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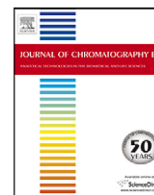

Multiresidue determination of fluoroquinolones, organophosphorus and *N*-methyl carbamates simultaneously in porcine tissue using MSPD and HPLC–DAD

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ABSTRACT

Increasing concerns on public health safety have led researchers to develop efficient methods to characterize veterinary drugs and pesticides residues simultaneously in animal products. This investigation presents a simple and rapid method to determine five types of fluoroquinolones (FQs), organophosphorus (OP) and *N*-methyl carbamates (NMCs) in porcine tissue simultaneously with the use of matrix solid-phase dispersion (MSPD), high performance liquid chromatography (HPLC) and diode array detection (DAD). The results show a recovery ratio of 60.1–107.7% with satisfactory relative standard deviations. The limits of detection (LOD) in porcine tissue are between 9 and 22 μg/kg. The applicability of the method to the multiresidue analysis in porcine tissue is reported in this contribution.

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1. Introduction

Along with the veterinary drugs, pesticides could be suspectable drug residues in animal products, when animals are exposed to them via air, water, food, etc. In Wang et al.'s [1] detailed investigation on the concerned field, the possible ways for the accumulation of pesticides residues in the body of livestock are listed as follows:

1. Direct exposure: Broad-spectrum pesticides such as organophosphorus (OP) and *N*-methyl carbamates (NMCs), are commonly applied by veterinarians to prevent livestock from epizoa. Pesticides could, therefore, be absorbed by these domestic animals with per cutem or per os.
2. Contamination from the environment: 40–60% pesticides are normally disposed in the soil and 5–30% are volatilized in the atmosphere when they are applied to the farmland, grassland, and forest.
3. Food chain transfer: The pesticides could be easily transferred and accumulated in the body of the livestock fed with crops which are potentially contaminated by pesticides residues. Fig. 1 shows the main routes of pesticides residues accumulated in animal products.

Therefore, it could be necessary and urgent to develop efficient methods to characterize veterinary drugs and pesticides residues in animal products simultaneously.

Recently a large number of studies have evidently shown that some veterinary drugs and pesticides are teratogenic, mutagenic, and reproductive toxicant [2–5]. Furthermore, the veterinary drugs can also increase the proliferation of antibiotic-resistant pathogens and thus pose threats to human health [6]. Therefore, some researchers proposed that the cocktail effect [7], combined effects of veterinary drugs, pesticides, and medicines to human health, should be focused upon for its chronic, potential and accumulated toxicity.

To provide safe food to consumers, both China and European Union (EU) have established the maximum residue limits (MRLs) for fluoroquinolones (FQs) ranging from 10 to 1900 μg/kg in the foods of animal [8,9]. The Positive List System of Japan has set MRLs of 50 μg/kg for ofloxacin (OFLX) in chicken [10]. Chinese MRL of dichlorvos (DDVP) is 100 μg/kg in porcine muscle [11]. And Food and Agriculture Organization/World Health Organization (FAO/WHO) has set an MRL for carbaryl (CAR) in bovine muscle at 200 μg/kg [12].

To ensure the safety of food supply, efficient methods are required for the simultaneous monitoring of residue levels of veterinary drugs and pesticides in animal products. A large number of available methods have been developed for determining separately veterinary drugs and pesticides residues in food. Many analytical procedures have been introduced to analyze FQs in animal products [13–16]. However, only a few literatures have reported the determi-

