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Simultaneous Determination of Trichlorfon and Dichlorvos Residues in Olive Flounder (*Paralichthys olivaceus*) by Liquid Chromatography-Mass Spectrometry: Validation and Application to Pharmacokinetics

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Research Article

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Abbreviations: MRM: Multiple Reaction Monitoring; AChE: Acetylcholinesterase; MRL: Maximum Residue Limit; DP: Declustering Potential; S/N: Signal-to-Noise Ratio (S/N); AUC: Area Under Plasma Concentration-time Curve; λ2: Lambda z; (t1/2) Elimination Half-Life; Cmax: Maximum Plasma Concentration; Tmax: Time to Reach Cmax; MRT: Mean Residence Time; CL/F: Total Body Clearance (CL/F); Vz/F: Volume of Distribution.

ABSTRACT

Trichlorfon, an organophosphate insecticide, is used in aquaculture to control parasitic organisms. This study evaluated a rapid, sensitive, and specific LC-MS/MS method for the determination of trichlorfon and dichlorvos residues in the tissues of olive flounder. Separation was carried out on an Eclipse Plus C18 column by gradient elution using water-acetonitrile with 0.1% formic acid at a flow rate of 0.3 mL min^{-1}. Detection was performed by electrospray ionization in the positive ion mode with nitrogen as the collision gas. In multiple reaction monitoring (MRM) mode, ion transitions were detected at m/z 259→109 (trichlorfon) and m/z 221→108.9 (dichlorvos). Linear calibration curves were obtained with good correlation coefficients. The limit of detection (LOD) values for trichlorfon and dichlorvos were 0.5 and 1.2 μg kg^{-1}, respectively, with corresponding limit of quantification (LOQ) values of 1.7 and 4.0 μg kg^{-1}, respectively. The average recoveries ranged from 88.2% to 114% at three spiked concentration levels (5, 10, and 100 μg L^{-1}) with relative standard deviations (RSDs) below 13.8% for plasma, muscle, and liver. The developed analytical method was applied to pharmacokinetic studies of trichlorfon and dichlorvos in olive flounder after administration by dipping at concentrations of 1 or 5 mg kg^{-1}.

INTRODUCTION

Owing to an increase of parasitic infections in aquaculture, fish disease and productivity are raising significant concern [1]. To reduce economic loss, fish farmers have utilized alternative measures to control infections, such as chemical reagents.

Organophosphorus compounds are extensively used as pesticides or weedicides worldwide. Trichlorfon (dimethyl(2,2,2-trichloro-1-hydroxyethyl)phosphonate) **(Figure 1)** is an organophosphate insecticide used to destroy various insect pests, such as fish parasites in aquaculture, and control ectoparasites and endoparasites of aquatic species [2]. Furthermore, trichlorfon is the most commonly used chemical treatment in several countries for controlling sea lice, trematodes, nematocides, taenia, and acanthocephalans [3,4]. The most frequently suggested treatment includes the application of 0.1 to 1 mg L^{-1} of trichlorfon for 1 day [5]. Trichlorfon is also used to treat Alzheimer's disease and bilharzial dysentery in humans, under the name metrifonate [6].

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When trichlorfon is used under unstable conditions, such as high temperature or pH<5.5 [7], in sunlight [8], or in aerated water [9], it rapidly decomposes to dichlorvos, which is dangerous and poisonous to aquatic animals, including fish, crab, and shrimp [10]. In addition, dichlorvos, which is a broad-spectrum pesticide and acaricide, exhibits higher toxicity than its parent compound and is more lipid-soluble.

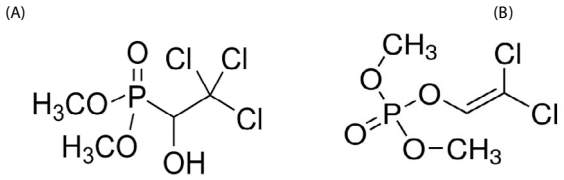

Figure 1. Chemical structures of trichlorfon (A) and dichlorvos (B).

Trichlorfon predominantly acts by inhibiting acetylcholinesterase (AChE) activity in the synaptic and neuromuscular junction of skeletal muscle, thereby altering the antioxidant defence system of an organism [11]. Moreover, trichlorfon has been reported to be effective for the treatment of various fish diseases in carp [12], Nile tilapia [10], sea bass [13], salmonid [14], and European eel [15], and fish farmers often use extensive amounts of trichlorfon in the aquatic environment for treatment. Therefore, trichlorfon, as well as its decomposition product, may exist in high concentrations, which in turn can cause intoxication and damage to human erythrocytes [16]. Because of the aforementioned possible health hazards to humans, regulatory levels have been established by the Food and Agricultural Organization/World Health Organization (FAO/WHO). In 2000, the FAO/WHO recommended maximum residue limits (MRLs) for trichlorfon in animal muscle, liver, kidney, and fat of 50 mg kg^{-1} [17]. However, only a few countries have established MRLs for trichlorfon in fish species. Thus, a method for monitoring trichlorfon and dichlorvos that are illegally stored in fish tissues is necessary to determine the hazards associated with human consumption.

