinations. [d] Below the established detection limit.

sensitivity of the electron capture detector to the phenol–DNPE derivatives.

Liquid chromatograms of hop sample extracts exhibited large amounts of UV-absorbing material (Figure 2) and the detection of the underivatized phenols (I, II, and IV) at trace levels was not possible by absorbance detection at 254 nm. For this reason, carbofuran residues were collected in predefined time intervals without regard to the detector response. The isocratic, reverse-phase separation was effective in isolating I, II, and IV from the bulk of the coextracted constituents, and increasing the solvent strength produced a noticeably colored effluent. The HPLC column cleanup was rapid, requiring approximately 0.5 h/sample, considerably less than sample cleanup by reverse-phase column chromatography on Nuchar. It may be feasible to combine column fractions in less complex matrices without sample interference.

The OV-210 gas chromatography column proved optimum for the analysis of each fraction, and its performance did not deteriorate after the analysis of a large number of hop samples. I-DNPE, IV-DNPE, and II-DNPE had retention times of 5.5, 8.0, and 9.2 min, respectively. With OV-1 the elution order of II-DNPE and IV-DNPE was inverted, and OV-17 did not separate them. Packings coated with less than 5% liquid phase required several large injections of II-DNPE to obtain the maximum detector response.

Recovery Determination and Limits of Detection. Green hop samples were fortified with I–III at 0.30 and 1.0 mg/kg to determine recoveries and demonstrate detection limits. Results appear in Table I. Analysis of control samples indicated little matrix interference for I and II, and detection limits of 0.3 mg/kg were realized; at this level mean recoveries were 77 and 75%, respectively. The recoveries averaged 86% for fortifications at 1.0 mg/kg.

The detection limit of III, however, was approximately 1.0 mg/kg due to a minor interference. The apparent residue of III in control samples varied between 0 and 0.3 mg/kg (Table I). This interference had retention properties similar of those to IV-DNPE on OV-1, OV-17, OV-210, and diethylene glycol adipate columns, demonstrating the similarity in their vapor pressures and polarities. Attempts to isolate the analyte from the natural product by deethoxylation of the sample in refluxing acid were ineffective as other coextractives were displaced that could not be separated from III-DNPE. With the HPLC cleanup procedure described the interference was sufficiently low that III could be accurately determined at levels as low as 1.0 mg/kg (Table I).

CONCLUSION

HPLC is a rapid and effective cleanup procedure for the isolation of carbofuran's phenolic metabolites from hops, a complex matrix containing large amounts of tannin (polyphenolic material) and acidic bitter principles. The methodology described may prove useful in the analysis of these pesticide residues in other crops and environmental samples.

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