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Determination of Multi-class Antimicrobials Residues in Soil by Liquid Chromatography–tandem Mass Spectrometry

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Antimicrobials residues in environmental matrices may result in the occurrence of antimicrobial-resistant bacteria in soil. In this paper, a new analytical method based on liquid chromatography-tandem mass spectrometry for multiresidue analysis of 24 antimicrobials of wide polarity range and variable physicochemical properties, including sulfonamides, tetracyclines, fluoroquinolones, macrolides, lincosamides and pleuromutilins in soil was developed. Samples were extracted with acetonitrile : Na_2EDTA-McIlvaine buffer (pH 4.0, 5:5, v/v) system and then re-extracted with 0.2 M sodium hydroxide solution. The extracts were purified using HLB solid phase extraction cartridge. Chromatographic separation of the components was performed on a Zorbax SB-Aq column using acetonitrile-0.1% formic acid as mobile phase. The method developed was linear in a concentration range from the limits of quantification to 200 μg kg^{-1}, with correlation coefficients higher than 0.99. The limits of detection and limits of quantification ranged from 0.01 to 2 μg kg^{-1} and 0.04 to 5 μg kg^{-1}, respectively. The overall average recoveries for target analytes were more than 60% except for tetracycline (59.3%) in three spiked levels of 1, 4 and 20 μg kg^{-1} with relative standard deviations less than 20%. The method was further applied

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on the determination of residual antimicrobials in real samples. Some target antimicrobials were detected at different levels and tetracyclines residues were dominant. 163.6 μg kg^{-1} of chlortetracycline was detected in a soil sample. The results indicate that the proposed method has a good feasibility.

1. Introduction

In recent decades, because large amount of drugs have been used in human and veterinary medicine,[1] they have been widely detected in a variety of environmental matrices such as water, soil.[2] Currently, pharmaceutical residues in the environment are of increasing worldwide concern. After administration, pharmaceuticals and their metabolites are excreted by animals and humans, and then the excretion of the faeces together with urine flows into the environment. Finally, these compounds accumulate in soil. Some hydrophilic drugs may be mobile in soil which can contaminate ground water,[3] and then they are introduced into the environment even into crops and the food supply.[4] So the existence of antimicrobials in water and soil may pose a risk to human health and environment ecology. In addition, the widespread use and environmental persistence of some veterinary or human drugs in the environment have raised concerns about the potential for the increase of antibiotic-resistant bacteria.[5] Bacteria resistant to antimicrobials have been found in aquatic environment and soil.[6-7] It becomes a hot research how to effectively assay the residues of antimicrobials in environment such as water body, soil and atmosphere.

Several methods for the analysis of the commonly used antimicrobials in water,[8] animal tissues,[9] milk,[10] and manure[11] have been described using liquid chromatography-tandem mass spectrometry (LC-MS/MS). However, because of the heterogeneity of solid matrices and the great diversity of pharmaceuticals with very different polarity and functionality, the determination of antimicrobials residues in soils is poorly documented. Their presence and distribution in the soil via land application are far from being fully understood, which is primarily due to a lack of appropriate analytical methodologies. In addition, most of the available multi-extraction

procedures and instrumental analytical methods for solid environmental samples cover only one[12] or specific classes of antimicrobials.[13,14] But none of these methods includes most common veterinary antimicrobials. Therefore, the development of a sensitive analytical method that allows for determining the residues of several classes of common veterinary drugs in soil is necessary.

The available information about the environmentally relevant concentrations of the commonly used antimicrobials is also limited; it is mostly due to analytical difficulties encountered. When trying to analyze these compounds at trace levels, various factors such as their polarity, solubility, pK_a, K_{ow} and stability in complex matrices shall be considered. As for soil matrix, the sample pre-treatment is the most difficult and time-consuming, and often involves one or more extraction and cleanup steps. Techniques of extraction such as pressurized liquid extraction (PLE),[6] microwave-assisted solvent extraction (MASE)[15] and supercritical fluid extraction (SFE)[16] have been introduced. The common advantages of all the techniques can be referred the improvement of rapidity and automation. However, some particular drawbacks must be considered. The PLE and SFE techniques require expensive apparatus and complicated optimization procedures. The MASE technique can improve extraction efficiency, but lacks extraction selectivity, thus, and it is required for a further cleanup step. Although the MASE technique is not easily automated, it can reduce the organic solvent consumption and no specialized laboratory equipment is required. After extraction, in common, purification has to be performed by solid-phase extraction (SPE), liquid–liquid extraction (LLE), gel-permeation chromatography (GPC) or semi-preparative liquid chromatography (LC). The SPE method is often preferred since it is faster, requires less solvent and has a lower risk of sample contamination. Due to the hydrophilic - lipophilic balance (HLB) properties and the effectiveness in the extraction of a wide range of acidic, basic and neutral

compounds from various matrices, Oasis HLB is one of the most widely utilized SPE sorbent for pharmaceutical extraction in soil samples. In this study, the extraction efficiencies of the C_{18} and MCX SPE cartridges were compared with that of the HLB SPE cartridge.

