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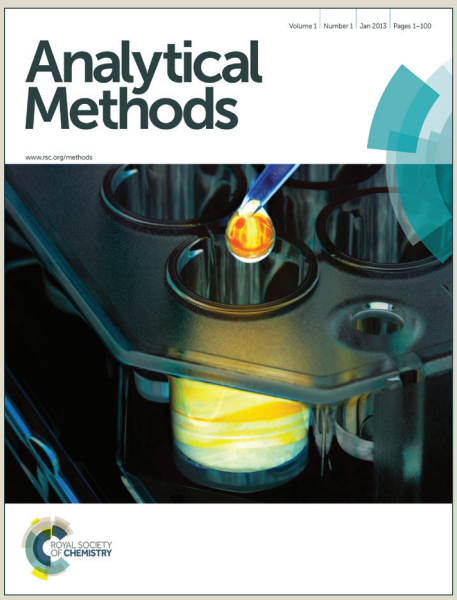

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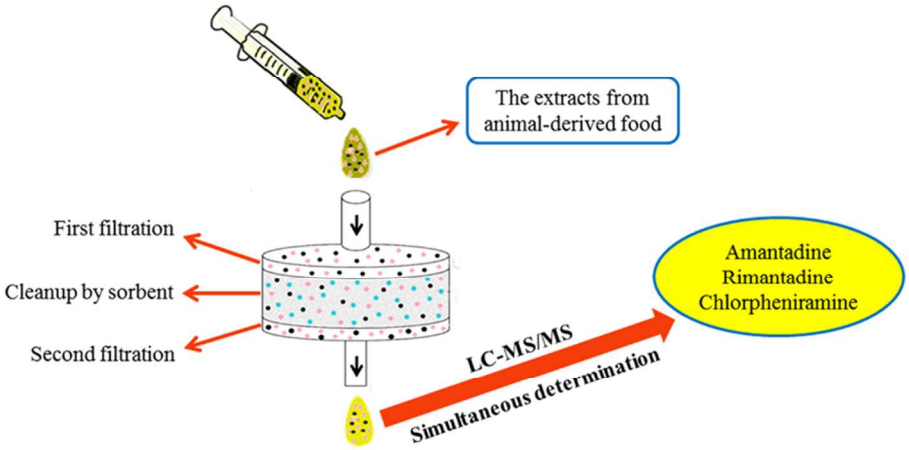

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Simultaneous Determination of Amantadine, Rimantadine and Chlorpheniramine in Animal-derived Food by Liquid Chromatography–Tandem Mass Spectrometry after Fast Sample Preparation

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Abstract A fast method for simultaneous determination of amantadine, rimantadine, and chlorpheniramine in seven animal derived samples including pork, chicken, duck, pig liver, chicken liver, pig kidney, and egg was developed with liquid chromatography-tandem mass spectrometry, and employed a new multifunctional syringe filter that makes the cleanup procedure simple and rapid based on the QuEChERS (quick, easy, cheap, effective, rugged and safe). The method was validated using amantadine-d_{15}, rimantadine-d_4 and chlorpheniramine-d_6 as internal standards for three analytes, respectively. Good linearities ($R^2 > 0.9938$) were obtained over the concentration range from 2 μg/L to 200 μg/L for amantadine and rimantadine, and from 0.2 μg/L to 20 μg/L for chlorpheniramine. The precision was evaluated by intra- and inter-day assays and the relative standard deviations were all within 9.85%. Mean recoveries ranged from 89.9% to 105%. The limits of detection and quantification were 0.5 and 1.0 μg/kg for both of amantadine and rimantadine, 0.05 and 0.1 μg/kg for chlorpheniramine, respectively. The application of the developed method in real samples showed that amantadine and chlorpheniramine were respectively detected with percentages of 3.7% and 0.3% in all tested samples.

