Determination of Phorate and Its Metabolite Phorate Sulfone Residues in the Egg by High Performance Liquid Chromatography-tandem Mass Spectrometry

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Abstract: In this study, we present a newly developed fast and sensitive method for the quantification of phorate and its metabolite phorate sulfone residues in the egg, using solid phase extraction and UPLC-MS/MS analysis.The eggs sample were extracted with acetonitrile under neutral conditions in this paper , cleaned up by the glass funnel plugged with cotton and salts equipped with anhydrous sodium sulfate and neutral alumina, and determinatied by waters high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). There are good linear correlations between the peak area and the corresponding concentration of phorate and its metabolite phorate sulfone in the range of 2.00-100.00 ng/mL and the linear coefficient of the three pesticides is above 0.9995. The recovery rate of phorate and its metabolite phorate sulfone ranged from 78.6% to 95.6%, and with the batch relative deviation ranged from 0.3% to 5.6%. The lower limit detection (LOD) of the phorate sulfone, phorate and phorate sulfoxide were 0.0005 mg/kg , 0.0005mg/kg and 0.0005 mg/kg, respectively. The phorate, phorate sulfone and phorate sulfoxide's quantitative（LOQ）were 0.0015 mg/kg, 0.0015 mg/kg and 0.0015 mg/kg, respectively. The results show that UPLC-MS/MS in conjunction with phorate and its metabolite phorate sulfone is a powerful tool for screening the inhibitory effects of eggs and can thus be used for safety control in food processing procedures.

1 INTRODUCTION

In normal cases, phorate and the metabolites of phorate sulfone are a pale yellow liquid, soluble in organic solvents, and the ability to dissolve in water is worse (Wang et al., 2017; Abbruzzese, 2002; Mo et al., 2011). It is relatively a stable and hydrolyses only in very basic or acidic conditions. It is very toxic both for target organisms and formammals including human and inhibits acetylcholinesterase and butyrylcholinesterase (Monu et al., 2015; Liu et al., 2017). Phorate and its metabolite phorate sulfone were most commonly applied in plant samples, which is non-biocumulative and has no residual action. But some metabolites may persist in soil. Phorate also damages some seeds, which is absorbed readily through all ways (Lucía et al., 2015; Pravin, 2015; Zhao and Huang, 2017). The toxicity of phorate is high. For example, oral LD50 was given to 8.0 mg/kg mice, and 1.1-3.2 mg/kg to rats (technical parameters) (Guan et al., 2008; Torres and Manes, 1996; Sun et al., 2006).

In China, it is forbidden to use phorate and the metabolites of phorate sulfone in food stuffs, well they have been found contaminating in different food products and their presence is regularly reported, which is harmful to the health of people and the survive of creatures (Maurice et al., 2007; Gerardo et al., 2014; Lucía et al., 2015). Drug abuse in the egg and food safety issues, of phorate and its metabolites phorate sulfone determination has need to adopt a simple, good reproducibility analysis method in various eggs (Kaushik et al., 2012). To the best our knowledge, phorate and its metabolite phorate sulfone determined simultaneously by the high liquid chromatography-tandem mass spectrometry in the eggs has not been found.

In this paper, the aim of this work is to introduce a reliable high liquid chromatography-tandem mass spectrometry method for quantification of phorate and its metabolite phorate sulfone in eggs from

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different habitats. Lastly, a sensitive, simple, re-enactment of a good and efficient liquid chromatography tandem mass spectrometry (UPLC-MS/MS), which is used for the selection of phorate and its metabolite phorate sulfone in the egg samples.

2 EXPERIMENTAL

2.1 Reagents and Standards

Through the Milli-Q water system (Millipore, Bedford, MA, USA) purification ultra-pure water, was used to prepare all the buffer and the sample solution. Acetonitrile, methanol, hexane and formic acid were of HPLC grade and purchased from Fisher Scientific (Fair lawn, New Jersey, USA). Technical grade phorate and its metabolite phorate sulfone, purity >95% were provided by the Agro-Environmental Protection Institute, Ministry of Agriculture (Tianjin, China). All other chemicals and solvents such as diethylamine, methanol, hydrochloric acid, neutral alumina and acetone were supplied by Guangzhou Chemical Reagent Factory (Guangzhou, China).