The present study focuses on developing a sensitive, selective and reproducible method for the simultaneous determination of 24 different antimicrobials including six sulfonamides (SAs), four tetracyclines (TCs), six fluoroquinolones (FQs), five macrolides (MLs), one lincosamides (LAs) and two pleuromutilins (PMs) in soils using LC-MS/MS with a triple quadrupole analyzer. Different extraction solutions, extract ratios and types of solid-phase extraction cartridges for soil sample preparation were discussed and optimized. Afterwards, the method developed was successfully applied to the determination of 100 soils samples randomly collected from different sources (35 piggeries, 25 vegetable fields, 20 living quarters, 20 orchards) in Guangdong Province, China.

2. Experimental

2.1. Reagents and materials

Reference standards of all pharmaceuticals including difluoxacin, sarafloxacin, enrofloxacin, ciprofloxacin, enoxacin, norfloxacin, chlortetracycline, oxytetracycline, doxycycline, tetracycline, sulfaquinoxaline, sulfaclozine, sulfamethoxydiazine, sulfamonomethoxine, sulfadimidine, sulfamethoxazole, tylosin, roxithromycin, kitasamycin, erythromycin, tilmicosin, clindamycin, valnemulin and tiamulin (purity>90%) were purchased from China Institute of Veterinary Drugs Control (Beijing, China) and J & K Chemical LTD (Beijing, China). HPLC-grade Methanol (MeOH), Acetonitrile (ACN) and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethylenedi-minetetraacetic acid disodium salt dihydrate ($Na_2EDTA{\cdot}2H_2O$), sodium

hydroxide pellets (NaOH), disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$) and citric acid monohydrate ($H_3Cit \cdot H_2O$), hydrochloric acid (HCl, 37%, w/v) and ammonia solution (25%, w/v) were purchased from the Guangzhou Chemical Reagent Company (Guangzhou, China). Ammonium acetate was purchased from TEDIA （Fairfield, OH, USA). Deionized water was obtained using a Millipore purification system Milli Q (Molsheim, France). Other chemical reagents were of analytical reagents grade.

Oasis HLB (hydrophilic-lipophilic balance, poly (divinylbenzene-co-N-pyrrolidone, 60 mg, 3 mL) SPE cartridge and Oasis MCX SPE cartridge (60 mg, 3 mL) were purchased from Waters Co. (Milford, MA, USA). Bond Elut-C_{18} SPE cartridge (200 mg, 3 mL) was purchased from Agilent Technologies Co. (Santa Clara, CA, USA).

A Na_2EDTA-McIlvaine buffer solution (0.1 M) was prepared by mixing 1000 mL of 0.1 M citric acid with 625 mL of 0.2 M disodium hydrogen phosphate (pH adjusted to 4.0±0.05 with NaOH or HCl as needed), and then 60.5 g of $Na_2EDTA \cdot 2H_2O$ was added into the above mixture.

Individual stock solutions were prepared at concentrations of 100 mg L^{-1} in methanol and stored at -20 ^{0}C. Mixed working standard solutions were prepared by the adequate mixing and dilution of the individual stock solutions.

2.2. Sample preparation and extraction

Blank soil sample selected for the establishment of the quantitative method was collected from a livestock farm at a depth of 0-10 cm. Soil samples were passed through a 3 mm sieve to remove plant detritus, root and gravel, and then stored at -20 ^{0}C until further analysis.

A sieved soil sample (5.0 g) was introduced into a 50 mL polypropylene centrifuge tube and spiked at 1, 4 and 20 μg kg^{-1} by the addition of 100 μL appropriate mixed working solutions. After

being stand at least 20 min, 15 mL of extraction buffer (ACN : Na_2EDTA-McIlvaine buffer (pH 4.0, 5:5, v/v) were added into the tube. The tube was vortex mixed to achieve homogeneity, and then the tube was ultrasonicated for 10 min, shaken for 20 min, finally centrifuged at 9000 rpm for 10 min. The supernatant was transferred to clean glassware and evaporated to below 7 mL in 45 ^{0}C water bath. The soil residue was extracted with 10 mL of 0.2 M NaOH again. The top aqueous layer was decanted to a new tube, adjusted pH to 4.0 with 1 M HCl, and centrifuged at 6000 rpm for 5 min. All the supernatant were combined prior to the cleanup step by solid phase extraction.