Over the past few years, several approaches have been developed for the determination of trichlorfon and dichlorvos in fruits, shrimp, wheat, vegetables, plants, and water, such as gas chromatography [18-20], high performance liquid chromatography (HPLC) [21-23], electrochemiluminescence [24], and chemiluminescence [25], as well as an amperometric AchE biosensor [26] and an electrochemical biosensor [27]. However, GC monitoring can result in incorrect quantification caused by the thermal degradation of trichlorfon in the heated injector. Moreover, HPLC analysis of water, soil, and oil samples does not provide high sensitivity for the quantification of organophosphorus pesticide residues. In addition, the use of HPLC with UV detection for the determination of trichlorfon has been reported to exhibit lower sensitivity because of the incomplete absorptivity of trichlorfon [28]. To minimize these issues, liquid chromatography-mass spectrometry (LC-MS) and LC-MS/MS have gained popularity for the analysis of pesticides, particularly for polar compounds, including biological fluids, which are problematic during analysis by GC or GC-MS [29]. Several studies have reported that LC-MS analysis can provide high sensitivity for the determination of pesticide residues in foodstuff and human serum [29-31]. Kawasaki et al. [32] screened 21 organophosphorus pesticides in blood by LC-MS, and Klein and Alder [33] screened a range of pesticide residues by LC-MS/MS using matrix-matched standards. In particular, [17] have developed an LC-MS/MS method for the simultaneous determination of trichlorfon and dichlorvos residues in animal tissues. However, thus far, few studies on the determination of these two pesticides in aquatic organisms using LC-MS/MS have been published. For this reason, a sensitive, rapid, and expeditious method to identify and quantify pesticide residues in aquatic organisms is needed.

Olive flounder (*Paralichthys olivaceus*) is one of the most common commercially cultured fish species in East Asia, including Korea, Japan, and China [34]. Although several aquaculture farms in Korea use trichlorfon to control sea lice in olive flounder or sea bream, there are only a few official studies monitoring its dosage and usage in marine fish.

This aim of this study was the development and validation of a new, rapid, and selective LC-MS/MS method for the simultaneous detection of trichlorfon and dichlorvos residues in olive flounder. Quality criteria such as specificity, selectivity, linearity, sensitivity, accuracy, precision, matrix effects, and stability were employed to validate the method. In addition, we assessed the practicability of applying this method to pharmacokinetic studies after administration of trichlorfon to fish by dipping.

MATERIAL AND METHODS

Reagents and Chemicals

Trichlorfon (C4H8Cl3O4P) and dichlorvos (C4H7Cl2O4P) standards were purchased from Sigma Chemical Co. (St. Louis, MO) and Fluka (Buchs, Switzerland). Trichlorfon used for administration was purchased from Daesung Microbiological Labs Co., Ltd. (Seoul, South Korea). HPLC-grade methanol, n-hexane, acetonitrile, and water were obtained from Merck (Darmstadt, Germany).

Preparation of Standard Solutions

Individual standard stock solutions of trichlorfon and dichlorvos were prepared at concentrations of 1 mg mL^{-1} in methanol and stored at -20°C in sealed vials. Multi standard working solutions (2, 5, 10, 50, and 100 µg L^{-1}) were prepared by dilution of each of the above stock solutions by HPLC-water with 0.1% formic acid. These solutions were used to spike blank samples and prepare matrix-matched calibration solutions.

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Animals

Olive flounder with a mean weight of 302 ± 5 g and, no prior exposure to antibiotics was obtained from a local fish farm (Pusan, Korea). For the experiments, the fish used in the analysis were maintained in circular aquariums (capacity, 2 ton) with flow-through filtered seawater at 22°C.

Sample Extraction and Clean-up

Plasma

First, 0.5 mL of acetonitrile was added to 200 µL of a plasma sample, which was then vortex mixed for 10 min. Second, the sample was centrifuged at 9,000 rpm at 4°C for 10 min. Third, the upper clear layer was filtered using a 0.2 mm membrane (Advantec, Tokyo, Japan), and then transferred to an auto sampler vial for LC-MS/MS analysis.

Muscle or liver

A 2 g aliquot of the muscle or liver sample was added to a test tube containing 20 mL of acetonitrile. After these samples were homogenized for 2 min, the tubes were subjected to shaking for 10 min using a vortex mixer, followed by centrifugation at 13,000 rpm at 4°C for 10 min. The supernatant was poured into a 200 mL pear-shaped flask and evaporated to dryness at 40°C using a rotary evaporator (Eyela, Tokyo, Japan). The obtained dry residue was reconstituted two times with 10 mL of acetonitrile-saturated n-hexane, transferred into a test tube, and then shaken for 10 min. The mixed solution was allowed to separate, and the hexane layer was removed. The eluate was collected and re-evaporated to dryness at 40°C using a rotary evaporator. The residue was reconstituted with 1 mL of 50% methanol and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatant was filtered using a 0.2 mm membrane (Advantec, Tokyo, Japan) prior to LC-MS/MS analysis within 24 h of preparation.

Chromatographic and Mass Spectrometer Operating Conditions

LC-MS/MS analysis of the samples was conducted on an Agilent liquid chromatographic system (Agilent 1290 Infinity) coupled with an Agilent 6430 Triple Quad LC/MS system (Agilent Technologies, Santa Clara, CA). The separation of trichlorfon and dichlorvos was performed using an Eclipse plus C18 column (2.1 × 100 mm, 1.8 µm, Agilent Technologies). Mobile phases A and B were degassed HPLC-water and acetonitrile, respectively, each with 0.1% formic acid. A and B were used according the gradient shown in **Table 1**, with a total run time of 17 min. Separation was carried out at a sampler temperature of 10°C and a column temperature of 40°C, with a flow rate of 0.3 mL min^{-1} and injection volume of 10 µL.

Table 1. Elution gradient for simultaneous determination of trichlorfon and dichlorvos.