Stock solution of phorate and its metabolite phorate sulfone were prepared by dissolving in 25 mL acetonitrile and stored in the dark at 4℃ in the dark for use within 2 month, respectively. The working solutions were prepared by direct dilution of the stock solution with acetonitrile immediately before use.

2.2 Apparatus

Equipped with electrospray ionization (ESI) interface (Waters Corp, Milford, MA, MA), a high liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis was carried out using an ACQUITY triple-quadrupole tandem mass spectrometer. Chromatographic separation was separated using a Waters C18 (50 mm × 2.1 mm, 1.7 μm) column.

2.3 UPLC-MS/MS Condition

The mobile phase consisted of A (acetonitrile) and B (0.1% formate acid water) at a flow rate of 0.30 mL/min. A linear gradient elution was performed as described below in the Table 1. The column and auto-sampler temperatures were set at 35℃ and 10℃, respectively.

For three compounds, the positive ion mode of electrospray ionization (ESI+) was chosen for phorate and its metabolite phorate sulfone. Quantification was achieved by multiple reactions monitoring (MRM) mode. Conditions of the ESI source were optimized as follows: the capillary was set at 4.0 kV, desolvation gas temperature was 600℃. The conditions of cone voltage, collision energies and the most sensitive ion transitions were performed as described below in the Table 2.

Table 1: Mobile phase gradient and flow rate.

Time(min)	A%	B%	Flow rate (mL/min)
0	5	95	0.30
0.50	5	95	0.30
3.00	70	30	0.30
3.50	5	95	0.30
5.00	75	25	0.30

Table 2: UPLC-MS/MS parameters for determination phorate and its metabolite phorate sulfone. Note: "*" referred to the qualitative ions.

Elution order	Analyte	Parent ion	Daughter ion	Cone(v)	Collision (v)
1	Phorate	261.100	111.100*	10	12
			124.800	10	32
2	Phorate sulfoxide	277.000	96.900*	15	32
			142.800	15	20
3	Phorate sulfone	293.100	143.100*	15	30
			171.100	15	24

2.4 Sample pretreatment

2.4.1 Extraction

An amount egg samples of 2.00 g spiked which were grounded up and homogenized through the corresponding national egg sample preparation standards were accurately weighed into a 50 mL polypropylene centrifuge tube. The egg samples were placed in room temperature for about 60 min before proceeding, and 10 mL acetonitrile solution were added into the tube. After vortexing for 3 min, the 50 mL polypropylene centrifuge tube was shaken vigorously on the vibrator for 10 min, and then centrifuged at 4℃, 10000 rpm for 10 min. The upper organic layer was then transferred to another 50 mL polypropylene centrifuge tube. The lower aqueous layer was extracted once again with 10 mL of acetonitrile. The extracts were combined and evaporated to dryness at 50℃. The residue was dissolved with 2 mL of acetonitrile for cleanup.

2.4.2 Cleanup

The glass funnel plugged with cotton and salts equipped with anhydrous sodium sulfate and neutral

alumina, which was conditioned with about 5 mL acetonitrile solution. The sample solution which have being purified were transferred into the separatory funnel. Finally, the analyte was eluted with 5 mL of acetonitrile. The elution was evaporated to dryness under a gentle nitrogen stream at 50℃. The residue was dissolved with 2 mL of acetonitrile: water (volume ratio: 1:1) and filtered through a 0.22 μm syringe filter for further UPLC-MS/MS analysis.

2.5 Preparation of Standard and Quality Control (QC) Samples

Standard stock solutions of phorate and its metabolite phorate sulfone were prepared by dissolving the accurately learned reference compounds in acetonitrile at the concentrations of 1000.0 ng/ml, respectively. A series of working standard solutions of phorate and its metabolite phorate sulfone were prepared by diluting standard stock solution with acetonitrile: water (volume ratio: 1:1) at appropriate concentrations. All solutions were stored in a 4 ℃ freezer and incubate at room temperature for about 30 min before proceeding.

Calibration samples and QC samples were prepared according to sample preparation item. The final concentrations of egg sample were 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/ml for phorate and its metabolite phorate sulfone. QC samples were obtained in the same manner with three levels of 10.0, 20.0 and 50.0 ng/ml for phorate and its metabolite phorate sulfone.