2.3. Solid phase extraction

Cleanup and enrichment were performed on the Oasis HLB cartridge, which was conditioned using 3 mL methanol followed by 3 mL ultrapure water and 3 mL Na_2EDTA-McIlvaine buffer. The supernatant was loaded into the cartridge at approximate 1 mL min^{-1}. The cartridge was then washed with 6 mL of 5% methanol in water and dried by applying a low positive pressure for 2 min, eventually the analytes were eluted with 6 mL methanol. The eluate was evaporated to near dryness under gentle nitrogen flux at 45 ^{0}C, and then re-dissolved in 1.00 mL of 20% methanol in 0.1% formic acid solution prior to analysis by LC - MS/MS.

2.4. LC–MS/MS analysis

The chromatographic system was composed of an Agilent 1200 series high-performance liquid chromatography (HPLC) system, including quaternary pump and autosampler (Milford, MA, USA). The mass system included Applied Biosystems API 4000 triple quadrupole mass spectrometer with electrospray ionization (ESI) interface and Analyst 1.5 software (Foster City, CA, USA).

Chromatographic separation was performed using an Agilent Zorbax SB-Aq C_{18} column (150

mm × 2.1 mm i.d., 3.5 μm). The mobile phase consisted of acetonitrile (A) and 0.1% formic acid in water (B). The mobile phase used in the gradient elution consisted of solvent A and solvent B. As described in our previous study,[26] the linear gradient developed for the analysis was performed as follows: 0 - 0.2 min 10% A; 0.2 - 1.0 min 10% - 20% A; 1.0 - 11 min 20% - 40% A; 11 - 15 min 40% - 90% A; 15 - 16 min 90% A; 16 - 18 min 90% - 10% A; 18 - 26 min 10% A. The total runtime was 26 min. The column was maintained at 35 ^{0}C. The flow rate was 0.2 mL min^{-1} and the injection volume was 5 μL.

The tandem MS analyses were carried out on API 4000 triple quadrupole mass spectrometer with electrospray ionization source. The turbo ion-spray source was used in positive mode with the following settings: Ion spray voltage (IS), 5000 V; Ion source temperature, 600 ^{0}C; Dwell time, 50 ms. The optimal collision energy (CE), declustering potential (DP) and transitions chosen for the multiple reaction monitoring (MRM) are listed in Table 1. Acquisition and analysis of data were performed through Analyst 1.5 software (Applied Biosystems) in Windows XP platform-based data-processing system.

2.5. Method validation

The performance characteristics of the developed method including selectivity, limit of detection (LOD), limit of quantification (LOQ), recovery and precision were evaluated.

The selectivity of the method was checked by analyzing 50 blank soil samples from different sources to evaluate possible matrix interferences. The results were evaluated by the presence of interfering substances around the analyte's retention time.

Linearity was evaluated by using of matrix-matched calibration curves. Seven-point ranging from the LOQ of each analyte to 200 μg kg^{-1} was prepared by spiking corresponding amounts of

target compounds into five gram blank soil extracts.

The LOD and LOQ for the analyte in soil were determined by signal to noise ratio *(S/N)* of 3 and 10, respectively. The most common method was based on the chromatographic response regarding the most intense ion transition for quantification and the ion transition ratio used for confirmation.

Recoveries and precision for the entire method were evaluated by spiking blank soil samples at three concentration levels (low, 1 μg kg^{-1}; medium, 4 μg kg^{-1}; and high, 20 μg kg^{-1}) for target analytes in six replicates at each level for three consecutive days. The recoveries of twenty-four analytes at the spiked samples were calculated by measuring the ratios of the predicted value obtained from the matrix-matched calibration curves to the corresponding spiked values. Intra-day precision was determined for the three concentration levels in six replicates for each concentration on the same day. Inter-day precision was determined for the three concentration levels in six replicates for each concentration on three different days. The intra-day and inter-day precisions were estimated by calculating the relative standard deviation (RSD, %) for the different concentrations.

Stability was expressed as a percentage of the initial value. Due to the significant difference of physicochemical properties of the 24 antimicrobials, the stability in pure solvent and sample solution should be checked prior to chromatographic investigations. This research mainly investigated the stability of the stock solution of the target analytes under -20 ^{0}C within 30 days and the short-term stability of the soil sample including room temperature (25 ^{0}C, in the autosampler) and 4 ^{0}C within 6, 12, 24 and 48 h. All stability studies were conducted in triplicate. The measured values were compared with those freshly prepared pure solvent and matrix standard

178 solutions at different concentrations.

179 **2.6. Matrix effects (ME)**

180 Matrix effects are common in LC-MS/MS analysis due to the molecules co-elute with the
181 compounds of interest and alter their ionization efficiency in the ionization interface, causing ion
182 suppression or enhancement.[14] The intensity of matrix effect was evaluated by the method of
183 post-extraction addition.[17] The percentage of ME is calculated as

184 $$ME\ (\%) = B/A \times 100$$

185 Where A and B represent the peak area of an analyte in pure solution and the analyte spiked after
186 extraction with 20 μg kg^{-1} of each compound, respectively. A ME value of 100% indicates that no
187 matrix effect is present. If the value is less 100%, there is signal suppression, whereas if the value
188 is above 100%, there is signal enhancement.