Keywords Amantadine · Rimantadine · Chlorpheniramine · Animal-derived food · Liquid chromatography–tandem mass spectrometry

1. Introduction

Amantadine hydrochloride and rimantadine hydrochloride (α-methyl-1-adamantane-methylamine hydrochloride) have been clinically used for therapy of infections caused by a broad range of RNA-containing viruses, especially on the influenza A virus [1]. Therefore, previously these drugs have been widely applied to treat animal diseases in the process of breeding. However, on an account of the potential resistance to these drugs for human beings [2], now they had been prohibited to use in livestock and poultry farming in many countries including USA [3] and China [4]. Chlorpheniramine (2-pyridinepropanamine, γ-(4-chlorophenyl)-*N*,*N*-dimethyl, (*Z*)-2-butenedioate, CP) is a powerful antihistaminic for its moderate degree of sedation [5], and popularly used in animal feeding. However, the influence of chlorpheniramine

may lead to accidental death of elderly people [6]. Their residues in animal tissues and egg also may be harmful for human health. Thus, it is necessary to develop an effective method for the analysis of these drugs in animal matrices.

Up to now, a number of assays have been reported for the determination of these drugs in biological fluids [7] and animal tissues, including the determination of rimantadine by capillary zone electrophoresis [8], amantadine by gas chromatography-mass spectrometry (GC-MS) [9], high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [10] or fluorescence detection (HPLC-Flu) [11]. The most of above methods usually require cumbersome derivatization treatment, time-consuming and laborious extraction procedures, and long chromatographic analysis time, but exhibited lower sensitivity. Compared with these, liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography- tandem mass spectrometry (LC-MS/MS) have the advantages of simple sample preparation without derivatization and high selectivity and sensitivity, and the increased utilization. A number of its application have been reported for the determination of amantadine[12, 13], rimantadine[13, 14] or chlorpheniramine[15]. The sample preparation methods for them were often liquid-liquid extraction or solid-phase extraction in plasma and urine, but not suitable for the more complex samples like animal matrices. QuEChERS method is a simple, rapid and promising sample preparation method and widely used for the multi-residue determination in different food matrices including plant and animal such as apple juice[16], chicken[13], bovine milk[17], liver[17], shrimps[18], fish [19] and so on, but it still has largely space for improvement. Thus, this study aims to develop an assay for simultaneous quantification of amantadine, rimantadine, and chlorpheniramine in different animal derived samples by LC-MS/MS, and used a new multifunctional filter based on a QuEChERS method to quickly prepare samples.

2. Experimental

2.1 Materials and chemicals

The standards of amantadine hydrochloride (purity 98%) was obtained from National Institute of Pharmaceutical and Biological products (Beijing, China), rimantadine (purity 99%) from Sigma Aldrich (St. Louis, USA) and chlorpheniramine (purity 99%)

from Dr. Ehrenstorfer (Augsburg, Germany). The internal standard of amantadine-d_{15} hydrochloride (purity 99%) was supplied by Toronto Research Chemicals. Inc (Toronto, Canada), chlorpheniramine-d_6 hydrochloride (purity 99%) and rimantadine-d_4 hydrochloride (purity 98%) were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Canada).

HPLC-grade acetonitrile, methanol, and *n*-hexane were purchased from Fisher Scientific (Fair Lawn, USA). Analytical-grade anhydrous sodium sulfate and acetic acid were supplied by Beijing Chemical Reagent Co. Ltd (Beijing, China). Ultra-pure water was prepared using a Milli Q-plus system (Billerica, MA, USA). The reference sorbents of primary second amine (PSA), octadecylsilane (C_{18}), florisil and neutral alumina (Al_2O_3) were all obtained from Agilent Technologies (California, USA).

The food of animal origin selected for this experiment included pork, chicken, duck, pig liver, chicken liver, pig kidney, and egg. All these animal tissues and eggs were all purchased from supermarkets in Chinese mainland.

2.2 ***Standard preparation***

Stock solutions (1000 μg/L) of individual compounds (amantadine, rimantadine, and chlorpheniramine) and their isotopic internal standards (amantadine-d_{15}, rimantadine-d_6, and chlorpheniramine-d_4) were individually prepared by dissolving appropriate amount of standards in methanol, and stored in the dark at -20℃. The mixed working solution (I) were obtained by diluting all stock solutions of individual compounds in the same volumetric flask, the working solution (II) of internal standards (IS) also was prepared in another volumetric flask with same method.

