Thesis for the Degree of Master of Science

Simultaneous determination of trichlorfon and dichlorvos residues in Olive Flounder (*Paralichthys olivecues*) by LC-MS/MS : Validation and application to pharmacokinetic study

by

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Pukyong National University

February 2016

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LC-MS/MS를 이용한 넙치 내 트리클로폰과 디클로보스 잔류량 동시분석법 개발 및 약물동태학적 연구

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By

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February 26, 2016

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LC-MS/MS를 이용한 넙치내 트리클로폰과 디클로보스 잔류량 동시분석법 개발 및 약물동태학적 연구

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요 약

Trichlorfon 은 어류의 기생성 질병의 치료에 사용되는 구충제로, choline-esterase 활성을 저하시키는 유기인계 물질이다. 흡충, 조충, 선충, 구두충 등 acetylcholine 을 신경전달물질로 이용하는 기생생물에 약효를 발휘한다. 이는 물속에서 점차 dichlorvos 로 전환되고 trichlorfon 보다 약 100 배이상의 강력한 AChE 억제제이다. Trichlorfon 의 dichlorvos 의 전환은 수중 내의 수온, pH, 주광 및 산소량에 크게 영향을 받는다. 국내에서 Trichlorfon 은 담수어인 잉어 및 뱀장어 사용 용법·용량이 설정되어 있으나, 해산어류에 대해서 용법·용량에 대한 연구없이 사용되고 있는 실정이다.

따라서 본 실험의 목적은 LC-MS/MS 를 이용한 넙치 내의 trichlorfon 과 dichlorvos 의 잔류량 분석법을 개발하고 이에 따른 약물동태학적 특성을 연구하였다. 분석용 Column 은 Eclipse Plus C18 column (2.1 x 100mm, 1.8μm)를 사용하였고, 이동상 용매조성으로는 0.1% formic acid 를 포함한 water 과 0.1% formic acid 를 포함한 acetonitrile 으로 flow rate 는 0.3mL min^{-1} 의 기울기(gradient)조건으로 분석하였다. 시료 전처리 과정에서는 유기용매, 원심분리기, 회전감압농축기를 사용하였다. Trichlorfon 과

dichlorvos 의 ESI-MS/MS spectrum 은 positive 이온화 모드에서 분석을 하였다. Trichlorfon 의 딸이온은 m/z 109, 221 및 79 로 나타났으며 어미이온으로부터 m/z 109 가 이온세기가 가장 크고 안정하기 때문에 Trichlorfon 의 정량이온(quantification)으로 선택하였다. Dichlorvos 의 딸이온은 m/z 108.9, 79.1 로 정량이온은 108.9 로 선택하였다. 표준곡선은 trichlorfon 과 dichlorvos 의 표준용액의 각 농도 (0.1 - 100 μg l^{-1}) 에 따라 측정한 후 얻은 상관계수 R^2 값은 0.999 의 높은 직선성을 보여주었다. Trichlorfon 과 dichlorovos 의 검출한계는 각각 0.5 μg kg^{-1}, 1.2 μg kg^{-1} 나타났고, 정량한계는 1.7 μg kg^{-1} 와 4.0 μg kg^{-1} 로 나타났다. Trichlorfon 과 dichlorvos 의 넙치 혈장, 근육 및 간의 평균회수율은 88.2 – 114%로 변동계수는 13.8% 이하로 나타났다. 그리고 분석방법을 적용하여 넙치에 trichlorfon 의 농도에 따른 침지법으로 시간에 따른 약물동태학적 특성을 알아보았다. 본연구에서 실험한 분석방법으로 수산용 동물용 의약품의 잔류허용기준을 설정하는데 실용적인 기본자료로 활용될 수 있을 것이다.

Ⅰ. 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, such as chemical reagents.