3 RESULTS AND DISCUSSION

3.1 Optimization of Extraction Procedure

In order to optimize the extraction procedure, several variables were screened for optimization of the extraction step, including extraction solvent, pH value and extraction temperature.

Based on the molecular structure and their solubility, phorate and its metabolite phorate sulfone were generally extracted from various matrices using organic solvents such as methylene chloride, ethyl acetate, methanol, or acetonitrile. Different extraction with different solvent systems were used to extract phorate and its metabolite phorate sulfone from egg samples spiked at 0.02 mg/kg. The results depicts the obtained recoveries of phorate and its metabolite phorate sulfone for three kinds of egg samples in Table 3. The results show that, when methylene chloride, ethyl acetate and methanol were used as the extraction solvents, low recoveries of phorate and its metabolite phorate sulfone were obtained. However, when acetonitrile as the extraction solvent in neutral environment, the higher recoveries from egg samples were obtained. However, acetonitrile gave more easily in operation and is less toxic. Thus, acetonitrile was chosen as extraction solvent in this study.

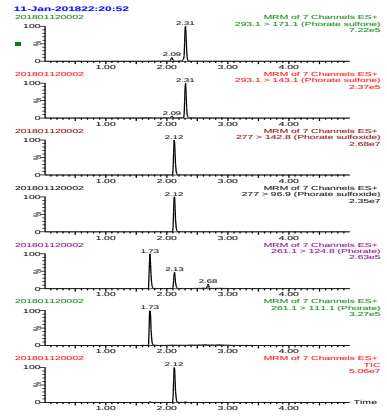
3.2 Optimization of Chromatographic Condition

3.2.1 Selection of Constant Volume of Solvent

For significantly improving the peak separation and shape efficiency during UPLC-MS/MS analysis, different solvents were used such as: methanol, acetonitrile, 0.1%, formic acid water, acetonitrile: water (volume ratio: 1:1) and 5 mmol/L, ammonium acetate solution. After several trials, it was found that the phorate and its metabolite phorate sulfone of spectra can not be effective separation when selecting the methanol, acetonitrile, 0.1%, formic acid water and 5mmol/L, ammonium acetate solution as constant volume of solvent.Therefore, it was subsequently acetonitrile and water (volume ratio: 1:1) as constant volume of solvent, which led to good resolution and satisfactory peak shape, the constant solvent results were shown in Figure 1.

Table 3: Recoveries of phorate and its metabolite phorate sulfone from different extracting solvents (0.02 mg/kg, n = 5).

Sample	Phorate		Phorate sulfoxide		Phorate sulfone	
	Recoveries (%)	RSD (%, n=5)	Recoveries (%)	RSD (%, n=5)	Recoveries (%)	RSD (%, n=5)
Methanol	33.8	2.1	22.5	1.8	26.2	3.1
Acetonitrile	88.6	1.6	86.2	0.9	92.1	2.5
Methylene chloride	32.3	0.7	43.1	1.2	39.2	2.8
Ethyl acetate	45.1	1.3	36.2	2.1	41.3	1.9



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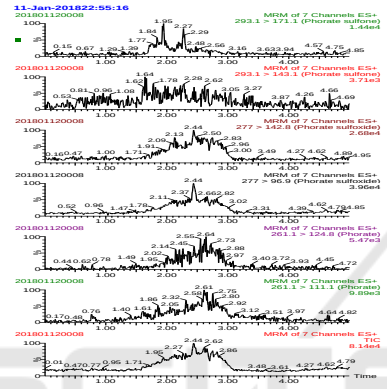

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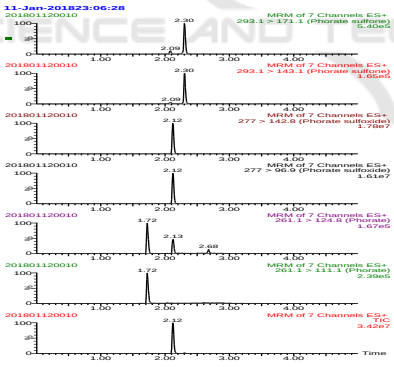

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Figure 1: Typical chromatograms of phorate and its metabolite phorate sulfone spiked at 0.02 mg/kg in egg samples (A: 20.0 ng/ml Hen's Egg standard sample, B: Hen's Egg blank sample, C: Hen's Egg add sample).