Time (min)	A (vol%)[a]	B (vol%)[b]
0	90	10
1	90	10
7	20	80
9.5	20	80
10	90	10
15	90	10

Notes: [a]HPLC-water with 0.1% formic acid. [b]acetonitrile with 0.1% formic acid.

The analytes were identified and quantified using a mass spectrometer equipped with electrospray ionization (ESI) source operating in the positive ionization mode. Multiple reaction monitoring (MRM) mode was selected for the quantification of trichlorfon and dichlorvos, with the following precursor to product ion transitions and corresponding parameters: trichlorfon, m/z 259 → 109 with a declustering potential (DP) of 70 V and a collision energy (CE) of 11 eV; dichlorvos, m/z 221 → 108.9 with a DP of 80 V and a CE of 12 eV. The first and most abundant MRM transition was used for quantification, whereas the other transitions were used for qualification. **Table 2** summarizes the optimized MRM conditions and retention times for trichlorfon and dichlorvos. The following ionization source parameters were employed: capillary voltage, 4000 V; nebulizer gas, N2; nebulizer gas flow rate, 11 L min^{-1}; nebulizer pressure, 40.0 psi; gas temperature, 350°C. Data acquisition and processing were carried out using the Mass Hunter software (ver. A.00.06.32; Agilent Technology).

Table 2. Optimized MS/MS operating conditions for the analysis of trichlorfon and dichlorvos.

Compounds	RT (min)	Parent ion (m/z)	MRM Transitions (m/z)	DP (V)	CE (eV)	Ionization
Trichlorfon	4.6	259	109[a]	70	11	ESI+
		259	221[b]	70	5	
		259	79[b]	70	9	
Dichlorvos	6.1	221	108.9[a]	80	12	ESI+
		221	79.1[b]	80	10	

Notes: RT, retention time; DP, declustering potential; CE, collision energy. [a]Transitions for quantitative analysis. [b]Transitions for qualitative analysis.

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Assay Validation

Selectivity

To assess interference by endogenous compounds, six sources of fish were screened and compared by utilizing chromatographic-MS/MS conditions with the retention times for the blank plasma or muscle sample. And a mixture of trichlorfon and dichlorvos was spiked with the blank plasma or muscle sample.

Calibration

Calibration curves were constructed using concentrations of 0.1, 1, 2, 5, 10, 20, 50, and 100 μg L^{-1}, which were obtained by serial dilution of a mixture containing trichlorfon and dichlorvos. The calibration curves constructed using matrix-matched standards were compared to those obtained from neat samples. The neat sample calibration curve was estimated using quality control (QC) samples. The QC samples were prepared using 0.2 mL of blank plasma at three concentrations (10, 50, and 100 μg L^{-1}) of trichlorfon and dichlorvos.

Sensitivity

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined as the minimum concentration of standard analytes in the spiked blank plasma. The LOD and LOQ for trichlorfon and dichlorvos were defined as the response at a signal-to-noise ratio (S/N) of 3.3 and 10, respectively.

Accuracy and precision

The precision of the method was tested by measuring both the intra-day and inter-day precisions of the standard solutions. The intra-day precision was determined from five replicates of QC samples spiked with mixtures of trichlorfon and dichlorvos at 10, 200, and 2000 μg L^{-1} on the same day (repeatability), whereas the inter-day precision was determined over five successive days (reproducibility). These two parameters were expressed as the relative standard deviation of the result (RSD%). The benchmark for the acceptability of the data was accuracy within ±15% of the theoretical concentration and precision within ±15%.

Extraction recovery and matrix effect

The recoveries of trichlorfon and dichlorvos were obtained by comparison of the mean peak areas obtained for the QC samples, which were post-extracted by the analytical procedure, with nominal concentration levels of 10, 50, and 100 μg L^{-1} trichlorfon and dichlorvos. The matrix effect was assessed by analysing standards of the two compounds dissolved in the mobile phase and standards spiked into the extracts of three matrices: plasma, muscle, and liver. The response peak area ratios of the two compounds from each matrix group were compared.

Stability

To determine the stability of the stock solution, three replicates of trichlorfon and dichlorvos stock solutions were freshly prepared. The response under different temperature conditions and times was compared with that obtained for the fresh stock solution in plasma. The plasma samples were subjected to three freeze-thaw cycles, as well as studies conducted utilizing short-term and long-term conditions. The stability of the auto sampler was evaluated by re-analyzing the extracted analyte to determine the effect of delays in analysis over 24 or 48 h at 4°C. All stability studies were conducted at concentration levels of 10, 200, and 2000 μg L^{-1} using three replicates of QC samples. The analyte was considered stable if the responses of the stored and fresh samples differed by less than 15%.

Application to pharmacokinetic study

To assess the applicability of the optimized method, a pharmacokinetic analysis of trichlorfon and dichlorvos in olive flounder was conducted by dipping. During the acclimation period, fish were maintained for 3 weeks at 22°C in seawater to ensure that all individuals were healthy and feeding. The fish were fed twice a day with commercial feed (Woosungfeed, Daejeon City, Korea), but they were starved 1 day prior to conducting studies. Control fish were kept separately in a clean tank under the same conditions. In each treatment group, 10 fish were maintained in a 100 L tank at trichlorfon concentrations of 1 and 5 mg kg^{-1} at 22°C for 1 h. After administration, each fish was removed from the dipping tank and immediately transferred into clean seawater. Each test tank was evaluated using 10 replicates, with blood collected from each fish at 6 h, 12 h, 24 h, 2 days, 4 days, 7 days, 14 days, and 21 days. Blood was collected from the caudal blood vessel using a heparinized 3 mL syringe within 1 min after administration. The plasma samples were immediately separated by centrifugation at 9,000 rpm for 10 min at 4°C and stored in a freezer at -70°C until analysis.

