Determination of Trichlorfon and Dichlorvos Residues in Shrimp Using Gas Chromatography with Nitrogen−Phosphorus Detection

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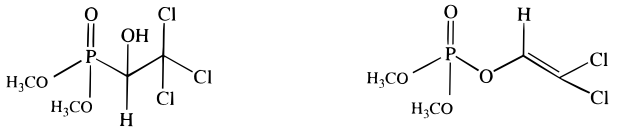
A method capable of quantifying trichlorfon and dichlorvos in shrimp at concentrations of 20−80 ng/g has been developed. Ground shrimp is homogenized in ethyl acetate and centrifuged. The supernatant is dried completely on a rotatory evaporator. The extract is dissolved in petroleum ether, concentrated on a solid phase extraction column, and analyzed by gas chromatography using a cool on-column inlet and a nitrogen−phosphorus detector. The analytes are separated from matrix components using a thermal gradient on a (cyanopropyl)phenyl-methylpolysiloxane column. The method was validated with control shrimp fortified at 20, 40, and 80 ng/g trichlorfon and dichlorvos. The average recoveries and intralaboratory coefficients of variations were 50−83% and 15−21% respectively.

Keywords: *Trichlorfon; dichlorvos; shrimp; gas chromatography; nitrogen−phosphorus detector*

INTRODUCTION

Trichlorfon and dichlorvos (Figure 1) are used among cultured aquatic species for controlling planktonic invertebrates and parasites (Brandal and Egidius, 1979). Treatment occurs by transferring the cultured species to baths containing the drug for a specific amount of time or by administering the drug directly into the rearing pen. The doses used range between 0.25 and 25 000 μg/mL, and the exposure times vary between 1 min and 4 days. In water, trichlorfon decomposes to the more toxic drug, dichlorvos, the likely active form of the former (Hofer, 1981; Samuelson, 1987). The decomposition rate depends on the aeration rate, the pH, and the temperature (Samuelson, 1987). Dichlorvos is more soluble in fat than trichlorfon and penetrates biological membranes more easily (Hofer, 1981). The extent to which both drugs may build up in the body before intoxication depends on the drug application rate, duration of treatment, lipid content, animal size, rate of metabolism, water temperature, and pH (Horsberg et al., 1989). Trichlorfon treatment has led to several incidents of high mortality in fish and juvenile crustaceans (Horsberg et al., 1989; Omkar and Shukla, 1985; Juarez and Rouse, 1983). Trichlorfon and dichlorvos have been found to be both teratogenic and mutagenic in mice and rats (Courtney et al., 1986; Wild, 1975). Due to the absence of adequate toxicological data, a safe level for the use of trichlorfon in cultured aquatic species marketed for human consumption has not been determined. Because of the potential for the presence of illegal residues, a method is required to monitor for trichlorfon and dichlorvos in shrimp.

Trichlorfon and dichlorvos are frequently analyzed by gas chromatography (GC) because they have a low ultraviolet absorbance. Trichlorfon is thermally labile and decomposes when analyzed by GC using a heated injector. Quantitation of trichlorfon has been achieved by monitoring the thermal breakdown products such as dichlorvos, dichloroacetaldehyde, and dimethyl phosphite (El-Refai and Giuffrida, 1965; Pieper and Richmond, 1976; Anderson et al., 1966; Devine, 1973). The completion and reproducibility of the breakdown process which varies daily depends on the size and position of glass wool inserted in the injection port liner to provide additional surface area for heat exchange. Since dichlorvos is a breakdown product of trichlorfon, it is difficult to accurately quantify the separate amounts of trichlorfon and dichlorvos that are present in a sample. Derivative methods such as methylation (Look and White, 1981), silylation (Bowman and Dame, 1974; Akhtar, 1982), and acylation (Anderson et al., 1966; Vilceanu et al., 1973; Look and White, 1981) have also been used for GC analyses of trichlorfon. To avoid the thermal decomposition of trichlorfon and the need for derivatization, trichlorfon and dichlorvos standards have been analyzed as separate compounds by GC using a cool on-column inlet and a flame ionization detector (FID) (Slahck, 1988). This GC procedure was adapted for the determination of trichlorfon and dichlorvos in shrimp using a nitrogen−phosphorus detector (NPD) because it is more specific and provides better sensitivity than the FID.



A. Trichlorfon B. Dichlorvos

Figure 1. Structures of (A) trichlorfon and (B) dichlorvos.