3.2.2 Selection of Mobile Phase

According to the references, according the process of phorate and its metabolite phorate sulfone chromatographic separation, however, in order to significantly improve the phorate and its metabolite phorate sulfone chromatographic separation, it primarily deployed 0.1% formic acid water and acetonitrile system as the mobile phase. In this experiment, it employed the gradient elution method for the purpose of receiving better separation effect and using shorter analysis time, the mobile phase gradient and flow rate results were shown in Table 1.

Therefore, it was subsequently employed the mobile phase gradient as mobile phase, which led to good resolution and satisfactory peak shape.

3.3 Method Validation

The validation of limit of detection, recovery, specificity, linearity and precision for the proposed method were determined. Specificity was checked by analyzing thirty blank egg samples. No interfering peaks could be detected at the retention time of phorate and its metabolite phorate sulfone (Figure 1). The calibration curve for the measuement of each compound was performed with six different concentrations in triplicate using the same UPLC-MS/MS method as described above. The linearity and regression study were performed for phorate and its metabolite phorate sulfone standard graph to generate calibration curve. The high linear coefficient (r = 0.9995) indicated good linearity over the concentrations ranged from 2.0 to 100.0 ng/ml for phorate and its metabolite phorate sulfone in egg samples. For the recovery study, each egg samples spiked at 0.01, 0.02 and 0.05 mg/kg were used for validation of the extraction procedure and cleanup. The peak areas for spiked samples were compared with those of standards to determine the recovery. Table 4 shows the results of recovery and repeatability of the method at spiked levels. Recoveries of phorate and its metabolite phorate sulfone from these samples were between 78.6% and 95.6% with relative standard deviations of less than 5.6%. The lower limit of detection (LOD) was determined as the concentrations at signal-to-noise(S/N) ratios of 3. The lower limit detection (LOD) of the phorate sulfone, phorate and phorate sulfoxide were 0.0005 mg/kg , 0.0005mg/kg and 0.0005 mg/kg, respectively. The phorate, phorate sulfone and phorate sulfoxide's quantitative （ LOQ ） were 0.0015 mg/kg, 0.0015 mg/kg and 0.0015 mg/kg, respectively.The data reported indicate that the above method for the analysis ofphorate and its metabolite phorate sulfone in egg samples can achieve good recovery and repeatability.

Table 4: Recoveries of phorate and its metabolite phorate sulfone from spiked egg samples (mg • kg^{-1}, n = 5).

Sample	Spiked level (mg/kg)	Phorate		Phorate sulfoxide		Phorate sulfone	
		Recoveries	RSD (%, n=5)	Recoveries	RSD (%, n=5)	Recoveries	RSD (%, n=5)
Hen's Egg samplesnol	0.01	83.2	1.5	85.1	2.3	78.6	5.6
	0.02	81.5	4.3	82.8	0.7	82.1	0.3
	0.05	82.5	2.5	91.6	2.8	82.3	1.5
Duck egg samples	0.01	88.6	1.4	84.3	3.2	81.3	2.1
	0.02	85.2	4.1	86.1	0.7	85.4	3.2
	0.05	95.6	2.2	82.1	2.6	81.6	1.4
Goose egg samples	0.01	87.1	2.4	84.1	3.2	81.2	1.8
	0.02	83.5	4.2	85.3	0.4	82.7	2.1
	0.05	81.7	2.8	91.3	3.5	85.3	3.5

4 CONCLUSIONS

In view of the need for a selective and sensitive method to determine phorate and its metabolite phorate sulfone in egg samples, in this work, a simple, sensitive and reproducible high liquid chromatography-tandem Mass spectrometry method was developed to determine phorate and its metabolite phorate sulfone residue in egg samples .The extraction method is cost effective and does not consume large amounts of organic and toxic solvents. The obtained results show that the proposed method can be used for pre-concentration and evaluation of trace amounts of phorate and its metabolite phorate sulfone in real egg samples and the LOD, the LOQ, linear range, selectivity, and precision is good. It was suggested that the electrostatic force (ionic interaction) was the dominant effect in the specific recognition of phorate and its metabolite phorate sulfone.

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