The pharmacokinetic parameters were calculated using WinNonlin 5.1 (Pharsight Corporation, Mountain View, CA) according to the manufacturer's directions. The area under the plasma concentration-time curve (AUC) from 0 to 720 h after administration was calculated using the linear trapezoidal rule and Simpson's rules (Pharmacologic Calculation System, Version 5.1, 2006). The data are expressed as mean ± standard deviation for all experiments.

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RESULTS AND DISCUSSION

Optimization of LC-MS/MS

The separation and simultaneous determination of the two target pesticides using LC-MS/MS was optimized. To achieve good peak shapes and short run times, we tested different mobile phases, such as acetonitrile and methanol, as the organic phase in preliminary experiments. We considered additives to water, such as formic acid, which are favourable for the electrospray process; such additives result in high ionization of pesticides and good retention times for polar compounds. The flow rate and gradient elution were utilized to obtain symmetric peaks and sufficient data points for each compound. A water-acetonitrile mobile phase including 0.1% formic acid provided symmetric peaks with efficient separation at a flow rate of 0.3 mL min^{-1}, with a total run time of 17 min. A C18 column was used for the separation because such columns have been reported to increase the retention time of trichlorfon [28-30].

The molecular structure of the targets was elucidated and quantified by confirmatory analysis with MRM. **Figure 2** shows the MS/MS product scan spectra of the target analytes obtained in the positive ion mode. To monitor the maximum response of the product ions and two or three precursor ions, we selected the parent ion in full scan mode and searched for the fragment ions by utilizing the declustering potential and collision energies. **Table 2** summarizes the optimized MS/MS conditions for analysis of trichlorfon and dichlorvos. Electrospray ionization of trichlorfon and dichlorvos produced $[M+H]^+$ ions at 259 and 221, respectively, in the positive ionization mode, which were used for quantification and confirmation. The protonated forms of trichlorfon and dichlorvos were monitored as precursor ions, and the fragment ions identified from the spectra at m/z 109 and m/z 108.9, respectively, were produced as the prominent product ions **(Figure 2).** To observe the maximum response, the fragmentation conditions and collision energy were optimized for each analyte. Therefore, a quantitative analysis was performed in MRM mode to obtain high sensitivity and selectivity: m/z 259→221 and 79 for trichlorfon, and m/z 221→79.1 for dichlorvos.

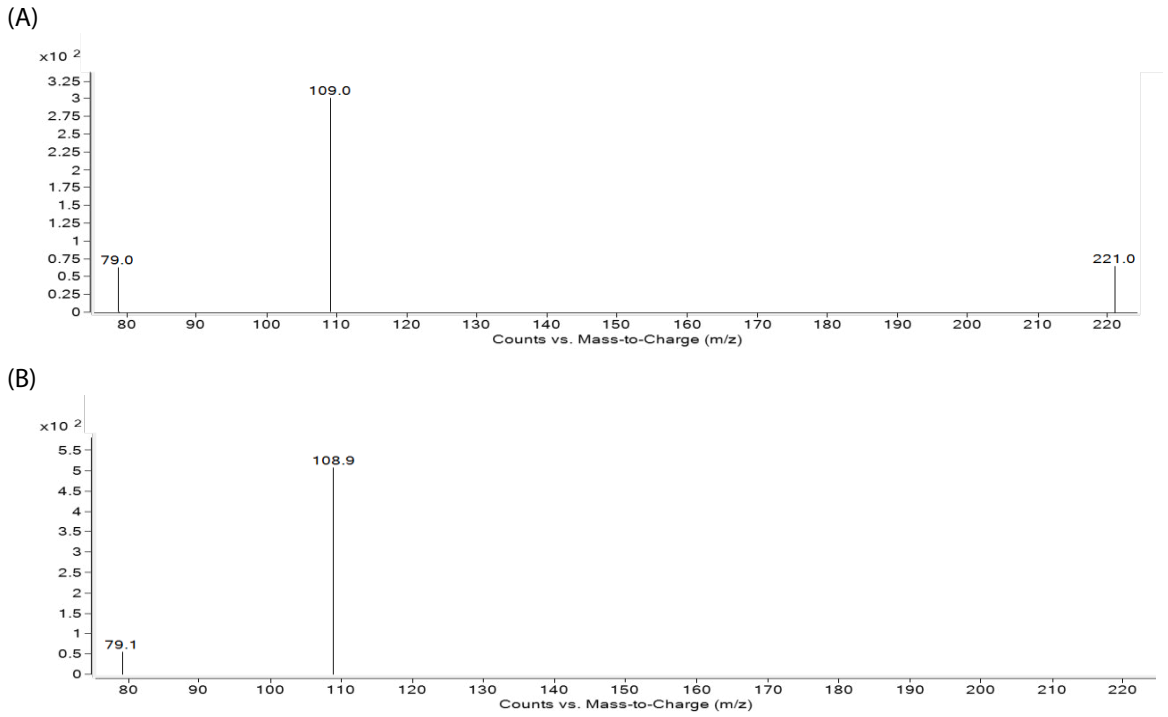

Figure 2. Mass spectra of trichlorfon (A) and dichlorvos (B) obtained using electrospray ionization in the positive ionization mode.