Organophosphorus compounds have been extensively used as pesticides or weedicides worldwide. Trichlorfon (dimethyl(2,2,2-trichloro-1-hydroxyethyl)phosphonate) (Fig. 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, it 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 1day [5]. Trichlorfon is also used to treat Alzheimer's disease and bilharzial dysentery in humans, under the name metrifonate [6]. 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 that of the main compound, and it is more lipid-soluble.

Trichlorfon predominantly acts by inhibiting acetylcholinesterase (AChE) activity in the synaptic and neuromuscular junction of skeletal muscle, thereby altering the antioxidant defense 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 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 that the maximum residue limits (MRLs) of trichlorfon in animal muscle, liver, kidney, and fat should be 50 mg kg^{-1} [17]. However, only a few countries have reported the MRLs of 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, monitoring by GC 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 been gaining popularity for the analysis of pesticides, particularly 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 [30] and human

serum [29]. Kawasaki et al. [31] screened 21 organophosphorus pesticides in blood by LC-MS. Klein and Alder [32] screened a range of pesticide residues by LC-MS/MS using matrix-matched standards. In particular, Wang et al. [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 commercially cultured fish species in East Asia, including Korea, Japan, and China [33]. 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 study aimed to develop and validate 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 its application to pharmacokinetic studies after administration of trichlorfon to fish by dipping.

Ⅱ. Materials and methods

1. Reagents and Chemicals

Trichlorfon ($C_4H_8Cl_3O_4P$) and dichlorvos ($C_4H_7Cl_2O_4P$) 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).

2. 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℃ in sealed vials. A multistandard working solution (2, 5, 10, 50, and 100 µg L^{-1}) was prepared by dilution of each of the above stock solutions by HPLC-water with 0.1% formic acid. This solution was used to spike blank samples and prepare matrix-matched calibration solutions.

3. 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℃.

4. Sample extraction and clean-up

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

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℃ for 10 min. The supernatant was poured into a 200 mL pear-shaped flask and evaporated to dryness at 40℃ 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 then separated, and the hexane layer was removed. The eluate was collected and re-evaporated to dryness at 40℃ using a rotary evaporator. The residue was reconstituted with 1 mL of 50% methanol and centrifuged at 12,000 rpm at 4℃ 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.

5. Chromatographic and mass spectrometer operating conditions

For sample analysis, LC-MS/MS analysis 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 C_{18} 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 mode shown in Table 1, with a total run time of 17 min. Separation was carried out at a sampler temperature of 10℃ and a column temperature of 40℃, with a flow rate of 0.3 mL min^{-1} and, injection volume of 10 µL.

The analytes were identified and quantified using a mass spectrometer equipped with an 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, while the others 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, N_2; nebulizer gas flow rate, 11 L/min; nebulizer pressure, 40.0 psi; gas temperature, 350℃. Data acquisition and processing were carried out using the Mass Hunter software (ver. A.00.06.32; Agilent Technology).

Table 1. Gradient elution for simultaneous determination of trichlorfon and dichlorvos

Time	A	B
0	90	10
1	90	10
7	20	80
9.5	20	80
10	90	10
15	90	10

6. Assay validation

6.1. 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.

6.2. Calibration

Calibration curves were constructed using concentrations of 0.1, 1, 2, 5, 10, 20, 50, and 100 μg L^{-1} 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 0.2mL blank plasma at three concentrations (10, 50 and 100 μg L^{-1}) of trichlorfon and dichlorvos.

6.3. 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.

6.4. 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 solutions at 10, 200, and 2000 μg L^{-1} during the same day (repeatability), and 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%.

6.5. 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 analyzing 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.

6.6. 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 autosampler was evaluated by reanalyzing the extracted analyte to determine whether delays occurred during analysis over 24 or 48 h at 4℃. 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%.

6.7. 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℃ 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 mg kg^{-1} and 5 mg kg^{-1} at 22℃ 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℃ and stored in a freezer at -70℃ 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 720h 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.

Ⅲ. Results & Discussion

1. Optimization of LC-MS/MS

The separation and simultaneous determination of the two target pesticides was optimized using LC-MS/MS. 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 favorable for the electrospray process; such additives result in high ionization of pesticides and exhibit 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, 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].