Several methods have been described for extracting dichlorvos and trichlorfon from tissue. Organic solvents such as acetonitrile, ethyl acetate, and a mixture of cyclohexane/acetone have been used for homogenization of tissue containing both drugs followed by liquid−liquid partitioning, gel permeation chromatography, or partition chromatography to remove coextractants (Anderson et al., 1966; Schultz et al., 1971; Devine, 1973; Horsberg and Hoy, 1990). However, these extraction methods are laborious and time consuming. To make the method less labor intensive, to minimize the use of organic solvents, and to provide a cleaner extract, solid-phase extraction (SPE) was incorporated into the sample cleanup.

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In this report, a GC method is described for the direct quantitation of trichlorfon and dichlorvos in shrimp as separate compounds. Ground shrimp containing sodium sulfate is homogenized in ethyl acetate. The extract is centrifuged, *n*-hexadecane is added to the supernatant, and the ethyl acetate is completely evaporated. The extract is dissolved in petroleum ether and loaded onto a preconditioned cyanopropyl SPE column. The SPE column is washed with dichloromethane/petroleum ether to remove some matrix components. The analytes are eluted from SPE column with methanol/diethyl ether. The eluent is dried completely and reconstituted in toluene. The extract is analyzed by GC using a cool on-column inlet and a NPD. The analytes are separated from further matrix components using a thermal gradient.

MATERIALS AND METHODS

Apparatus. Gas chromatography was performed using a Hewlett-Packard 5890 Series II gas chromatograph equipped with pressure programmable cool on-column inlet and a NPD (Wilmington, DE) or equivalent system. Separation was achieved using a DB-1701 column, 0.25 mm × 0.25 μm × 30 m (J & W Scientific, Folsom, CA) connected to a deactivated fused silica tubing used as a retention gap, 1 m × 0.53 mm (Hewlett-Packard, Wilmington, DE). The SPE column used was a cyanopropyl column, 500 mg × 6 mL (Supelco, Bellefonte, PA).

Reagents and Solutions. Methanol was HPLC grade (Burdick & Jackson, Baxter, Columbia, MD); ethyl acetate, petroleum ether, and dichloromethane were organic residue analysis grade (J. T. Baker, Phillipsburgh, NJ). Ethyl acetate and diethyl ether (Burdick & Jackson, Baxter, Columbia, MD) were dried over granular sodium sulfate (ACS reagent, J. T. Baker, Phillipsburg, NJ). *n*-Hexadecane was 99% pure (Sigma, St. Louis, MO). The wash solution on the SPE column was dichloromethane/petroleum ether (10 + 90, v/v) and the elution solution was methanol/diethyl ether (2 + 98, v/v). Both solutions were dried over sodium sulfate. Trichlorfon and dichlorvos reference standards were obtained from Ultra Scientific (North Kingstown, RI). After correcting for purity, 100 μg/mL stock standard solutions of each reference standard were prepared using toluene. The stock standard solutions were stored at 4 °C for up to 1 month. The intermediate standard solution, 10 μg/mL of each pesticide in toluene, was prepared by diluting the stock standard solution. Trichlorfon and dichlorvos intermediate standard solutions were used to prepare combined working standard solutions at levels of 0.13, 0.25, 0.5, or 1 μg/mL trichlorfon and dichlorvos. The intermediate and working standard solutions were stored at 4 °C for up to 5 days. Extracts from control tissue were used as a diluent for calibration standards. Four control (drug-free) shrimp samples were prepared using the extraction procedure. Two hundred microliters of a working standard containing trichlorfon and dichlorvos was used to reconstitute dry control shrimp extract. The concentrations of the two drugs in tissue matrix were 10, 20, 40, or 80 ng/g.

Animal Treatment. Ecuadorian white shrimp, *Penaeus vannamei*, were purchased from Edisto Shrimp Company, Edisto Island, SC. The crustaceans, which weighed approximately 12 g each, were housed in salt water tanks held at room temperature (20−23 °C) and a pH of 7−8. They were fed a commercial shrimp diet while acclimating for a week prior to the study. Trichlorfon or dichlorvos was administered to the shrimp by water exposure at concentrations of 0.2−3 μg/mL in a 10-gallon container. The exposure solution was prepared by adding the appropriate amount of trichlorfon or dichlorvos to water to give the desired test concentration. The shrimp were added to the exposure solution and maintained there for 1 to 3 h. At the end of the dosing period, the shrimp were removed from the exposure solution, rinsed in salt water, sacrificed, beheaded, and placed on ice. The shell, fins, and tail were removed. The shrimp meat was ground twice and mixed to achieve homogeneity. A sample was analyzed; the remaining ground shrimp were frozen at ≤−60 °C for future analysis. Control shrimp from other geographical regions were also analyzed during the development of the method.