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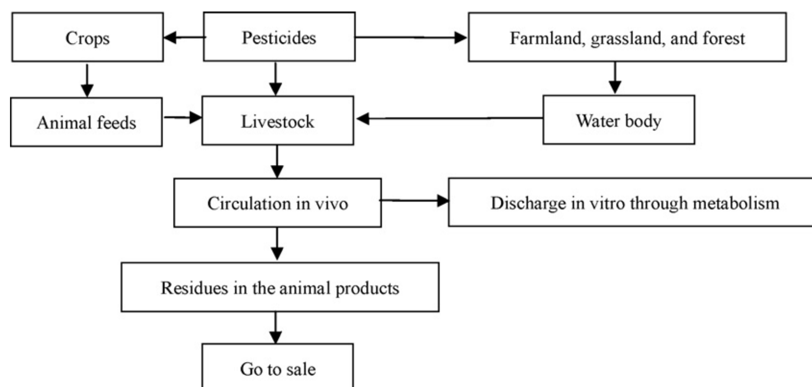

Fig. 1. Main routes of pesticides residues accumulated in food animal products.

nation of OP or NMCs in animal products [12,17,18]. To the best of our knowledge, there is no report on the simultaneous determination of veterinary drugs and pesticides residues in animal products up to date. So, this contribution presents the study for the pre-processing and simultaneous determination of veterinary drugs and pesticides residues in animal tissues for the first time. In this study, a method using matrix solid-phase dispersion (MSPD) and high performance liquid chromatography (HPLC) is developed to determine five types of FQs, OP, and NMCs simultaneously in porcine tissue. The veterinary drugs [i.e. FQs such as enoxacin (ENO), ofloxacin (OFLX), and lomefloxacin (LOM)], one OP pesticide [dichlorvos (DDVP)] and one NMC [carbaryl (CAR)] were selected as target contaminants in this study. Fig. 2 shows the chemical structures of these contaminants.

2. Experimental

2.1. Materials

Enoxacin (ENO, 91.1%), ofloxacin (OFLX, 98.6%) and lomefloxacin (LOM, 90.0%) were obtained from the National Institute