189 **3. Results and discussion**

190 **3.1 Sample extraction**

191 In order to develop an effective sample extraction step, several extraction solvents including its
192 volume and ratio of the buffer in solvent system were evaluated.

193 Many minerals and organic matter in the soil matrix may form kinds of interactions (such as
194 complexation, hydrogen bonding, hydrophobic interaction and ion-exchange) with the analytes, so
195 that the extraction of the compounds of interest from soil becomes difficult and complex.
196 Therefore, an appropriate sample pretreatment method is very important for an accurate
197 determination of target analytes in soil samples. On basis of the physicochemical properties of the
198 target compounds and the extraction approaches of similar sample matrix in literatures,[5,13,14,18,19]
199 several preliminary experiments were performed to extract the antimicrobials residues from soil

samples. Thus the following five extraction solvent systems were tested:

- M_1=ACN/MeOH (1:1, v/v).

- M_2=ACN/acetate buffer (1:1, v/v, pH 4.0).

- M_3=ACN/acetate buffer (1:1, v/v, pH 4.0) and 0.5 g Na_2EDTA.

- M_4=ACN/citrate buffer (1:1, v/v, pH 4.0) and 0.5 g Na_2EDTA.

- M_5=ACN: Na_2EDTA-McIlvaine buffer (5:5, v/v, pH 4.0).

Blank soil samples were spiked with 100 μL of 0.2 mg L^{-1} (each) mixed working standard solution to evaluate the mean recoveries based on the mentioned extractive method above. The recoveries are summarized in Fig. 1. The results demonstrate that good yields (more than 80%) were obtained only for SAs, clindamycin, roxithromycin and tiamulin when using the M_1 system, however, the recoveries of the other compounds were very low (most analytes less than 20%). Salvia et al.[5] suggested that the acetate-based method could result in better recoveries, particularly for veterinary antimicrobials such as sulfonamides and macrolides. Therefore, the M_2 and M_3 systems were also chosen as the extraction solvent. The results show that the high recoveries (70% above) were obtained for major target analytes such as SAs, MLs and LAs. However, the measured recovery ratios of 4 TCs and 6 FQs were all below 60%, and the recoveries of the ten analytes obtained by the M_2 were slightly lower than those by the M_3 (the addition of Na_2EDTA).

TCs and FQs have a strong adsorption capacity to the soil since the polarity/ionic functional groups existed in their chemical structures. So for improving the extraction efficiency of TCs and FQs from soil samples, a complexation agent (Na_2EDTA buffer and (or) citrate buffer), which can abate the chelate effect, was added to avoid the complexation of these analytes with divalent cations such as Mg^{2+} or Ca^{2+} in soil [20] and facilitate the extraction of bound compounds. As shown

222 in Fig. 1, the recovery ratios of five of the six FQs (except difloxacin) and one (tetracycline) of the
223 four TCs were below 40% when the M_4 system was used as the extraction solvent. In contrast, the
224 M_5 system achieved relatively high recoveries for all the analytes except FQs (12%-36%). Thus,
225 the M_5 could be used to extract most target analytes from soil samples. Further, the volume ratio of
226 ACN in the Na_2EDTA-McIlvaine buffer（for example, 9:1, 7:3, 5:5 and 3:5, v/v）was investigated.
227 The experiments show that the recoveries of most analytes (except for FQs) increased with the
228 decrease of acetonitrile in the extraction solvent. The higher recoveries (more than 60%) were
229 obtained with the 5:5 ratio of ACN to Na_2EDTA-McIlvaine buffer than both the 9:1 and 7:3.
230 However, too low ACN (3:5, v/v) in M_5 system resulted in low recoveries for MLs and PMs.
231 Several volumes of the M_5 system (10, 15 and 20 mL) were subsequently tested. The results
232 indicate that the volume of 15 mL gave higher recoveries than the volume of 10 mL, especially for
233 TCs. On the other hand, compared to 15 mL, the 20 mL did not significantly increase the
234 recoveries for most of the analytes. Therefore, in order to get the higher recoveries, while
235 minimizing the consumption of solvent and time, the volume of 15 mL M_5 was chosen for the
236 following experiments.