2.3 ***Sample preparation***

All tissue samples were finely chopped and homogenized using a kitchen blender, and stored in -20 ℃. Poultry tissue or egg (2 g) spiked in 20 μL of IS working solution II was extracted with 1% acetic acid in acetonitrile (10 mL) by vortex for 2 min. After centrifugation at 3000 r min^{-1} for 5 min, the supernatant was transferred into a 50-mL centrifuge tube. The sample was extracted again with the same method and the supernatants were combined. After the addition of 3 g of anhydrous sodium sulfate and 10 mL of *n*-hexane, the extract was vortexed for 1 min and centrifuged at 3000 r

min^{-1} for 5 min. The available acetonitrile phase was transferred into a 100 mL of heart bottle and dried on a vacuum rotary evaporator at 40 ℃. Then the residue was redissolved with 1 mL of methanol and cleaned up by passing an MSF [20] filled with 50 mg of PSA using uniform speed, and injected into LC-MS/MS directly.

2.4 ***Apparatus and chromatographic conditions***

Chromatographic conditions were carried out with an Agilent 1200 HPLC system equipped with a G1322A degasser, G1311A quatpump, G1316B column compartment, G1315C diode array detector, G1329A autosampler, and a 20-μl sample loop (Wilmington, DE, USA). The separation of analytes was performed on a XDB-C_{18} column (2.1 mm×150 mm, 3.5 μm particle size) from Agilent at a room temperature and a gradient elution at a flow rate of 0.3 mL/min. The mobile phase consisted of eluent A (0.1% volume ratio of formic acid in water) and eluent B (methanol). The percentage of A was started at 90%, linearly decreased down to 30% in 2 min, held constant for 4 min, returned to the initial ratio in 1 min, and equilibrated for 3 min. The total time for one run was 10 min. The injection volume was 10 μL.

Mass spectrometry analysis was achieved using an API 5000 triple quadrupole tandem mass spectrometry (Applied Biosystems, USA). The instrument was operated in positive electrospray with a voltage of 4.0 kV and source temperature of 500 ℃. Nitrogen was used as the collision gas. The instrumental operation and data analysis were performed using the Analyst 1.4.2 software. Multi-reaction monitoring (MRM) parameters for three target analytes are summarized in Table 1.

2.5 ***Method validation***

Calibration curves were conducted using the working standard solution I by plotting the peak area to concentrations of 2, 4, 10, 20, 40, 100, and 200 μg L^{-1} for amantadine and rimantadine, 0.2, 0.4, 1, 2, 4, 10, and 20 μg/L for chlorpheniramine. The concentrations of all IS were 20 μg/L. The matrix effect was investigated according to the slope ratios of the matrix-matched standard calibration to standard solution calibration. The sensitivity of this method was evaluated by the limit of detection (LOD) and limit of quantification (LOQ), defined as spiked concentrations that produced the signal-to-noise ratio (S/N) of 3 and 10, respectively. The accuracy and

precision were estimated by recoveries of three analytes with 5 replicates at three spiked concentration levels in different matrices. The spiked samples were prepared by adding appropriate volumes of working standard solutions into each blank matrix, and setting for 30 min after vortexing for 30 s for sufficient stability. The intra-day and inter-day relative standard deviations (RSDs) were measured for the repeatability of this method.

3. Results and discussion

3.1 Optimization of the cleanup methods

Typically, the traditional QuEChERS (TQ) method was applied widely on the extraction and cleanup of pesticides from various matrices for its simpleness, convenience, and speediness [20]. First of all, various sorbents such as PSA, C_{18}, florisil and Al_2O_3 were all tested for this method. The extracts were firstly purified by using *n*-hexane to remove fat in matrices. Then the residue after drying and redissolution as described above was transferred into a 2-mL centrifuge tube, and the sorbents of 50 mg were added. After vortex and centrifugation, the supernatant were filtered through 0.22-μm filter for instrumental analysis.