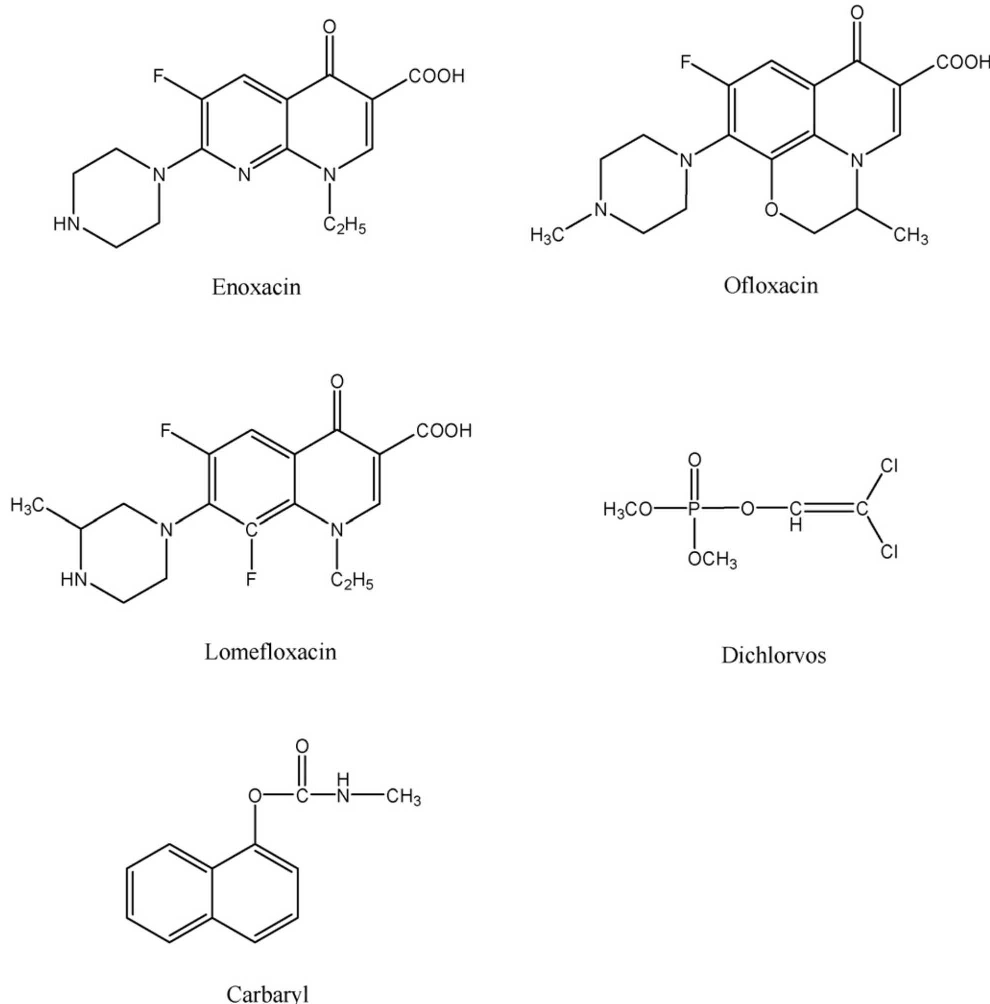

Fig. 2. Chemical structures of the selected drugs in this study.

for the Control of Pharmaceutical and Biological Products (Beijing, China). Dichlorvos (DDVP) and carbaryl (CAR) were obtained from the Institute of Environmental Protection of Agriculture (Tianjin, China). HPLC-grade methanol and acetonitrile (ACN) were obtained from Burdick & Jackson (Muskegon, MI, USA), and HPLC-grade orthophosphoric acid (H_3PO_4) was obtained from Kermel Chemical Reagents Development Centre (Tianjin, China). Silica C_{18} (50 μm) was obtained from Baseline Chrom. Tech. (Tianjin, China). Triple distilled water (18.3 MΩ cm resistivity) was prepared by a Molelement water purification system (Molecular, Shanghai, China). All solutions prepared for HPLC were filtered through a 0.45 μm filter.

For recovery studies, porcine muscle was purchased from a local food market and kept deep-frozen at −18 °C prior to the analysis.

2.2. Standard solutions

Individual standard stock solutions of ENO, OFLX, LOM, DDVP and CAR with the concentration of 100 μg/ml were diluted with methanol and stored in the dark at 4 °C. A fortification mixture of ENO, OFLX, LOM, DDVP and CAR (10 μg/ml) in methanol was freshly prepared right before analysis from these stock solutions. When the lower level of fortification solution is required, additional dilution with methanol was conducted.

2.3. The procedure of matrix solid-phase dispersion (MSPD)

A 0.5 g blended tissue homogenate was weighed in an analytical scale and fully immersed in the standard mixture solution. The tissue sample and 2.0 g amount of C_{18} were then placed in a glass mortar with external diameter of 90 mm and gently ground to obtain a homogeneous material. Afterwards, 1.0 g anhydrous sodium sulfate, 0.25 g C_{18}, the C_{18}/tissue matrix blend and 0.5 g anhydrous sodium sulfate were introduced in order into a 10-ml syringe barrel pre-plugged with a filter disc and placed on a vacuum manifold. The flow rate was controlled at 0.5 ml/min and the C_{18}/tissue matrix blend was washed with 6 ml *n*-hexane, followed by 8 ml ACN. Afterwards, the eluate was evaporated to dryness under a gentle stream of N_2, and the residue was dissolved in 1 ml methanol. The final solution of 20 μl was injected onto the HPLC column after they were filtered through a 0.45 μm disposable syringe filter unit.

2.4. HPLC–DAD analysis

The LC analyses were accomplished using a LC-10Avp (Shimadzu, Japan) HPLC system consisting of a LC-10ATvp secondary pump system, DGU-12A on-line degasser, CTO-10ASvp thermostatted column compartment, and SPD-M10Avp diode array detector. CLASS-VP software was used to control the LC components and to process ultraviolet data. A Kromasil C_{18} chromatography column (150 mm × 4.6 mm, 5 μm) was used.