The molecular structure of the target was elucidated and quantified by confirmatory analysis with MRM. Fig. 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 optimal 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 (Fig. 2). To observe the maximum response, the fragmentation conditions and collision energy were optimized for each analyte. Therefore, the 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.

(A) **(B)**

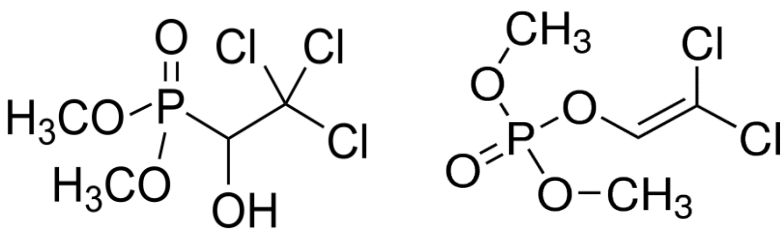
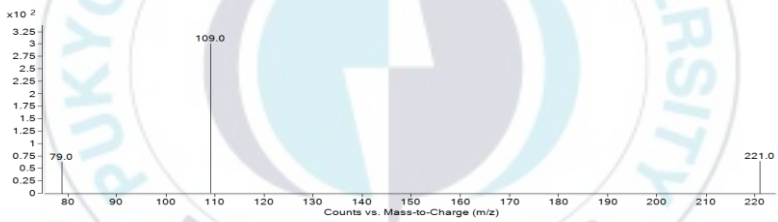


Fig 1. Chemical structures of trichlorfon (A) and dichlorvos (B)

(A)



(B)

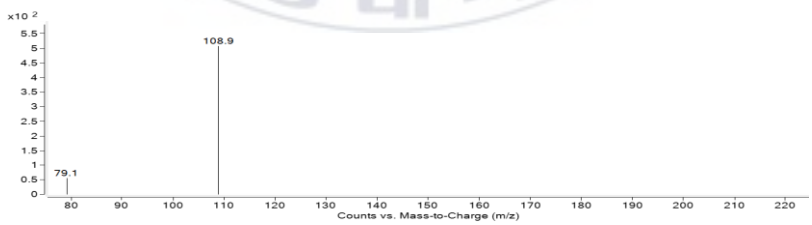


Fig 2. Mass spectra of trichlorfon (A) and dichlorvos (B) in positive ionization mode**.**

Table 2. MS/MS optimal operational 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		259	109[a]	70	11	ESI+
		259	221[b]	70	5	
		259	79[b]	70	9	
DDVP		221	108.9[a]	80	12	ESI+
		221	79.1[b]	80	10	

RT: Retention time

DP: Declustering portential

CE: Collision energy

[a]: Transitions for quantitative peaks

[b]: Transitions for qualitative peaks

2. 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 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 maintained at 4℃.