Fig. 1 gives the cleanup effects of the four sorbents for the three analytes in two kinds of typical matrices, pork and pig liver, which were selected as representative for optimization of cleanup sorbent. The data of this investigation were processed by the external standard method for quantification. The results showed that these sorbents had little influence on recovery of chlorpheniramine compared with other analytes, but C_{18} and florisil could absorb amantadine and rimantadine partly with the approximate recoveries of 70%-80%. For other two analytes, the recoveries using PSA were all a little higher than those using Al_2O_3. In addition, it was satisfying for the baseline noise when using PSA for the sorption of the pigment in the matrices of liver and egg. Therefore, compared to C_{18} [21] that used as the cleanup sorbents in chicken muscle, PSA was selected for the cleanup of the extract in this method.

To simplify the sample preparation, a new multifunctional syringe filter (MSF) designed by Qiu et al. [22] and processed by Tianjin JinTeng Experimental equipment Co., Ltd was introduced in the study. Table 2 gives the mean recoveries of the

traditional QuEChERs (TQ) and the above modified QuEChERs (MQ) using MSF for the analytes in different matrices. Compared to TQ, the new filter simplified the cleanup procedure, accelerated the speed of sample preparation and improved the work efficiency by integrating cleanup process and solution filtering in one step. The results indicated that the two cleanup methods were all satisfying, but in contrast MSF was more convenient for the preparation of large amounts of samples. The recoveries of amantadine in this study were better than those in previous report using solid-phase extraction (SPE) [23] and more stable in the same matrix.

3.2 ***Matrix effect***

The animal-derived food including different animals (pork, chicken, duck), different parts (meat, liver, kidney, egg) frequently consumed by the majority of people in China was selected as target samples for this method. To evaluate matrix effect, the slopes obtained in the matrix-matched calibration (MMC) were compared with those obtained with the standard solution calibration (SSC). As evidenced by the slope ratios[21], the matrix effect was negligible when the ratio was within ±10% of the slope ratio of 1.0 but was significant at the ratio of >±10%.

The slope ratios in all investigated matrices were within ±10% except those in pig liver and pig kidney. In these two matrices, the signal enhancements of three analytes were obviously observed with higher slope ratios of 62.1%-83.1%. Therefore, the isotopic internal standards were used to reduce the matrix effects. The slope ratio for each compound (Table 3) was all within 10%, indicates the matrix effect could be negligible and SSC was available to accurately quantify three analytes in all matrices.

3.3 ***Linearity and sensitivity***

Calibration curves of internal standard method which constructed with a linear regression with 1/*x* weighting were partly shown in Table 4. They all exhibited good linearity with relative coefficients (R^2) higher than 0.9938 for three analytes. The LODs and LOQs respectively were 0.5 and 1.0 μg/kg for both of amantadine and rimantadine, indicates higher sensitivity than previous study of Yan et al. [21]. They reported that LODs were 1.02 and 0.67 μg/kg, and LOQs were 3.40 and 2.21 μg/kg for amantadine and rimantadine in pork, respectively. Yun et al. [23] also used

LC-MS/MS coupled with MCX SPE column to detect amantadine in animal tissues and obtained LOD of 5.0 μg/kg, which is lower sensitive and more complicated compared to present developed method. For chlorpheniramine, LODs and LOQs were 0.05 and 0.1 μg/kg in all matrices (Table 4), respectively. Until now few reports involved in the detection of chlorpheniramine in animal-derived food although there are some references about its detections in pharmaceutical formulations[24], human[25] and animal plasma[26, 27] for pharmacokinetic study. Thus, present method will firstly provide a important measure to monitoring its residue in animal tissues and egg. These indicates that this method is very sensitive for all target analytes, and higher sensitivity 10 times for chlorpheniramine than that for amantadine and rimantadine.

3.4 ***Accuracy and precision***

The accuracy and precision of the developed method were described by intra- and inter-day variability assays at three spiked levels of 0.1, 1, and 10 μg/kg for chlorpheniramine and 1, 10, and 100 μg/kg for amantadine and rimantadine. Table 5 gives an overview of recoveries and RSDs of three analytes in seven different matrices. The average recoveries ranged from 89.9% to 105% with intra-day RSDs within 10.7%, and inter-day RSDs within 9.98%, indicates good accuracy and precision (RSD≤20%) for the developed method.