Extraction of Sample. Ground shrimp (2.5 g) was placed in a 50-mL centrifuge tube. Five control shrimp samples and one control shrimp sample fortified at 40 ng/g dichlorvos and trichlorfon were analyzed with each sample set. Four control shrimp samples were used to prepare a matrix standard curve. To calculate recovery, 100 μL of working standard containing 1 μg/mL dichlorvos and trichlorfon was added to control shrimp. Fifteen grams of Na_2SO_4 and 30 mL of dry ethyl acetate were added to the shrimp sample. The sample was homogenized for 30 s and centrifuged at 1500*g*, 25 °C for 10 min. The supernatant was poured into a 200-mL pear-shaped flask containing 100 μL of *n*-hexadecane. Twenty milliliters of dry ethyl acetate was added to the extract in the centrifuge tube. The extract was homogenized and centrifuged as before. The supernatant was combined in the same pear-shaped flask. Ethyl acetate was evaporated completely using a rotatory evaporator and an oily residue was left in the pear-shaped flask. *n*-Hexadecane prevents the extract from going to dryness. The extract was dissolved in 5 mL of petroleum ether. A cyanopropyl SPE column was conditioned with 3 mL of diethyl ether followed twice by 5 mL of petroleum ether. The flow control valve on the SPE manifold was closed, and 1 mL of petroleum ether was added to the column. The extract was added to the SPE column, and the flow rate was adjusted to 1−2 drops per second. The pear-shaped flask was rinsed with 1 mL of petroleum ether, and the rinse was added to the SPE column. The SPE column was washed with 2 mL of dichloromethane/petroleum ether (10 + 90). A vacuum of 5 in. Hg was applied for 10 s to completely drain the SPE column. The analytes were eluted from the SPE column using 3 mL of methanol/diethyl ether (2 + 98) collected into a 15-mL glass centrifuge tube. A vacuum of 5 in. Hg was applied for 20 s to completely drain the SPE column. The extract was evaporated to dryness gently under nitrogen. The residue was reconstituted in 200 μL of toluene and analyzed within 24 h of preparation.

Chromatographic Conditions. The GC was operated using a constant flow rate of 1.8 mL/min (electronic pressure control) of helium. The injection volume was 1 μL. The analysis time was 30 min with an equilibration time of 5 min between analyses. The NPD temperature was 250 °C, and the bead current was 20−30 pA. The gas flow rates were air at 100 mL/min, helium make-up at 25 mL/min, and hydrogen at 3.5 mL/min. The column oven temperature was set at 80 °C for 1 min after sample injection. It was heated at 5 °C/min to 130 °C and held for 1 min. It was further heated at 5 °C/min to 190 °C, then at 20 °C/min to 230 °C, and maintained at 230 °C for 4 min. The cool on-column inlet temperature was set at 90 °C for 1 min. It was heated at 40 °C/min to 230 °C and held for 25 min. The purge activation time was 1 min. One blank solvent injection was made to equilibrate the system prior to the analysis of standards and samples.

System Suitability. The signal to noise ratio for 0.5 μg/mL trichlorfon and 0.5 μg/mL dichlorvos was typically ≥60:1. Noise levels in the five control shrimp samples were determined at the retention times for each drug and used to calculate the limit of detection (LOD) and limit of quantitation (LOQ) (*U.S. Pharmacopeia-National Formulary 1990*, 1989). The LOD represents a peak response of approximately 3 times the noise level . The LOQ represents a peak response of approximately 10 times the noise level. An LOD of approximately 3 ng/g was calculated for dichlorvos and 8 ng/g was calculated for trichlorfon. An LOQ of approximately 8 ng/g was calculated for dichlorvos and 14 ng/g was calculated for trichlorfon.