237 For enhancing the recoveries of FQs, further optimization of extraction protocols was needed.
238 According to the properties of these compounds and the corresponding literatures on the analysis
239 of FQs residues, several extraction solvents including acidic, basic and different buffer solutions
240 were evaluated. Blank soil samples were spiked with 100 μL of 0.2 mg L^{-1} (each) mixed working
241 standard solution to evaluate the extraction recoveries of different solvents. The results are
242 summarized in Table 2. The pH value of the extraction solvent had a great influence on the
243 extraction efficiency of FQs. 0.1 M HCl, 0.05 M orthophosporic acid and 5% formic acid in

244 acetonitrile did not extract any FQs. The phosphate buffer (pH 3.2) - acetonitrile (1:1, v/v) system
245 and phosphate buffer (pH 7.4) also gave very poor recoveries (all below 40% for the six FQs).
246 Delepine et al.[21] used 0.05 M phosphate buffer solution (pH 7.4) to extract FQs from muscle.
247 Good recoveries for FQs were obtained. But in our experiments, perhaps because there are a great
248 number of divalent metallic elements and organic matters in soil matrix, very low recoveries were
249 obtained when the phosphate buffer solution was used to extract FQs from soil. Turiel et al.[22]
250 reported that the high recoveries for FQs could be obtained when the 50% (w/v) $Mg(NO_3)_2$
251 solution containing 4% of ammonia was used to desorb and extract FQs from soil on basis of the
252 formation of fluoroquinolones-Mg^{2+} complexes. In this study, good recoveries were also obtained
253 using this extraction solution. Nevertheless, because Mg^{2+} in the extracts formed precipitation
254 with the Na_2EDTA-McIlvaine buffer solution, resulting in blockage of the SPE cartridge in the
255 cleanup step. Fortunately, good recoveries for FQs were achieved when using strong basic solution
256 as an extraction solvent. One reason was due to FQs (as anionic form) being dissolved in sodium
257 hydroxide solution. Another reason was that in alkaline condition the carboxyl of FQs was
258 negatively charged, which has an electrostatic repulsion to the negative charge on the surface of
259 the soil.

260 Thus, the concentration and volume of NaOH were further optimized. Firstly, the influence of
261 the concentration of NaOH on the extraction efficiency was investigated in the concentration
262 range of 0.01 - 0.5 M. The results reveal that the extraction efficiency of FQs increases with the
263 increase of NaOH concentration. However, if the concentration of NaOH was too high, the
264 recoveries of the other analytes decreased, especially up to 0.5 M, the recoveries of TCs, SQ and
265 SCZ were significantly lowered. Secondly, the different volumes of NaOH solution were tested.

The results show that the recoveries for FQs increased with the increase of the volume of NaOH solution. On the contrary, the recoveries for the other target analytes such as SAs and MLs decreased. The results are shown in Fig. 2. For a compromise, the 10 mL of 0.2 M NaOH was used for the following experiments.

Finally, the ACN : Na_2EDTA-McIlvaine buffer (5:5, v/v, pH 4.0) system (M_5) in combination with 0.2 M NaOH was selected to extract target analytes in soil samples.

3.2 Cleanup

In complex environmental samples, for example sediments and soils, some matrix components can mask analytes in the chromatographic separation and in the final detection system.[6] Therefore it is very necessary to choose the ideal SPE sorbents giving an acceptable recovery for all target compounds with different physicochemical properties. At present, the most commonly used SPE cartridges, which allow large sample volumes to be concentrated and purified in one step, are HLB[14], C_{18}[23] and MCX[24] cartridges. In this study, three types of SPE (Bond Elut-C_{18} SPE C_{18}, Oasis MCX and Oasis HLB) were evaluated. Each type of cartridge was processed at its optimal conditions. As shown in Fig. 3, recoveries less than 50 % for most of the target analytes were obtained with both C_{18} and MCX cartridges, especially for SAs (below 10%). However, the HLB cartridge achieved the best recoveries (75-104%) for all analytes except for valnemulin (67%). So the HLB cartridge was chosen as the optimized SPE cartridge.

3.3. Optimization of LC-MS/MS conditions

The electronic spray ionization-tandem mass spectrometer offers a high sensitivity and improved selectivity through multiple reactions monitoring acquisition to detect antimicrobials in real samples. The optimization of MS parameters for each compound was performed by direct infusion

288 of pure reference standards (1 mg L^{-1}) into the MS/MS compartment at 10 μL min^{-1} by a syringe
289 pump (Harvard Apparatus, Holliston, MA). In the positive ion mode, the protonated molecules
290 $[M+H]^+$ were observed for all compounds on their full-scan mass spectra. These ions were
291 selected as precursor ions to further produce product ions, and the corresponding parameters
292 including declustering potential and collision energy in MRM mode were optimized. The results
293 are listed in Table 1. For each analyte, two ion transitions were monitored; the first transition
294 corresponding to the highest abundance was used for quantification and the second one for
295 confirmation. Ion logarithms were selected in accordance with the 2002/657/EC requirements
296 (IPs≥4)[25].