Solvent A (0.003 mol/l H_3PO_4) and solvent B (acetonitrile) were combined in a gradient as follows: 13–20% B (5 min), 20–38% B (2 min), 38% B (8 min), 38–64% B (5 min), 64–13% B (2 min). The flow rate was 1.0 ml/min, and the column heater was set at 25 °C. The investigated analytes were eluted within 22 min, and a 15-min post time allowed re-equilibration of the column. ENO, OFLX and LOM were monitored at the absorbance wavelength of 280 nm (Fig. 3(a)), and 220 nm for DDVP and CAR (Fig. 3(b)). Retention times for the analytes are shown in Table 1.

3. Results and discussion

3.1. The procedure for MSPD

MSPD involves homogenizing and dispersing of a small amount of matrix with adsorbent (usually C_{18} or C_8), and the mixture was

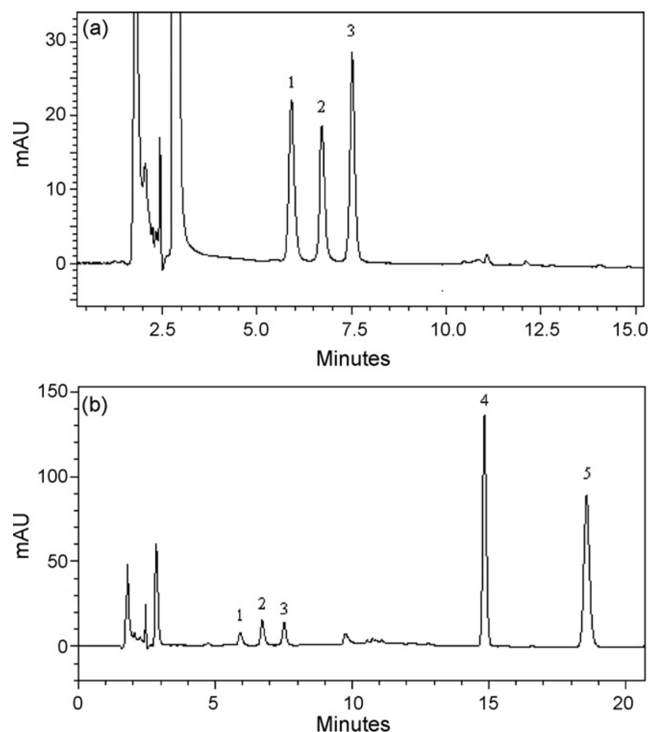

Fig. 3. HPLC–DAD chromatograms of a 5 μg/ml standard solution: (a) λ = 280 nm; (b) λ = 220 nm; 1—ENO, 2—OFLX, 3—LOM, 4—DDVP and 5—CAR.

washed with a small amount of solvent before eluting to extract a wide range of compounds. This technique was developed by Staren Barker et al. (1989) to isolate drug residues from tissues and has been widely used for biological samples. The MSPD method can include sample homogenization, cellular disruption, extraction, fractionation, and purification in a single process.

3.1.1. Optimization of the rinsing and eluting conditions

In the present study, solid-phase extraction on C_{18} (Alltech) stationary phases for purification of the five veterinary drugs and pesticides residues in porcine muscle has been investigated. The results show that the recoveries of all tested contaminants range from 16.3 to 60.5% with high relative standard deviations. In order to improve the experimental condition, MSPD was used and the result demonstrates that the MSPD-based method can shorten analysis time and reduce the amount of required solvent waste without affecting the detecting results.

Based on 2.0 g C_{18} and 0.50 g porcine muscle, the effect of rinsing and eluting conditions were investigated (the spiking level was 0.4 μg/g). To optimize the conditions, *N*-hexane, acetoacetate, the mixture of ACN and acetic acid, and ACN were used in this study. The recoveries of the analytes under different rinsing and eluting conditions are shown in Table 2.