Extraction

Because of the high amount of organic matter in biological tissues, the selective extraction of pesticides is complicated. Owing to the polar and thermally labile character of the target compounds, acetonitrile was chosen as the solvent, which, owing to its polarity, resulted in good extraction. As fish muscle and liver contain fat matrices, it is necessary to remove lipids by clean extraction. The most commonly used solvent is n-hexane, which can dissolve fat, and this solvent provided good recoveries for the pesticides after sample homogenization. Trichlorfon is easily converted into dichlorvos at high temperatures, and hence, the experiments were conducted at 4 °C.

Assay Validation

Specificity and selectivity

A specificity study was conducted to confirm the absence of endogenous substances at the retention times of the studied analytes. **Figure 3** shows typical chromatograms of blank fish plasma or muscle samples spiked with trichlorfon and an olive

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flounder sample after dipping for the pharmacokinetic study. The retention times of trichlorfon and dichlorvos are approximately 4.6 and 6.1 min, respectively. Moreover, the retention times in the blank plasma and muscle samples were the same as in the samples after dipping at a dose 1 mg kg^{-1}. No interfering peaks from endogenous or exogenous compounds were observed in the chromatograms of the blank plasma or muscle at the retention times of trichlorfon and dichlorvos.

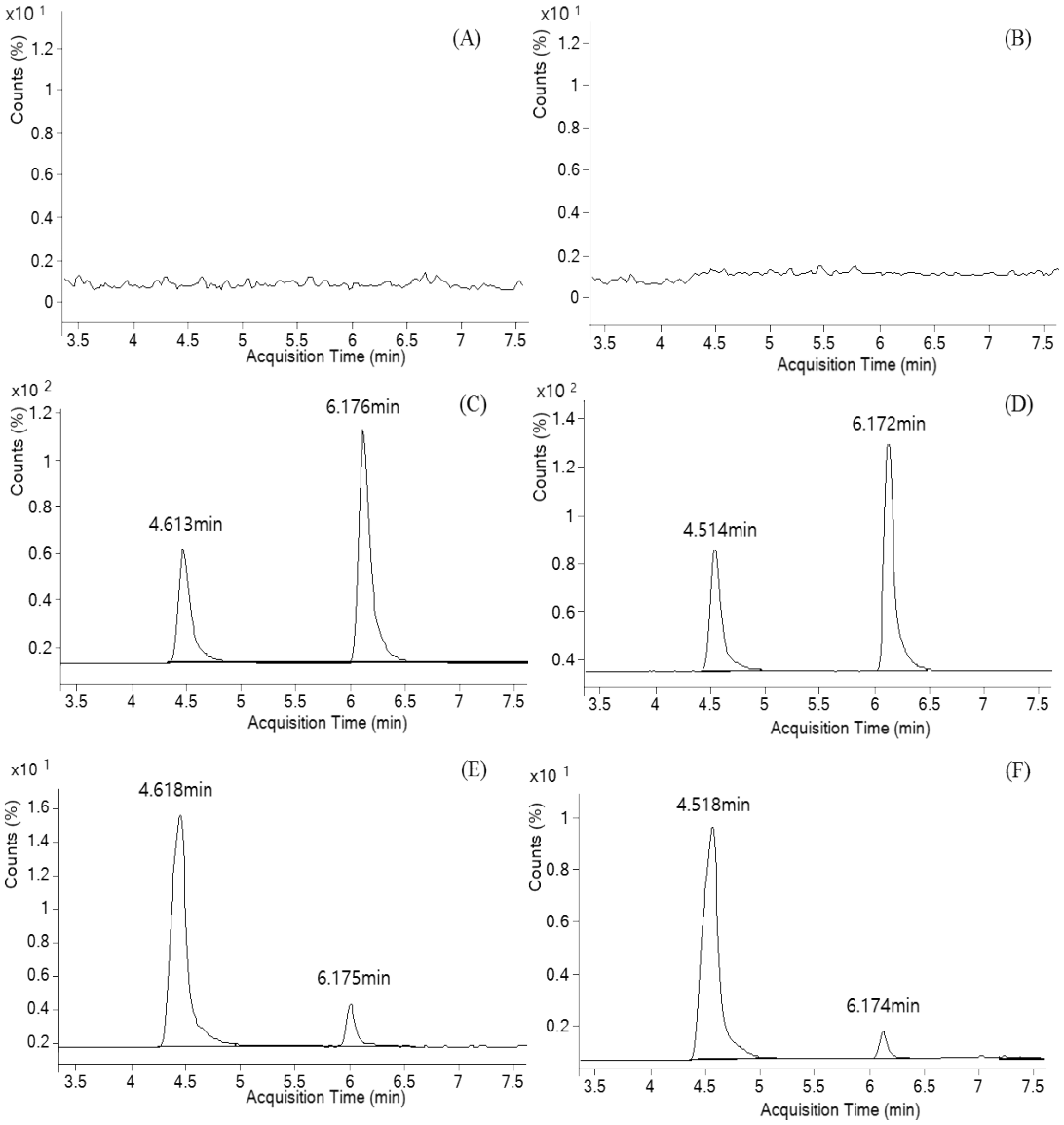

Figure 3. MRM LC-MS/MS chromatograms of trichlorfon and dichlorvos: (A) blank fish plasma, (B) blank fish muscle, (C) blank fish plasma, (D) blank fish muscle spiked with 100 ng mL^{-1} of trichlorfon and dichlorvos, and (E) plasma, (F) muscle sample 6 h after administration of 1 mg kg^{-1} trichlorfon by dipping

Calibration and linearity

Calibration curves were obtained by analysing the peak area of the analytes in the chromatograms. A mixture of trichlorfon and dichlorvos standards was serially diluted to obtain samples in the range from 0.1 to 100 µg L^{-1}, which were analysed using the optimized method. The equation of the trichlorfon calibration curve was y = 295.5x + 144.2 (y: peak area, x: trichlorfon concentration, n=5) with a coefficient of determination r2=0.999, and the equation of the dichlorvos calibration curve was y=690.8x + 111.4 (y: peak area, x: dichlorvos concentration, n=5) with a coefficient of determination r2=0.999. These results show that good linearity was observed for both trichlorfon and dichlorvos.