297 The chromatographic separation of the target compounds was performed using HPLC. The
298 Zorbax SB-Aq column, which was proved to be superior to other chromatographic columns in our
299 laboratory,[26] was used for LC separation of the twenty-four analytes. In brief, acetonitrile was
300 selected as eluent A and 0.1% formic acid in Milli Q water was selected as eluent B. The linear
301 gradient program was referred to the gradient program previously reported in section 2.4.

302 **3.4. Validation of the analytical method**

303 **3.4.1. Specificity**

304 Specificity is the ability to assess unequivocally the analyte in the presence of endogenous
305 compounds. It was checked by analyzing 50 different blank soil samples to verify the absence of
306 interfering substances. The results show that this method could effectively extract and recover all
307 the target analytes spiked in the soil samples and no interfering peaks within the 2.5% margin of
308 the relative retention time of the 24 analytes. Typical MRM chromatograms in the positive ESI
309 mode obtained from the blank soil extracts are illustrated in Fig. 4a.

310 **3.4.2. Linearity**

311 Since sample matrices tend to affect (either reduce or enhance) the ion intensities of target
312 analytes, matrix-matched calibration curves are used to determine the analytes concentrations. The
313 linearity of the method was determined by seven values (not excluding blank values) from the
314 expected range of concentrations with six replicates of each. As shown in Table 3, the soil matrix
315 for the prepared matrix-matched calibration curves was from piggeries. The calibration curves
316 were linear for all compounds over a wide range of concentrations from the LOQ to 200 μg kg^{-1}
317 with a correlation coefficient (*r*) higher than 0.99.

318 **3.4.3. Recovery and precision**

319 Recovery and precision (repeatability and within-laboratory reproducibility) were determined by
320 processing independently the eighteen spiked samples at three levels (1, 4 and 20 μg kg^{-1}) in three
321 different days. As shown in Table 3, the average recoveries for most antimicrobials increases with
322 the increase of the spiking levels and the overall average recoveries for target analytes are more
323 than 60% except for tetracycline (59.3%) in three spiked levels. The higher recoveries were
324 obtained for macrolides and lincosamides, and low recoveries were obtained for polar
325 tetracyclines, fluoroquinolones and sulfonamides. There is a certain difference within different
326 spiked levels for several target analytes. In low level (1 μg kg^{-1}), the average recoveries for
327 tetracycline and sulfaclozine are less than 55% (53.2% and 54.4%, repectively); in medium and
328 high levels, the average recoveries for most target analytes exceeded 60% except that the
329 recoveries of three compounds including chlortetracycline, tetracycline and sulfaquinoxaline are
330 almost near 60% (58.2%, 58.6% and 59.4%, respectively). Although all the relative standard
331 deviations are below 20%, the inter-day RSDs are larger than the intra-day RSDs, suggesting there

is a certain difference within intra-day recoveries. The results are satisfactory for the detection of multi-class antimicrobials residues in soil samples. Typical MRM chromatograms in the positive ESI mode obtained from the blank soil extracts spiked at a concentration level of 4 μg kg^{-1} are illustrated in Fig. 4b.

3.4.4. LOD and LOQ

The LOD was calculated as a *S/N* of 3:1 and the LOQ was defined as a *S/N* of 10:1. The results showed that clindamycin was higher sensitivity (0.01 μg kg^{-1} LOD) in the optimized LC-MS/MS conditions. The LODs of all target compounds ranged from 0.01 μg kg^{-1} to 2.0 μg kg^{-1} and the LOQs ranged from 0.04 to 5.0 μg kg^{-1} (Table 3). The developed method is sensitive enough for the determination of trace antimicrobials in soil samples.

3.4.5. Stability

The results of stability test show that 24 analytes were stable at -20 ^{0}C in the stock solution within 30 days, no degradation was observed in pure methanol solvent. Most analytes in the fortified soil extracts remained stable for 48 h at 4 ^{0}C except that tetracycline and roxithromycin were stable within 36 h. In addition, stability test in the autosampler showed that no significant loss of the compound was observed in matrix extracts solution at 25 ^{0}C for 24 h. However, the significant decrease was observed for TCs, especially for tetracycline (near 40%) and 2 MLs (kitasamycin, 30% and roxithromycin, 35%) in 48 h. Therefore, the prepared sample solution must be analyzed within 2 days for ensuring accuracy and precision.

3.5. Matrix effects

Matrix effects were evaluated at the concentrations of 20 μg kg^{-1}. The matrix effects for each compound in soil from piggeries are summarized Table 3. Most antimicrobials experienced weak

matrix suppression. There was matrix suppression at moderate intensity level (62.6% - 76.1%) for FQs and TCs except oxytetracycline (86.9%) and obvious matrix suppression for sulfaquinoxaline (56.8%). Although spiking appropriate internal standards and isotope dilution technique would eliminate for the matrix effects, large varieties of target compounds and the cost of isotope internal standard make this unfeasible. Therefore, this research adopted the matrix matching standard curve method to further compensate for matrix effects.