As indicated in Table 2, the analytes could not be eluted by *n*-hexane at 6 ml. When eluting the analytes with 8 ml acetoacetate,

Table 1
The maximum UV-detection wavelengths and retention time of the five analytes.

Analytes	λ (nm)	Typical retention time (min)
ENO	269	5.79
OFLX	295	6.73
LOM	288	7.46
DDVP	211	14.84
CAR	221	18.56

ENO: Enoxacin; OFLX: ofloxacin; LOM: lomefloxacin; DDVP: dichlorvos; CAR: carbaryl.

Table 2
Recoveries (%) of the analytes in different ringing and eluting conditions ($n = 5$).

Rinsing and eluting solvents	ENO	RSD	OFLX	RSD	LOM	RSD	DDVP	RSD	CAR	RSD
6 ml C_6H_{14}	n.d.	–	n.d.	–	n.d.	–	n.d.	–	n.d.	–
8 ml $CH_3COOCH_2CH_3$	28.5	3.9	19.7	8.3	34.3	6.2	48.1	5.7	35.8	7.9
8 ml CH_3CN/CH_3COOH (v/v = 99:1)	96.3	3.5	75.0	5.7	92.9	3.8	60.3	7.1	68.8	5.6
8 ml CH_3CN	94.6	3.3	80.1	3.9	88.5	4.2	75.9	6.5	94.2	4.5

n.d.: Not detected; RSD: relative standard deviations.
ENO: Enoxacin; OFLX: ofloxacin; LOM: lomefloxacin; DDVP: dichlorvos; CAR: carbaryl.

the recoveries of all the analytes were not acceptable. However, when eluting the analytes with 8 ml ACN, the recoveries of most compounds surpassed the results obtained by eluting the analytes with 8 ml ACN–acetic acid except for ENO and LOM. Furthermore, ACN–acetic acid eluent will cause extra interferences in the chromatograms. Therefore, the proper rinsing and eluting solvents were 6 ml *n*-hexane and 8 ml ACN respectively. According to the above analysis, the rinsing and eluting conditions for porcine muscle were tested, and similar results were obtained. Under the optimal conditions, the recoveries of the analytes were above 80% except for that of DDVP, which was approximately 70%.

3.1.2. Optimization of the eluting solvent dose

The effect of the eluting solvent dose was investigated at the 0.4 μg/g spike level. The recovery for each analyte is increased rapidly to ≥60% with a slow increase in eluent volume from 4 ml to the equilibrium value of 8 ml. Therefore, the eluent volume used for subsequent studies was set at 8 ml.

3.1.3. Effects of the ratio of sample to C_{18} on the recoveries of the analytes

The interactions observed in MSPD involve the analyte with the solid support, the solid phase of cartridges, the dispersed matrix, as well as the matrix with solid support and with the solid phase. Eventually, all of the preceding components interact with the elution solvents.

It is, therefore, very crucial to investigate the relations between the ratio of sample to C_{18} and the recoveries of the analyte. An amount of 0.5 g tissue sample was used for each test, 0.5, 1.0, 2.0 and 3.0 g of C_{18} were used in the MSPD procedure to give different ratios of sample to C_{18}. 0.2 μg standard solutions of the analytes were fortified in all experiments.

It has been observed in the study that with 0.5 and 1.0 g of C_{18} matrix, the average recoveries for most of the analytes were 36.8–57.0% and with the relative standard deviations (RSD) between 11.4 and 26.2%. This result showed that large amounts of sample to C_{18} made it difficult to obtain a homogeneous packing column for the MSPD procedure, and thus with a lower reproducibility.

However, when the amount of C_{18} was increased to 2.0 and 3.0 g, the RSD was between 3.3 and 9.6%, the average recoveries of the analytes increased to the level between 63.9 and 95.8%. The results were satisfactory when the C_{18} weights were 2.0 or 3.0 g, although the large amount of C_{18} would consume more eluting solvent. As a result, 0.50 g sample and 2.0 g C_{18} were proved to be suitable for the extraction and purification in the MSPD process.