Sensitivity

The LOD and LOQ values for trichlorfon and dichlorvos were determined using the minimal accepted S/N values of 3.3 and 10, respectively **(Table 3).** The LOD values of trichlorfon and dichlorvos were 0.5 and 1.2 µg kg^{-1}, respectively. The LOQ values of trichlorfon and dichlorvos were 1.7 and 4.0 µg kg^{-1}, respectively. The LOD values obtained by our assay were lower than those obtained in the studies by Hem et al. [29] and Zhu et al. [30] whereas the LOQ values were higher than those obtained in a previous study by Wang et al. [17]. Although higher sensitivity could be obtained by using an extremely sensitive mass spectrometer, these limits are sufficient for analysis of the target compounds in olive flounder.

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Table 3. Validation results from analysis of spiked plasma samples analysed in trichlorfon and dichlorvos

Compounds	LOD (µg/kg^{-1})[a]	LOQ (µg/kg^{-1})[b]	Calibration Curve[c]	R2	Recovery[d]	Mean (µg/kg^{-1}) ± RSD(%)[e]
Trichlorfon	0.5	1.7	y=295.5x + 144.2	0.999	103.1	103.5 ± 2.2
Dichlorvos	1.2	4.0	y=690.8x + 111.4	0.999	108.4	101.8 ± 2.5

Notes: [a]Limit of detection; [b]Limit of quantification; [c]x=concentration of trichlorfon or dichlorvos (µg/kg-1), y= intensity; [d]Accuracy was studied using the recoveries of the compounds. The recoveries were determined by spiking blanks samples (plasma) at a level of 100 µg kg^{-1} with the standard mixture solution. [e]Relative standard deviation for n=3.

Accuracy and precision

QC samples at three different concentrations (10, 200, and 2000 µg L^{-1}) were assessed in five replicates to determine the intra- and inter-day precision and accuracy. **Table 4** summarizes the intra- and inter-day precision and accuracy for olive flounder plasma samples. The intra-day accuracies for trichlorfon ranged from 98.8%-101.4%, and the intra-day precision was ≤2.1%. The inter-day accuracies for trichlorfon ranged from 99.1%-112%, and the precision was ≤3.2%. Moreover, the intra-day accuracies for dichlorvos ranged from 97.7%-114%, and the intra-day precision was ≤2.6%. The inter-day accuracies for dichlorvos ranged from 96%-101.9%, and the precision was ≤3.1%. The accuracy and precision values were found to be satisfactory as they are within a good range (80%-120%).

Table 4. Accuracy and precision of trichlorfon and dichlorvos in olive flounder plasma.

Concentration (µg L^{-1})		Intra-day (n=5)			Inter-day (n=25)		
		Measured concentration	Accuracy, mean recovery (%)	Precision (RSD, %)	Measured concentration	Accuracy, mean recovery (%)	Precision (RSD, %)
Trichlorfon	10	10.5	105	2.1	11.2	112	1.7
	200	202.7	101.4	1.4	205.3	102.7	3.2
	2000	1975.6	98.8	0.7	1981.5	99.1	2.8
Dichlorvos	10	11.4	114	2.6	9.6	96	0.4
	200	195.4	97.7	1.1	203.7	101.9	2.9
	2000	1981.2	99.1	0.9	1992.9	99.6	3.1

Extraction recovery and matrix effect

Matrix effects caused by endogenous interference in the samples not detected by MS/MS could decrease or increase the ion intensity of the analyte. To assess the matrix effect on analysis quantitatively, the area obtained for a neat solution was compared to that of the area obtained for a blank matrix sample spiked with the pesticide after extraction. By assessing these response ratios, the suppression or enhancement of the signal can be quantitatively evaluated [35]. These effects were evaluated by comparing the plotted area with the concentration of the extracts from three matrices (plasma, muscle, and liver) after spiking with three different concentrations of analytes in five replicates **(Table 5).**

Table 5. Recoveries of trichlorfon and dichlorvos and matrix effects in spiked samples (n=5).

Compounds	Matrix	Spiking levels (µg L^{-1})	Mean recovery (%, n=5)	RSD[a], range (%)
Trichlorfon	Plasma	5	101.2	1.1
		10	105.0	2.1
		100	98.8	2.3
	Muscle	5	88.4	4.3
		10	95.2	6.5
		100	91.7	5.2
	Liver	5	106.4	10.4
		10	108.3	13.8
		100	98.1	9.7
Dichlorvos	Plasma	5	108.2	1.8
		10	114.0	2.6
		100	115.2	2.9
	Muscle	5	102.1	5.6
		10	98.7	6.1
		100	95.8	6.3
	Liver	5	88.2	8.5
		10	93.7	10.4
		100	92.5	12.7

Note: [a]RSD, relative standard deviation.