3.6. Method application

A liquid chromatography-tandem mass spectrometric method based on the ESI multiple reaction monitoring mode for multiresidue analysis of 24 antimicrobials in soil was developed. Firstly, samples were extracted with acetonitrile-McIlvaine buffer system and 0.2 M sodium hydroxide solution, and then purified by solid phase extraction cartridge. Chromatographic separation was carried out on the Zorbax SB-Aq column using acetonitrile-0.1% formic acid as mobile phase with gradient program. For evaluating the applicability and performance of the proposed method, 100 soils samples collected from different sources (35 piggeries, 25 vegetable fields, 20 orchards and 20 living quarters) were examined. None of the target compounds was detected in the samples collected from the living quarters. However, other soil samples were found to be contaminated with at least four antimicrobials. The TCs were dominated antimicrobials detected in soil samples, especially the soils from piggeries with maximum level of 163.6 μg kg^{-1} chlortetracycline, followed by FQs (0.7 - 40.7 μg kg^{-1}). Four analytes (kitasamycin, tiamulin, doxycycline and tilmicosin) were detected in the orchard soils at concentrations ranging from 1.5 μg kg^{-1} to 5.9 μg kg^{-1}. Eight analytes (tiamulin, chlortetracycline, oxytetracycline, tetracycline, doxycycline, tilmicosin, enrofloxacin and sulfamonomethoxine) were found at concentrations ranging from 0.5

376 μg kg^{-1} to 18.3 μg kg^{-1} and ciprofloxacin and norfloxacin at levels of the quantification limits in
377 the vegetable fields. The findings obtained in this study indicate that animal manure can cause
378 veterinary pharmaceuticals contamination of agricultural soil. Some antimicrobials detected at
379 relatively high concentrations in soil may be inferred that the animals were long-term
380 adiminstration and the pharmaceuticals were excreted through animal body as parent compounds.

381 **4. Conclusions**

382 In this study a robust, sensitive and selective method has been developed and validated for the
383 determination of 24 pharmaceuticals in soil matrices. The method has enabled accurate
384 multiresidue determination of the target analytes in soil at μg kg^{-1} levels. The acceptable absolute
385 recoveries were above 60% for most of the target compounds. This methodology was successfully
386 applied to four different sources of soils including piggeries, vegetable fields, orchards and living
387 quarters. Several commonly used antimicrobials such as chlortetracycline, enrofloxain and
388 tilmicosin were detected at different concentration levels. Even though some antimicrobials are
389 detected at relatively low concentrations, there are high risks of their potential harms to human
390 health.

391 **Acknowledgments**

392 The authors thank the financial support by the Program for Changjiang Scholars and Innovative
393 Research Team in University (No. IRT13063) and the Joint Project of National Natural Science
394 Foundation of China and Natural Science Foundation of Guangdong Province, China (U0631006)
395 for this work.

396

397 **References**

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Figure captions

Fig. 1. Influence of the extraction solvents on the recoveries of the target compounds

DIF, difluoxacin; SAR, sarafloxacin; ENR, enrofloxacin; CIP, ciprofloxacin; ENO, enoxacin; NOR, norfloxacin; CTC, chlortetracycline; OTC, oxytetracycline; DC, doxycycline; TC, tetracycline; SQ, sulfaquinoxaline; SCZ, sulfaclozine; SMD, sulfamethoxydiazine; SMM sulfamonomethoxine; SM2, sulfadimidine; SMZ, sulfamethoxazole; TYL, tylosin; ROX, roxithromycin; KIT, kitasamycin; ERY, erythromycin; TIL, tilmicosin; CLI, clindamycin; VAL; valnemulin; TIA, tiamulin. M_1, ACN/MeOH (1:1, v/v); M_2, ACN/acetate buffer (1:1, v/v, pH 4.0); M_3, 0.5 g Na_2EDTA and ACN/acetate buffer (1:1, v/v, pH 4.0); M_4, 0.5 g Na_2EDTA and ACN/citrate buffer (1:1, v/v, pH 4.0); M_5, ACN : Na_2EDTA-McIlvaine buffer (5:5, v/v, pH 4.0). Error bars represent standard deviation of the individual compound spiked at 4 $\mu g\ kg^{-1}$ ($n = 3$).

Fig. 2. Influence of the concentration (a) and amount (b) of NaOH on the recoveries of 24 antimicrobials at the spiked 4 $\mu g\ kg^{-1}$ each

The abbreviations are the same as **Fig. 1**.