3.2. HPLC–DAD

3.2.1. HPLC conditions

As a consequence of the presence of acidic and basic functional groups, the FQs are prone to chemical tailing due to interactions with free silanols groups from stationary phase [19]. In the study, the pH value and the organic modifier content of the mobile phase employed for HPLC analysis have been examined. The combination of an acidic pH (2.80) and triethylamine (TEA) in the mobile phase was necessary to give short retention time to FQs (<8 min) as well as a good peak shape. However, adding amine modifier TEA in the mobile phase would cause baseline drift seriously and decompose DDVP and CAR. In this study, the analytes could be separated well when an ACN–H_3PO_4 gradient was used. In addition, replacing ACN by methanol will lead to serious baseline drifting while maintaining the peak of the chromatographic resolution of FQs.

Furthermore, several binary or even ternary eluents were tested by adopting different proportions of solvents, such as ACN, methanol, H_3PO_4, citric acid, phosphate buffer, and acetic acid buffer. In this study, solvent A (0.003 mol/l H_3PO_4) and solvent B (ACN) were chosen as the optimal chromatographic condition in a gradient. A gradient was set as follows: 13–20% B (5 min), 20–38% B (2 min), 38% B (8 min), 38–64% B (5 min), and 64–13% B (2 min).

The influence of H_3PO_4 was tested. It was reasonable that the retention time of FQs was shortened as pH increased to 3.20 while the resolution of FQs was unsatisfactory. When pH was lower than 2.40, the baseline was drifted obviously since it might adversely influence the chromatographic column. The retention time of DDVP and CAR remains unchanged with pH changed between 2.40 and 3.20. It was also found that pH 2.80 gives the best selectivity to experimental results in terms when processed samples were analyzed. HPLC analysis of the tissue samples was performed within 22 min.

3.2.2. Identification

The advantage for the application of the HPLC–DAD method in this study allows both the retention time and the spectrum as the means for separation and identification of the analytes (see Table 1). Using a photodiode array detector, the absorption spectra of ENO, OFLX, LOM, DDVP, and CAR standards in the mobile phase could be recorded via selecting the HPLC monitoring wavelength. The measurement was conducted at 280 nm, which resulted in an average maximum absorbance for all the FQs, and 220 nm for that of the pesticides.

3.3. Method validation [20]

3.3.1. Linearity

Seven-point calibration curves were quantitation utilized the UV peak area for each analyte. The calibration curves were found to be linear in the 0.01–10 μg/ml range studied (0.01, 0.05, 0.1, 0.5, 1, 5, 10 μg/ml levels were used). The linear equations, correlation coefficients, and detection limits of pharmaceuticals are presented in Table 3. The results indicate that correlation coefficients are between 0.9976 and 0.9999.

3.3.2. Intra-day and inter-day repeatability

The analysis of the calibration standards was used to determine the intra-day (three repetitions of each concentration) and inter-day repeatability (three repetitions of each concentration, three days). The results (for three levels) are listed in Table 4. The intra-day RSD were lower than 6.9% which is lower than 8.1% for inter-day analysis.

Table 3
Regression equations, coefficients of determination (r^2) and detection limits of analytes.

Analytes	Regression equation	r^2	Linear range (μg/ml)	LOD (μg/l)	LOQ (μg/l)
ENO	$y = 3.6 \times 10^4x - 3.2 \times 10^3$	0.9980	0.01–10	4	14
OFLX	$y = 2.4 \times 10^4x + 2.1 \times 10^3$	0.9952	0.01–10	5	17
LOM	$y = 4.3 \times 10^4x - 1.1 \times 10^3$	0.9984	0.01–10	2.5	9
DDVP	$y = 2.4 \times 10^5x + 2.7 \times 10^3$	0.9996	0.01–10	6	20
CAR	$y = 2.4 \times 10^5x + 9.6 \times 10^3$	0.9998	0.01–10	5	17

y, Peak area; *x*, concentration (μg/ml).
ENO: Enoxacin; OFLX: ofloxacin; LOM: lomefloxacin; DDVP: dichlorvos; CAR: carbaryl.