3. Assay validation

3.1. Specificity and selectivity

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

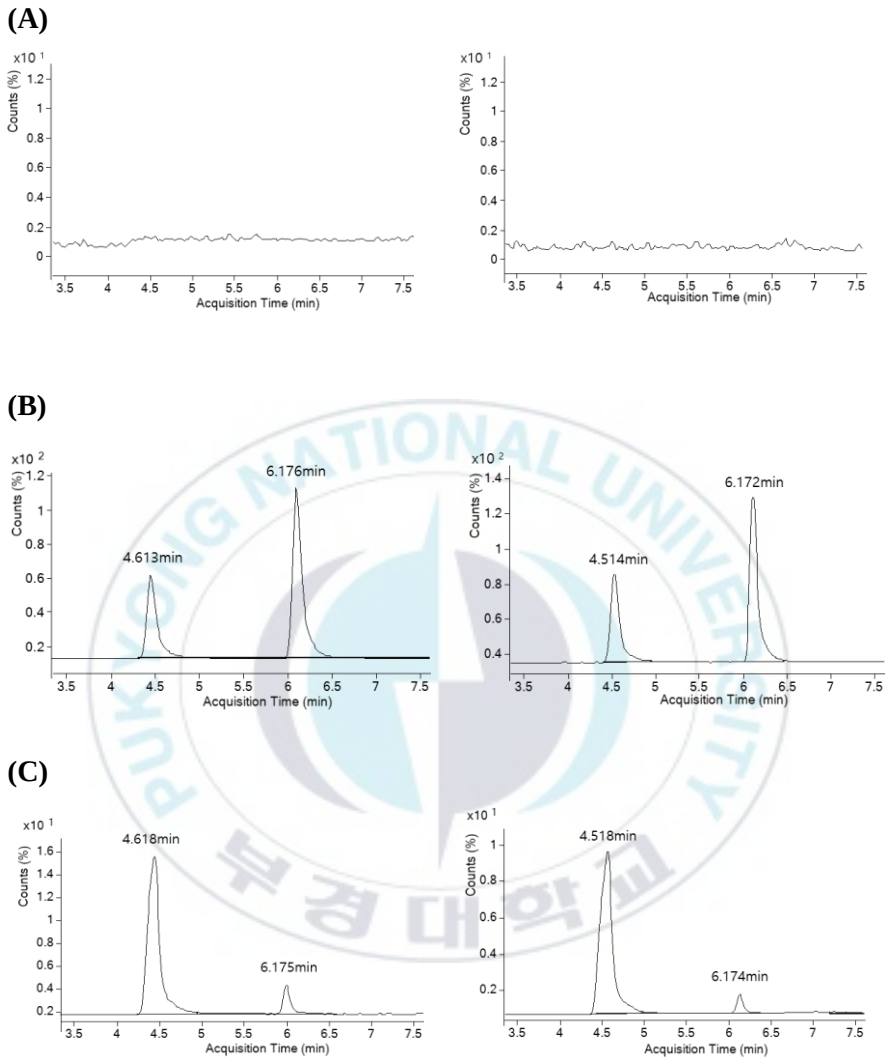


Fig 3. MRM LC-MS/MS chromatograms of trichlorfon and dichlorvos: (A) Blank fish plasma/muscle, (B) Blank fish plasma/muscle spiked with 100ng/mL of trichlorfon and dichlorvos and (C) a plasma/muscle sample at 6h after administration of dipping at a dose of 1mg/kg trichlorfon.

3.2 Calibration and Linearity

Calibration curves were obtained by analyzing 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 analyzed using the optimized method. The equation of the trichlorfon calibration curve was y = 295.5 x + 144.2 (y: peak area, x: trichlorfon concentration, n = 5) (Fig. 4) with a coefficient of determination r^2 = 0.999 and the equation of the dichlorvos calibration curve was y = 690.8 x + 111.4 (y: peak area, x: dichlorvos concentration, n = 5) (Fig.5) with a coefficient of determination r^2 = 0.999. These analysis results showed that good linearity was observed for both trichlorfon and dichlorvos.

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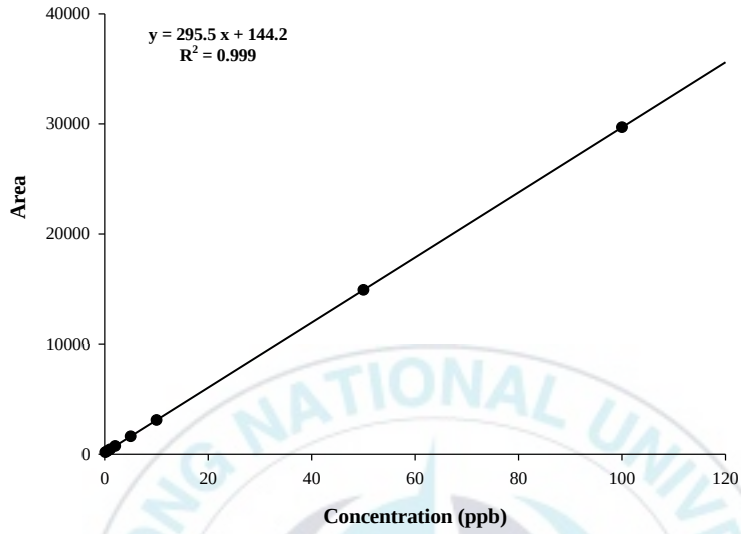


Fig. 4. Calibration curve used for the quantification of trichlorfon level.