Calculations. Using peak heights and amounts of trichlorfon or dichlorvos on-column (ng), a standard curve was constructed by applying linear regression. The correlation coefficient was routinely ≥0.99 and the standard curves were

Table 1. Average Percent Recovery of Trichlorfon and Dichlorvos from Fortified Shrimp

fortification level (ng/g)	no. of samples analyzed	trichlorfon average % recovery	trichlorfon % CV	dichlorvos average % recovery	dichlorvos % CV
20	7	79	19	67	18
40	8	75	16	68	19
80	10	83	15	50	21

always linear. The concentration of dichlorvos or trichlorfon was determined using the following equation:

$$\frac{\text{ng}}{\text{g}} = \frac{A}{V_\text{i}} \frac{V_\text{f}}{W_\text{s}}$$

where A = nanograms of trichlorfon or dichlorvos on-column from standard curve; V_i, injection volume (μL); V_f, final volume (μL); W_s, sample weight (g).

RESULTS AND DISCUSSION

Table 1 demonstrates the performance of the method. The average percent recoveries and coefficients of variation (CVs) for shrimp fortified at 20, 40, and 80 ng/g trichlorfon were 79% (19%), 75% (16%), and 83% (15%), respectively. The average percent recoveries and CVs for shrimp fortified at 20, 40, and 80 ng/g dichlorvos were 67% (18%), 68% (19%), and 50% (21%), respectively. Control shrimp samples did not exhibit any interfering matrix peaks in the retention time windows of dichlorvos or trichlorfon. The method was used to analyze shrimp treated with trichlorfon or dichlorvos. The average concentration of trichlorfon residues obtained when shrimp were exposed to water containing 2 μg/mL trichlorfon for 2 h was 79 ng/g (n = 5) with a CV of 12%. The concentration of trichlorfon residues in shrimp increased with the exposure time (1−3 h) and the concentration in the exposure solution (0.2−3 μg/mL). There were no detectable residues of dichlorvos when shrimp were exposed to water containing 0.3 μg/mL trichlorfon for 1−3 h. Yet after 3 h, approximately half of the initial amount of trichlorfon in solution had degraded. The amount of dichlorvos measured in the exposure solution after 3 h was 0.1 μg/mL. Shrimp exposed to water containing 2 μg/mL of dichlorvos for 3 h resulted in 100% mortality. When shrimp carcasses were analyzed, the concentration of dichlorvos residues measured was close to the limit of quantitation. Dichlorvos may have been rapidly metabolized, or it may not have accumulated in shrimp muscle. Figure 2 shows chromatograms of (A) trichlorfon and dichlorvos matrix standard, 80 ng/g; (B) control shrimp; (C) fortified shrimp, 80 ng/g trichlorfon, and 80 ng/g dichlorvos; and (D) incurred shrimp, at 80 ng/g trichlorfon.

To test for specificity of the method, the chromatographic system was used to evaluate veterinary drugs with known use in aquaculture. The drugs analyzed include the following classes of compounds: sulfa drugs, tetracyclines, β-lactams, macrolides, nitrofurans, chloramine-T, methyltestosterone, trimethoprim, benzocaine, chloramphenicol, methylene blue, gentian violet, and malachite green. None of the drugs tested were found to interfere with the analysis of dichlorvos or trichlorfon. In addition, extracts of domestic and imported white shrimp and black tiger (*Penaeus monodon*) were analyzed using the chromatographic system. Analyses of black tiger shrimp resulted in higher recoveries for both trichlorfon and dichlorvos. Also the chromatograms acquired for extracts of black tiger shrimp showed fewer peaks from endogenous compounds than for extracts of white shrimp. No interfering matrix peaks were noted for the analyses of extracts of domestic control shrimp. An interfering matrix peak was observed in the retention time window for dichlorvos during the analyses of extracts of imported control shrimp. These extracts were reanalyzed by using a splitless inlet set at 150 °C and the same column oven temperature previously mentioned. The splitless inlet was not sensitive to the matrix effect observed with on-column injection. The peak shapes of both dichlorvos and trichlorfon in fortified control shrimp were very sharp and mirrored the peak shapes of the standards in neat solvent. Since dichlorvos is a decomposition product of trichlorfon, the fortified extracts containing both drugs and analyzed with a splitless inlet, were not quantified. These extracts were analyzed on a splitless inlet and an on-column inlet to compare the resolution of the interference peak from imported control shrimp in the chromatographic window of dichlorvos. The interference peak observed with on-column injection was resolved from dichlorvos using splitless injection.