Table 4
Intra-day and inter-day repeatability.

Analytes	Amount injected (ng)	Intra-day repeatability RSD ($n = 3$) (%)	Inter-day repeatability RSD ($n = 9$) (%)
ENO	1.0	2.0	2.7
	0.4	4.3	5.0
	0.1	6.7	7.9
OFLX	1.0	3.0	3.8
	0.4	5.4	6.1
	0.1	6.8	8.0
LOM	1.0	2.9	3.8
	0.4	3.1	4.2
	0.1	3.5	4.9
DDVP	1.0	1.3	2.5
	0.4	4.6	4.8
	0.1	5.4	6.2
CAR	1.0	1.1	1.6
	0.4	2.6	3.5
	1.0	3.2	3.9

ENO: Enoxacin; OFLX: ofloxacin; LOM: lomefloxacin; DDVP: dichlorvos; CAR: carbaryl.

3.3.3. Selectivity

The selectivity of the proposed method was evaluated by performing the extraction, HPLC–DAD analysis of porcine tissue samples were considered negative in terms of veterinary drugs and pesticides. No interferences were observed in these samples and no peak above a signal-to-noise ratio of 3 was detected at the retention times of the selected analytes, showing the good selectivity of the proposed method.

3.3.4. Accuracy

The accuracy of the method was tested by fortification of porcine samples at three known levels of 0.1, 0.4, and 1.0 μg/g. Extraction, analysis, and determination of the recovery were performed for each analyte. The percentage of recovery (%) was calculated as indicated by the Government Standard of People's Republic of China (see formula (1)) [20]. Fig. 4 shows chromatograms of a representative blank and spiked porcine tissue at 0.1 mg/kg level and Table 5 summarizes the recoveries and the RSD obtained for each analyte. The results indicate that average recoveries are between 60.1 and 107.7% and RSD of the peak areas change from 1.0 to 8.3%.

$$X = \frac{A \cdot Cs \cdot V}{As \cdot M} \qquad (1)$$

where *X* is the residue of each analyte in porcine tissue (mg/kg); *A* is the peak area of corresponding drug in sample solution; As is the peak area of corresponding drug in standard solution; Cs is the concentration of corresponding drug in standard solution (μg/ml); *V* is the sample volume diluted by methanol in MSPD (ml); *M* is the quality of the sample (g).