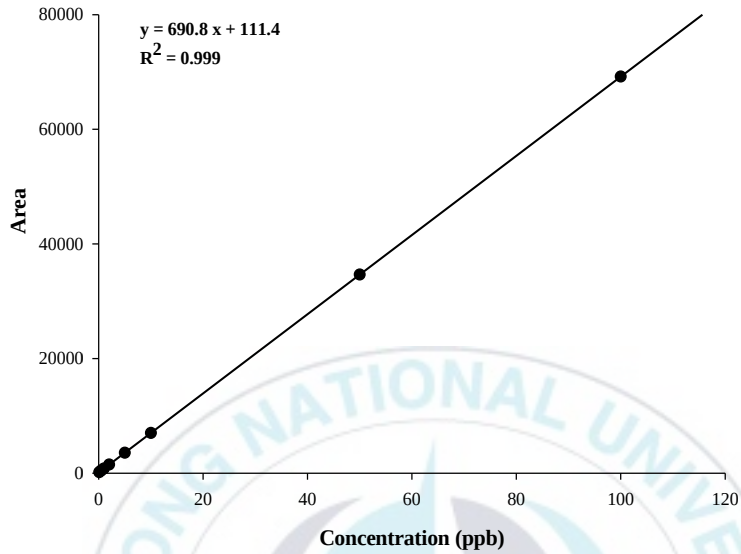


Fig. 5. Calibration curve used for the quantification of dichlorvos levels.

3.3. 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. [34] and Zhu et al. [35], 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.

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

Compounds	LOD(μg/kg)[a]	LOQ(μg/kg)[b]	Calibration curve[c]	R^2	Recovery[d]	Mean (μg/kg)±RSD(%)[e]
Trichlorfon	0.5	1.7	y= 295.5 x + 144.2	0.999	103.1	103.5±2.2
DDVP	1.2	4.0	y = 690.8x + 111.4	0.999	108.4	101.8±2.5

[A] Limit of detection

[B] Limit of quantification

[C] X= Concentration of trichlorfon or dichlorvos (μg/kg), Y= intensity

[D] Accuracy was studied by the determination of the recoveries of the compounds. Recoveries was determined by spiking at the level of 100μg/kg standard mixture solution to the blank samples (plasma)

[E] Relative standard deviation for n=3

3.4. 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% to 101.4%, and the intra-day precision was ≤2.1%. The inter-day accuracies for trichlorfon ranged from 99.1% to 112%, and the precision was ≤3.2%. Moreover, the intra-day accuracies for dichlorvos ranged from 97.7% to 114%, and the intra-day precision was ≤2.6%. The inter-day accuracies for dichlorvos ranged from 96% to 101.9%, and the precision was ≤3.1%. The accuracy and precision values were found to be satisfactory and indicative of a good range from 80% to 120%.

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

Concentration ($\mu g\ l^{-1}$)		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

3.5. 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 from a neat solution was compared to that of the area obtained from spiking a blank matrix sample with the pesticide after extraction. By assessing these response ratios, the suppression or enhancement of the signal can be quantitatively evaluated [36]. 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). If variation is observed with respect to response and precision (i.e., the plotted peak area is <85% or >120%), then a matrix effect exists.

However, the recoveries for trichlorfon ranged from 98.8% to 105.0% in plasma, 88.4% to 98.8% in muscle, and 98.1% to 108.3% in liver. Moreover, the recoveries observed for dichlorvos ranged from 108.2% to 115.2% in plasma, 95.8% to 102.1% in muscle, and 88.2% to 93.7% in liver. In all cases, the RSD ranges were <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.