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A matrix effect is considered to exist if variation of the response and precision is observed (i.e. the plotted peak area is <85% or >120%). However, the recoveries for trichlorfon ranged from 98.8%-105.0% in plasma, 88.4%-98.8% in muscle, and 98.1%-108.3% in liver. Moreover, the recoveries observed for dichlorvos ranged from 108.2%-115.2% in plasma, 95.8%-102.1% in muscle, and 88.2%-93.7% in liver. In each case, the RSD range was <14%, indicating the absence of matrix effects. Thus, this method is applicable for the detection of residues from different samples, and the use of this method can result in both time and cost savings.

Stability

Table 6 shows the stability of trichlorfon and dichlorvos in the plasma samples of olive flounder following exposure to different storage conditions at three concentration levels (10, 200, and 2000 µg L^{-1}) in three replicates. The plasma samples were stable after three freeze-thaw cycles, with concentrations ranging from 99.2%-103.6% trichlorfon and 98.6%-104.2% dichlorvos. The stability of the auto sampler was investigated over different times, and the concentrations of trichlorfon and dichlorvos in the processed samples at 24 h ranged from 83%-99.4% and from 90.4%-104.7%, respectively. Moreover, the concentrations of trichlorfon and dichlorvos in the processed samples at 48 h ranged from 92.7%-109.5% and from 103.1%-113.47%, respectively. The plasma samples showed short-term stability, with 86.4%−107.6% trichlorfon and 87.1%−110.9% dichlorvos, and long-term stability, with 100.5%−114.7% trichlorfon and 98.4%−97.0% dichlorvos. These results show that the plasma samples do not undergo any significant loss of trichlorfon and dichlorvos, indicating that these compounds are stable under typical treatment, processing, and storage conditions.

Table 6. Stability of trichlorfon and dichlorvos under different storage conditions (n=3).

Storage conditions Nominal concentration (µg L^{-1})	Stability (%)	
	Trichlorfon	Dichlorvos
Freeze/thaw stability (3cycles)		
10	99.2 ± 0.4	100.5 ± 0.1
200	102.3 ± 1.2	98.6 ± 1.7
2000	103.6 ± 1.5	104.2 ± 2.2
Auto-sampler stability (24h at 4 °C)		
10	93.9 ± 0.4	100.3 ± 1.4
200	83 ± 1.2	90.4 ± 2.5
2000	99.4 ± 3.3	104.7 ± 4.7
Auto-sampler stability (48h at 4 °C)		
10	95.1 ± 0.8	103.1 ± 2.2
200	92.7 ± 2.9	113.47 ± 1.5
2000	109.5 ± 4.6	110.3 ± 8.6
Short-term stability (4h at room temperature)		
10	86.4 ± 1.1	87.1 ± 0.9
200	91.2 ± 3.7	94.8 ± 4.8
2000	107.6 ± 8.5	110.9 ± 9.3
Long-term stability (4weeks at -80 °C)		
10	100.5 ± 0.7	98.4 ± 0.6
200	108.1 ± 7.2	99.2 ± 5.1
2000	114.7 ± 10.1	97.0 ± 8.2

Application to pharmacokinetic study

The developed assay was applied to the detection and determination of the residues in real samples that had been administrated trichlorfon. **Figure 4** shows the plasma concentration-time curves of trichlorfon and dichlorvos that were obtained following administration of the pesticides by dipping at a dose of 1 or 5 mg kg^{-1}. **Table 7** summarizes the pharmacokinetic parameters, such as lambda z (λ_2), which is estimated from linear regression of the terminal data points, elimination half-life ($t_{1/2}$), which is calculated using $t_{1/2}=0.693/\lambda_2$, maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), mean residence time (MRT), area under the plasma concentration-time curve from 0 to infinity ($AUC_{0-\infty}$), estimation of the total body clearance (CL/F), and volume of distribution (V_z/F).

The results of the optimized assay confirmed the presence of trichlorfon and dichlorvos residues in treated olive flounder. Following administration of 1 mg kg^{-1} trichlorfon, C_{max} of trichlorfon was 3.1 ± 0.5 ng mL^{-1} with T_{max} of 6.0 ± 0.0 h. The $t_{1/2}$ and $AUC_{0-\infty}$ values were 19.6 ± 4.1 h and 93.3 ± 15.7 ng mL^{-1} h, respectively. However, dichlorvos residues were not detected after administration of 1 mg kg^{-1} trichlorfon. Following administration of 5 mg kg^{-1} of trichlorfon, C_{max} of trichlorfon was 31.8 ± 3.7 ng mL^{-1} with T_{max} of 6.0 ± 0.0 h. The $t_{1/2}$ and $AUC_{0-\infty}$ values were 14.4 ± 2.8 h and 616.2 ± 25.2 ng mL^{-1} h^{-1}, respectively. C_{max} of dichlorvos was 4.36 ± 0.8 ng mL^{-1} with T_{max} of 6.0 ± 0.0 h. The $t_{1/2}$ and $AUC_{0-\infty}$ values were 6.7 ± 1.3 h and 42.37 ± 8.5 ng mL^{-1} h^{-1}, respectively.