3.5 ***Application to real sample***

The method described above was practically applied to the simultaneous determination of three analytes in 300 samples for seven matrices obtained from local supermarkets in China at random. Amantadine with a concentration range from 1.79 to 12.8 μg/kg was found in 2 samples among 86 chicken samples and in 9 samples among 66 egg samples. Chlorpheniramine was detected in only one sample among 66 egg samples with concentration of 1.28 μg/kg. Amantadine and chlorpheniramine were not detected in other matrices, and rimantadine were not detected in all tested samples.

4. Conclusion

In this work, a new method coupled with LC-MS/MS was developed and applied for simultaneous determination of amantadine, rimantadine, and chlorpheniramine in

seven animal-derived food. A new multifunctional filter based on the traditional QuEChERs method was introduced to simplify the cleanup procedure in the sample preparation for the first time. The results from assay validation suggest the developed method provides good accuracy, precision, and sensitivity. Its successful application in detection of real samples showed that this method is simple, fast, and sensitive for analysis of amantadine, rimantadine, and chlorpheniramine in multiclass tissues and egg of animal origin.

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Table 1 Multi-reaction monitoring parameters for three analytes

Compound	MRM ion pair (m/z)	Declustering potential (DP)/V	Collision energy (CE)/eV
Amantadine	152.0>135.0[a]	50	18
	152.0>93.0	48	40
Amantadine -d_{15}	167.3>150.3[a]	48	35
Rimantadine	180.2>81.0[a]	60	42
	180.2>163.2	65	18
Rimantadine-d_4	184.2>167.0[a]	60	18
Chlorpheniramine	275.0>202.0[a]	60	42
	275.0>230.0	60	18
Chlorpheniramine-d_6	281.2>230.0[a]	60	18

[a] means ion pair for quantification

Table 2 Recoveries of the traditional (TQ) and the modified QuEChERs (MQ) methods in different matrices (n=3)[a]

Matrix	Amantadine		Rimantadine		Chlorpheniramine	
	TQ (%)	MQ (%)	TQ (%)	MQ (%)	TQ (%)	MQ (%)
Pork	96.7±6.12	103±6.12	95.3±3.93	99.3±2.34	96.2±3.72	101±4.14
Pig liver	93.9±4.32	97.9±3.19	101±2.36	89.9±3.59	97.1±4.28	97.5±5.89
Pig kidney	97.1±8.34	104±6.19	91.4±8.17	102±7.65	93.8±5.88	94.5±6.21
Chicken	99.0±3.02	98.5±3.28	94.3±6.18	104±6.13	98.1±4.21	96.5±3.30
Chicken liver	98.2±7.62	93.1±6.76	98.2±3.74	97.1±6.71	104±9.11	96.1±8.19
Egg	94.3±3.17	97.0±2.64	99.0±4.21	96.4±4.56	102±1.93	97.0±2.66
Duck	97.1±2.84	102±1.49	96.3±2.28	95.8±2.36	93.7±2.91	98.6±3.50

[a] The value is the average recovery ± RSD at spiked concentration of 10 μg/kg.

Table 3 Slope ratios of matrix-matched calibration and standard solution calibration

Matrix	Amantadine		Rimantadine		Chlorpheniramine	
	RE[a] (%)	RI[b] (%)	RE (%)	RI (%)	RE (%)	RI (%)
Pig liver	-67.1	6.42	-66.0	2.75	-83.1	-7.92
Pig kidney	-62.1	5.88	-65.0	3.85	-82.2	-2.92

[a] RE is the slope ratio of MMC and SSC using the external standard method minus 1.0.

[b] RI is the slope ratios of MMC and SSC using the internal standard method minus 1.0.