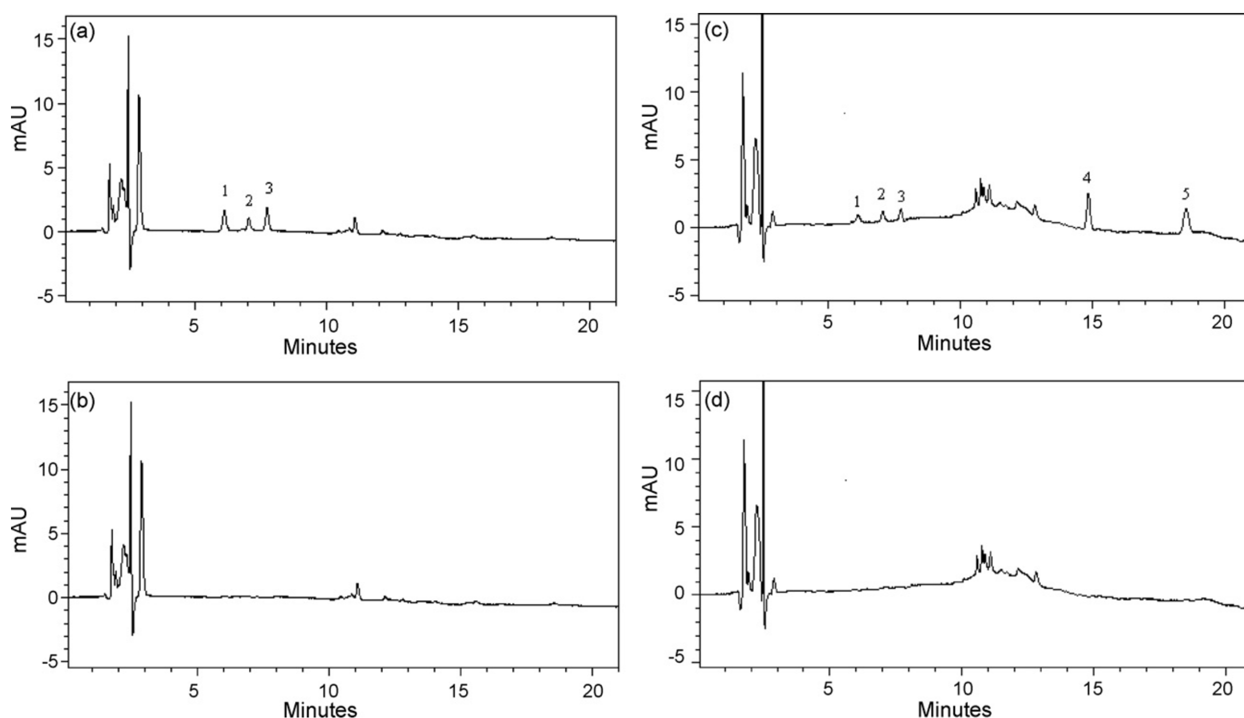

Fig. 4. Chromatograms of: (a) extracted sample from spiked porcine tissues with 0.1 mg/kg, λ = 280 nm; (b) blank extract, λ = 280 nm; (c) extracted sample from spiked porcine tissues with 0.1 mg/kg, λ = 220 nm; (d) blank extract, λ = 220 nm; 1—ENO, 2—OFLX, 3—LOM, 4—DDVP and 5—CAR.

Table 5
Average recoveries and repeatabilities of the analytes at three levels of spiking ($n = 5$).

Analytes	Added (mg/kg)	Average recovery (%)	RSD (%)	LOD (μg/kg)	LOQ (μg/kg)
ENO	1.0	95.1	7.8	10	34
	0.4	95.8	7.0		
	0.1	60.1	5.9		
OFLX	1.0	64.0	7.0	15	50
	0.4	63.9	5.9		
	0.1	60.9	3.0		
LOM	1.0	107.7	7.7	9	30
	0.4	80.0	6.4		
	0.1	75.3	5.9		
DDVP	1.0	72.8	1.2	22	74
	0.4	74.4	3.3		
	0.1	62.4	6.5		
CAR	1.0	105.6	4.4	18	60
	0.4	92.3	8.3		
	0.1	95.5	1.0		

ENO: Enoxacin; OFLX: ofloxacin; LOM: lomefloxacin; DDVP: dichlorvos; CAR: carbaryl.

With porcine muscle samples of 0.50 g, the MSPD method gives the limit of detection (LOD) in the range of 9–22 μg/kg and the limit of quantification (LOQ) between 30 and 74 μg/kg.

4. Conclusions

For the first time, the determination of the veterinary drugs and pesticides residues in animal tissues simultaneously were conducted successfully. The proposed MSPD method is relatively simple, faster and more economical in comparison with the SPE. It is especially suitable for multiresidue analysis of the concerned analytes in porcine muscle. In addition, simultaneous multiresidue determination method established in this contribution offers obvious advantages. For example, it needs less samples, use less amount chemical reagent and solvent. This method can also be used for screening, which can detect the ENO, OFLX, LOM, DDVP, and CAR in animal product tissues at the MRLs level and estimate the quantities of these chemicals.

In future work, improving the sensitivity and accuracy of the method and simultaneous analysis of a wide range of veterinary drugs and pesticides residues in animal products will be conducted.

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