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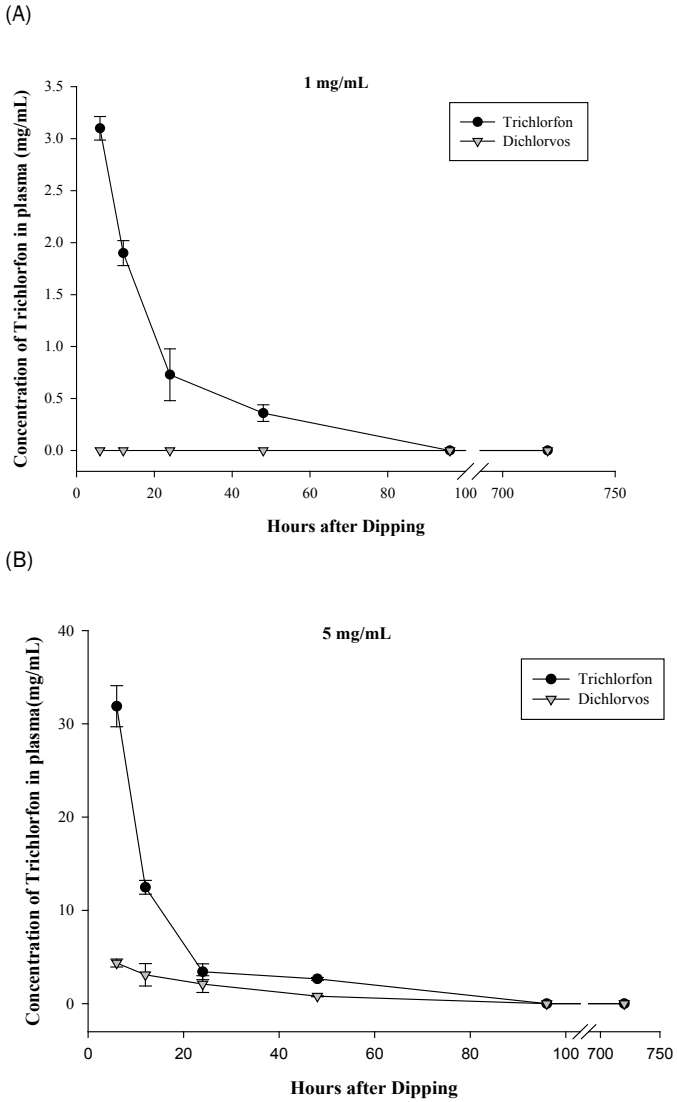

Figure 4. Mean plasma concentration versus time profiles of trichlorfon and dichlorvos after administration of trichlorfon to olive flounder by dipping at 1 mg kg^{-1} (A) and 5 mg kg^{-1} (B) (mean ± SD, n=10)

Table 7. Pharmacokinetics parameters measured for administration of trichlorfon to olive flounder by dipping at a dose of 1 and 5 mg kg^{-1} (mean ± SD; n=10).

Parameter	Trichlorfon		Dichlorvos	
	1 mg kg^{-1}	5 mg kg^{-1}	1 mg kg^{-1}	5 mg kg^{-1}
λ_2(1/h)	0.035 ± 0.001	0.048 ± 0.002	N.D	0.102 ± 0.003
$t_{1/2}$(h)	19.6 ± 4.1	14.4 ± 2.8		6.7 ±1.3
C_{max}(ng/mL)	3.1 ± 0.5	31.8 ± 3.7		4.36 ± 0.8
T_{max}(h)	6.0 ± 0.0	6.0 ± 0.0		6.0 ± 0.0
MRT(h)	31.6	23.15		9.92
$AUC_{0-\infty}$(ng/mL h)	93.3 ± 15.7	616.2±25.2		42.37 ± 8.5
CL/F(mL/h)	0.01	0.008		0.19
V_z/F(mL)	0.3	0.16		0.01
Note: N.D., not detected.				

These results indicate that trichlorfon and dichlorvos residues, which are mainly utilized as organophosphorus insecticides, are detected in the plasma samples when either a low or a high concentration of trichlorfon is administered. Currently, there is little published data on the pharmacokinetic properties of trichlorfon residues in olive flounder. However, Eškinja [36] reported that trichlorfon and dichlorvos residues in blood samples from rats are not detected 60 min after the administration of 300 mg kg^{-1} of trichlorfon and 2.5 mg kg^{-1} dichlorvos by i.v. inoculation. Koyama [37] reported that the concentration of trichlorfon is ten times higher than that of dichlorvos in blood from dogs 6 h after administering 200 mg kg^{-1} of trichlorfon. Although the 1 and 5 mg kg^{-1} dosage of trichlorfon in live flounder in this study is smaller than those used in previous studies, the results confirm similar observations with respect to the pharmacokinetic parameters. These results suggest that the validated method is appropriate for assessing pharmacokinetic studies of the administration of trichlorfon and dichlorvos in marine fish [36,37].

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CONCLUSION

A specific, sensitive, and rapid LC-MS/MS method for the simultaneous determination of trichlorfon and dichlorvos in olive flounder was developed and validated. The analytical method was validated using criteria such as specificity, selectivity, linearity, sensitivity, accuracy, precision, matrix effects, and stability. The method has a short running time and simple sample preparation procedure.

Using this method, pharmacokinetic studies were conducted following administration of a 1 or 5 mg kg^{-1} trichlorfon dosage to olive flounder by dipping. Although both trichlorfon and dichlorvos were detected in the plasma initially, the compounds had a short duration of effect (<96 h). Moreover, the LOQ was sufficient to detect the residues in the terminal time.

It is necessary to obtain data related to the application of organophosphorus compounds to different fishes. The data acquired from monitoring can be utilized to determine MRLs for residues in fish species. Our results confirm the need for continuous monitoring of trichlorfon residues in marine fish, and these results can aid in developing guidelines for using this pesticide in aquaculture.

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