312 **Table 4** Regression data and sensitivity of the developed method

Analyte	Linear equation	Concentration Range (μg/L)	R^2	LOD (μg/kg)	LOQ (μg/kg)
Amantadine	y=0.0371x+0.0744	2-200	0.9938	0.5	1.0
Rimantadine	y=0.0182x+0.0127	2-200	0.9970	0.5	1.0
Chlorpheniramine	y=0.0024x+0.00155	0.2-20	0.9964	0.05	0.1

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Table 5 Intra-day and inter-day precisions for three analytes in all matrices (n=5) [a]

Matrix	Spiked level [b] (μg/kg)	Amantadine		Rimantadine		Chlorpheniramine	
		Intra-day (%)	Inter-day (%)	Intra-day (%)	Inter-day (%)	Intra-day (%)	Inter-day (%)
Pork	1 (0.1)	96.4±6.21	98.4±8.79	97.4±5.25	94.4±7.80	99.4±3.63	94.5±5.45
	10 (1)	103±6.12	96.3±7.85	99.3±2.34	97.9±5.86	101±4.14	102±9.87
	100 (10)	99.0±6.12	96.7±7.20	95.0±6.34	97.4±7.62	97.0±6.14	97.7±8.22
Pig liver	1 (0.1)	97.6±3.25	94.6±6.78	94.5±3.12	94.6±5.79	92.1±3.17	94.6±4.80
	10 (1)	97.9±3.19	98.2±5.91	89.9±3.59	92.2±5.12	97.5±5.89	98.2±6.93
	100 (10)	99.8±2.30	100±7.00	96.7±4.31	100±5.01	95.3±2.21	98.4±6.02
Pig kidney	1 (0.1)	97.7±8.75	98.4±9.85	93.3±4.76	102±8.67	95.4±7.23	99.0±7.34
	10 (1)	104±6.19	104±9.98	102±7.65	95.5±5.78	94.5±6.21	99.5±8.19
	100 (10)	105±8.60	102±9.15	99.6±4.61	98.8±4.86	97.5±5.19	97..2±8.17
Chicken	1 (0.1)	96.5±6.82	92.3±7.92	94.2±4.33	97.1±6.53	98.6±7.38	101±8.45
	10 (1)	98.5±3.28	97.1±7.78	104±6.13	97.1±7.77	96.5±3.30	99.1±7.22
	100 (10)	95.1±6.09	96.9±9.01	94.8±5.37	97.2±8.02	95.6±6.11	96.9±7.37
Chicken liver	1 (0.1)	93.7±7.69	96.5±8.77	95.8±5.67	97.6±6.56	93.7±10.7	96.5±8.79
	10 (1)	93.1±6.76	93.7±8.47	97.1±6.71	101±7.74	96.1±8,19	97.2±9.17
	100 (10)	88.8±3.96	91.0±6.92	98.8±4.47	95.3±9.39	104±3.98	93.8±7.39
Egg	1 (0.1)	101±4.44	99.3±5.79	97.7±4.45	99.2±5.44	104±3.66	99.3±9.81
	10 (1)	97.0±2.64	98.1±9.07	96.4±4.56	98.8±9.52	97.0±2.66	97.2±6.49
	100 (10)	96.0±5.97	97.8±9.22	96.3±3.34	97.2±8.23	96.0±5.99	95.2±8.17
Duck	1 (0.1)	94.2±3.91	93.6±8.78	96.3±7.42	95.6±7.79	94.2±3.48	97.5±8.80
	10 (1)	102±1.49	101±4.29	95.8±2.36	99.7±6.19	98.4±3.15	101±4.16
	100 (10)	93.5±3.15	94.6±3.70	100±4.72	95.8±6.49	101±4.35	98.4±5.78

[a] The value is the average recovery ± RSD.

[b] The spiked levels are 1, 10, and 100 μg/kg for amantadine and rimantadine, and 0.1, 1, and 10 μg/kg for chlorpheniramine, respectively.