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

Compounds	Matrix	Spiking levels ($\mu g\ l^{-1}$)	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
Dichlorovos	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	88.4	8.5
		10	93.7	10.4
		100	92.5	12.7

[a]RSD : Relative standard deviation

3.6. 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, ranging from 99.2% to 103.6% trichlorfon and 98.6% to 104.2% dichlorvos. The stability of the autosampler was investigated over different times, and the concentrations of trichlorfon and dichlorvos in the processed samples at 24 h ranged from 83% to 99.4% and 90.4% to 104.7%, respectively. Moreover, the concentrations of trichlorfon and dichlorvos in the processed samples at 48 h ranged from 92.7% to 109.5% and 103.1% to 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 showed that the plasma samples do not undergo any significant loss of trichlorfon and dichlorvos, which were observed to be stable under typical treatment, processing, and storage conditions.

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

Storage conditons 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℃)		
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℃)		
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℃)		
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

3.7. Application to pharmacokinetic study

The developed assay was applied to the detection and determination of the residues in real samples that were administrated trichlorfon. Fig. 6 shows the plasma concentration-time curves of trichlorfon and dichlorvos that were obtained following administration of the pesticides at a dose of 1 or 5 mg kg^{-1} by dipping. Table 7 summarizes the pharmacokinetic parameters, such as the lambda z (λ_2), which is estimated by 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 ($AUC_{0-\infty}$), estimate of the total body clearance (CL/F), and volume of distribution (V_z/F).

With the optimized assay, the results confirmed the presence of trichlorfon and dichlorvos residues. Following administration of 1 mg kg^{-1} trichlorfon, C_{max} of trichlorfon was 3.1 ± 0.5 ng/mL 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 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 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 h, respectively. C_{max} of dichlorvos was 4.36 ± 0.8 ng/mL 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 h, respectively.

These results indicate that trichlorfon and dichlorvos residues, which are mainly utilized as organophosphorus insecticides, are detected in the plasma samples when a low or high concentration of trichlorfon is administered. Currently, there is little published data on the pharmacokinetic properties of trichlorfon residues in olive flounder. However, Eškinja [37] 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 [38] reported that the concentration of trichlorfon is ten times higher than that of dichlorvos in blood from dogs 6 h after administration of 200 mg kg^{-1} of trichlorfon. Although the dosage of 1 and 5 mg kg^{-1} of trichlorfon in live flounder in this study is smaller than those reported in previous studies, the observed results confirm similar observations with respect to the pharmacokinetics parameters. These results suggest that the validated method is appropriate for assessing pharmacokinetic studies of the administration

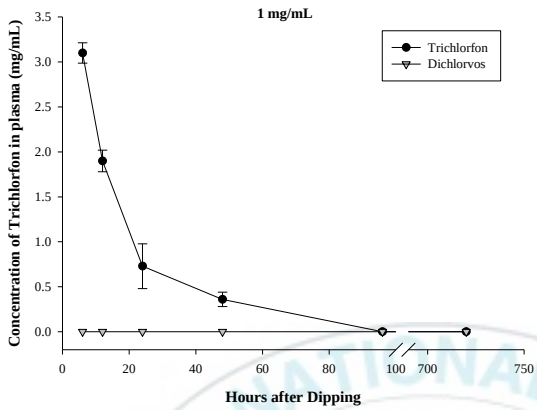
of trichlorfon and dichlorvos in marine fish.