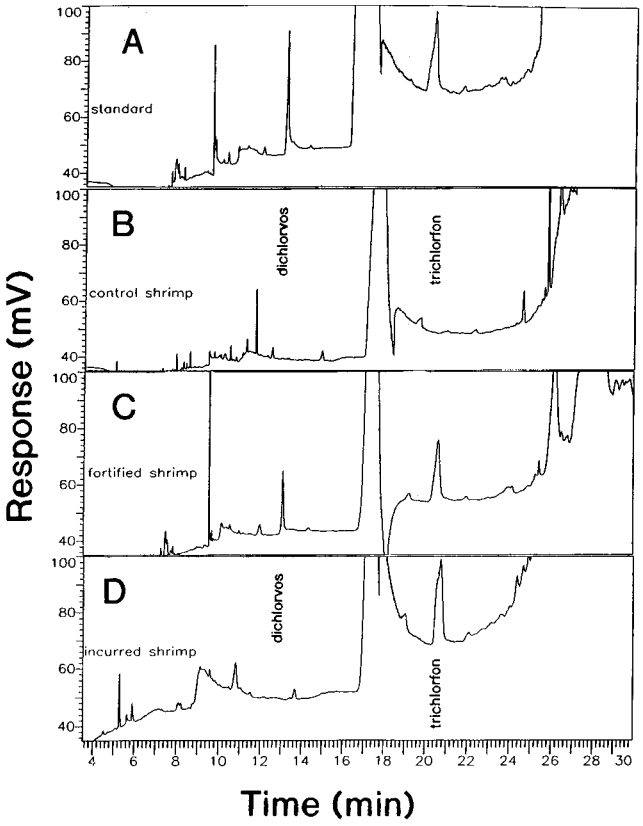

Figure 2. Chromatograms of (A) trichlorfon and dichlorvos matrix standard, 80 ng/g; (B) control shrimp; (C) control shrimp fortified at 80 ng/g dichlorvos and 80 ng/g trichlorfon; and (D) incurred shrimp, at 80 ng/g trichlorfon.

Dichlorvos is a very volatile compound. Use of a rotatory evaporator and a water bath at a temperature of 22 ± 3 °C to completely dry the ethyl acetate extract resulted in major losses of dichlorvos (Akhtar, 1982; Ivey and Claborn, 1969). The problem was overcome by using *n*-hexadecane to prevent the extract from drying completely. The elution solvent from SPE column, methanol/diethyl ether (2 + 98), was carefully evaporated using a slow stream of nitrogen. Nitrogen flow was stopped as soon as the extract was completely dry.

During development, various capillary columns were tested for the analyses of shrimp extracts. The most common stationary phases, methylpolysiloxane, (5% phenyl)-methylpolysiloxane, and (50% phenyl)-meth-

ylpolysiloxane microbore columns did not provide adequate separation. Good separation was achieved with a (14% (cyanopropyl)phenyl)-methylpolysiloxane microbore column (DB-1701, J & W Scientific). Extracts were injected directly into a retention gap, using a cool on-column inlet to prevent thermal decomposition of trichlorfon. Nonvolatile components from the matrix interacted with the retention gap causing broadening of trichlorfon and dichlorvos peaks compared to the peaks observed in neat solvent. This effect caused errors when standards prepared in neat solvent were used for quantitation. The peak areas were not used for quantitation because the dichlorvos peak showed some tailing in the matrix and also there was a small endogenous peak at the tailing end of the dichlorvos peak. To over come the matrix effect, the calibration standards were prepared in a tissue matrix. The retention gap was changed at the end of a series of analyses, and 5 cm was cut off the front end of the analytical column. The inlet and oven temperature programs were optimized to improve on the degree of separation of the analytes peak from the endogenous peaks and to eliminate sample carryover.

The GC was operated using a constant flow rate to maintain stability of the NPD during temperature programming. An applied bead current of 20−30 ρA, a detector temperature of 240 °C, and the following detector gases flow rates, air 100 ± 10 mL/min, hydrogen 3 ± 1 mL/min, and helium makeup gas 20 ± 5 mL/min, provided optimum conditions for the analysis of trichlorfon and dichlorvos. At higher applied currents and detector temperature, the background response was enhanced and the bead life was shortened. An applied current lower than 20 pA did not provide enough sensitivity for the analyses of dichlorvos.

The direct simultaneous identification and quantitation of dichlorvos and trichlorfon individually in shrimp is practical with GC analysis using a cool-on column inlet.

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