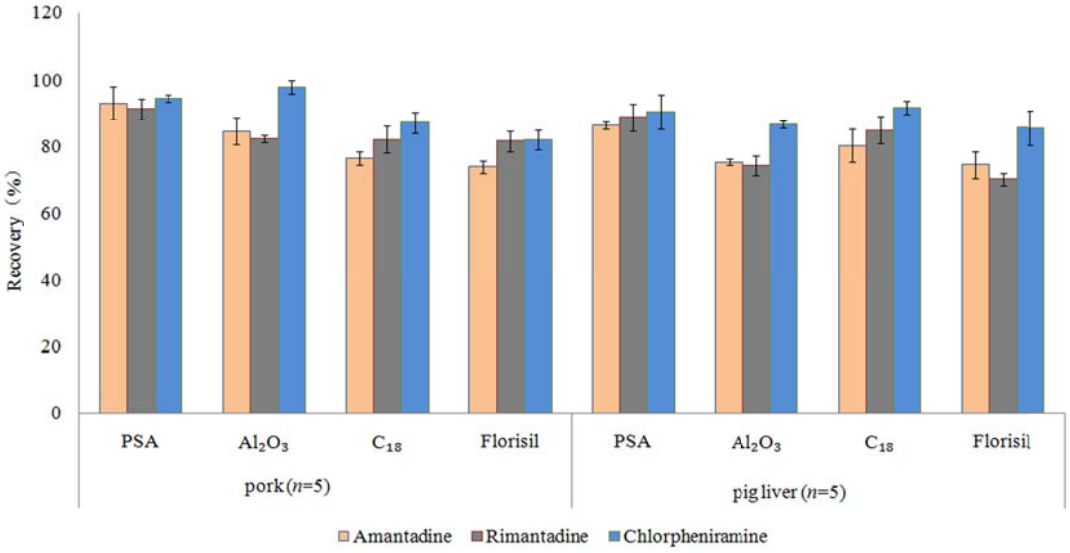

Fig.1 Purification effects of four sorbents for three analytes in two typical matrices

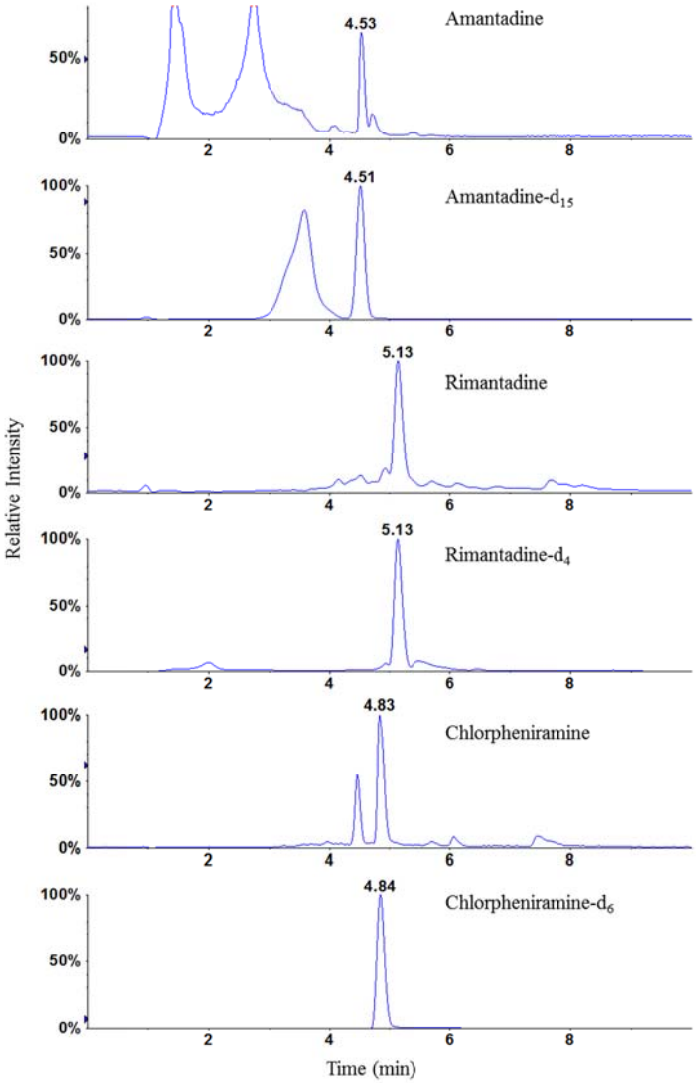

Fig. 2 The typical chromatogram of pork spiked with amantadine and rimantadine at 1.0 μg/kg, and chlorpheniramine at 0.1 μg/kg