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

Parameter	Trichlorfon		Dichlorvos	
	1mg kg^{-1}	5mg kg^{-1}	1mg kg^{-1}	5mg 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

N.D: Not detected

(A)



(B)

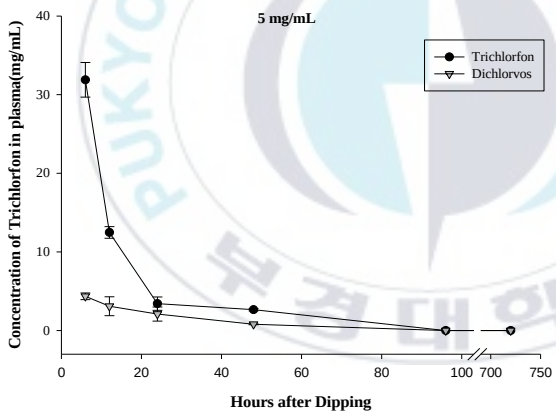


Fig. 6. Mean plasma concentration versus time profiles of trichlorfon after administration of dipping at 1mg kg^{-1} (A) and 5mg kg^{-1} (B) to olive flounder (mean ± SD, n=10).

Ⅳ. 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 administering a trichlorfon dosage of 1 of 5 mg kg^{-1} to olive flounder by dipping. Although both compounds were detected in the plasma initially, the resulting values had a short duration (within 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 confirmed 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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분자생물학실험실에 김무상박사님, 안상중박사님 감사드립니다. 클로닝 단어조차 모르는 저에게 차근차근 설명해주신 그 배려심, 본받겠습니다. 그리고 저에게 실험을 가르쳐준 분생실험실 모든 선배들께 감사드립니다.

과학원 방역과 언니들과 오빠들, 1년동안 분기별로 찾아갔을때마다 함께 밥도 같이 먹고, 얘기도 많이하고, 진짜 저도 방역과 인줄 알았어요 ㅋㅋ 이렇게 소중한 인연을 또 만들어가네요. 감사합니다. 그리고 준영이오빠, LC에 L자도 모르는 저에게 스탠다드 잡는방법부터 가르켜주시고 정말 감사합니다. 잘되실거에요!

우리 영원한 10동기들, 우리 다같이 졸업하는구나. 이것도 인연인가봐. 하씨, 니덕분에 실험실에 무거운짐들 반은 다 옮겼다.고마워! 준성이오빠, 먼저 취직할줄 몰랐네ㅋ 열심히 일해서 사장되라~ 인기오빠, 기장에서 은대구랑 오래있지말고 얼른나와서 미오랑만두랑겨울이랑 한번 다같이 놀아요. 권총, 우리방에서 캡스톤할려고 왔을때 못받아줘서 미안하다~ 몇 안되는 우리 여자 동기들. 수진아 나도 졸업한다! 기방에 니가 있어야되는데ㅋ 개미, 취직 축하한당~ 가은이, 나의 평생 약사가 되주렴. 소미, 니덕분에 3개월동안 진짜 편안하게 실험실에서 웃었다. 내가 진짜 고마워하는거 알제? 그리고 사랑하는 내 남자친구, 신후오빠. 오빠덕분에 여기까지 무탈하게 온것같아. 힘들때마다 도와주고, 위로와 응원덕분에 내가 변할 수 있었어, 많이 사랑하고 고마워.

그리고 나 자신에게 자랑스럽다는 말을 전하고 싶습니다. 혼자서 씩씩하게 실험하고 데이터를 정리하고 논문투고까지 해냈습니다. 제 바램과 달리 실험실 사람들이 다 나가게 되었고, 혼자 감당해야 할 일과 책임감에 많이 힘들고 날카로워지고 지쳐갔지만, 저를 사랑해주는 선배들과 동생들, 친구들, 10동기들 그리고 사랑하는 남자친구가 없었더라면 아마 전 어디선가 방황하고 있겠죠. 인간관계에 대해서도 많이 알게되었고, 세상엔 다들 이런사람, 저런사람이 있구나 하고 뼈저리게 느낀 석사생활이었습니다. 이제 저는 힘든 길을 걸어갈려고 합니다. 제가 선택한 길이 옳다고 믿으며 열심히 저의 20대를 살아가겠습니다. 비온뒤에 땅이 굳어지듯이 어떤 상황이 닥치더라도 중심을 잃지 않고 잘 헤쳐나가는 사람이 되겠습니다. 다시 한번 많은 분들께 감사드리고 사랑합니다.