Fig. 3. Influence of the different types of SPE columns on extraction efficiency for 24 antimicrobials at the spiked 4 $\mu g\ kg^{-1}$ each

457 The abbreviations are the same as **Fig. 1**.

458

459

460 **Fig. 4.** Typical MRM chromatograms obtained from the blank soil extracts (a) and blank soil

461 extracts spiked at 4 μg kg^{-1} each (b)

462 The abbreviations are the same as **Fig. 1**.

463

TABLES

Table 1. LC-MS/MS conditions for the analytes by SRM in positive ion mode

Compounds	Abbr.	Precursor ion $[M+H]^+$	Product ion	DP (V)	CE (eV)	R_t (min)	Compounds	Abbr.	Precursor ion	Product ion	DP (V)	CE (eV)	R_t (min)
Fluoroquinolones	**FQs**						Sulfamethoxydiazine	SMD	281.2	156	60	25	11.7
Difluoxacin	DIF	400.4	382.3	60	28	12.1				215.1*		25	
			356.2*		28		Sulfamonomethoxine	SMM	281.2	156	60	25	12.7
Sarafloxacin	SAR	386.4	368.2	60	28	11.8				215.1*		26	
			342.3*		28		Sulfadimidine	SM2	279.2	186	60	25	10.6

Enrofloxacin	ENR	360.6	316.4	60	30	10.7				156*		28	
			245.1*		37		Sulfamethoxazole	SMZ	254.2	156	53	23	13.7
Ciprofloxacin	CIP	332.4	314.2	60	25	9.9				91.7*		40	
			288.3*		25		**Macrolides**	**MLs**					
Enoxacin	ENO	321.1	303.2	63	28	9.4	Tylosin	TYL	916.6	174.3	101	52	16.1
			234.2*		28					772.6*		41	
Norfloxacin	NOR	320.4	302.3	50	26	9.6	Roxithromycin	ROX	837.8	679.5	60	33	17.6
			276.6*		16					158.2*		55	
Tetracyclines	**TCs**						Kitasamycin	KIT	772.4	109.1	90	78	17.7
Chlortetracycline	CTC	479.3	444.2	71	29	11.5				174.2*		50	
			462.1*		24		Erythromycin	ERY	734.7	158	64	43	14.8
Oxytetracycline	OTC	460.7	426.1	65	26	8.7				576.5*		27	
			443.3*		17		Tilmicosin	TIL	869.6	696.4	130	66	12.8

Doxycycline	DC	445.2	410.2	65	27	9.5				174.2*		60	
			427.2*		19		**Lincosamides**	**LAs**					
Tetracycline	TC	445.2	428.2	70	25	12.2	Clindamycin	CLI	425.2	126.2	72	37	11.9
			153.9*		44					377.3*		27	
Sulfonamides	**SAs**						**Pleuromutilins**	**PMs**					
Sulfaquinoxaline	SQ	301.3	156	62	24	16.5	Valnemulin	VAL	565.5	263.1	80	25	18.1
			91.7*		44					164.2*		44	
Sulfaclozine	SCZ	285.2	155.9	60	23	16.1	Tiamulin	TIA	494.5	192.2	48	29	17.3
			107.7*		38					119.2*		55	

Abbr., abbreviations; DP, declustering potential; CE, collision energy; R_t, retention time.

* for identification.

Table 2. Recoveries for FQs obtained with different extractive solvents (%, *n* = 3)

Solvent	Difloxacin	Sarafloxacin	Enrofloxacin	Ciprofloxacin	Enoxacin	Norfloxacin
0.1 M HCl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.05 M orthophosporic acid	0.6	17.8	5.4	16.9	22.7	19.5
5% HCOOH in acetonitrile	n.d.	0.1	n.d.	n.d.	n.d.	n.d.
0.1 M phosphate buffer-acetonitrile (1:1, v/v, pH 3.2)	34.3	34.5	22.3	17.5	4.5	7.4
0.02 M phosphate buffer (pH 7.4)	8.2	33.7	37.5	34.0	9.6	13.2
4% $NH_3 \cdot H_2O$ in 50% $Mg(NO_3)_2$ solution	64.6 ± 4.9	83.0 ± 5.2	78.2 ± 4.3	117 ± 6.6	101 ± 3.5	56.5 ± 6.7
0.1 M NaOH	85.6 ± 3.3	89.6 ± 3.7	88.8 ± 1.4	87.9 ± 3.2	87.1 ± 6.0	89.6 ± 5.5

n.d., not detected; spiking level, 4 μg kg^{-1} each.

Table 3. Linearity, LOD, LOQ, recovery and precision of the developed method and matrix effects from piggeries soil

Group	Analyte	Linearity	LOD	LOQ	Intra-day recovery, (%, n = 6)			Intra-day RSD, (%, n = 6)		
		(r)	(μg kg^{-1})	(μg kg^{-1})	1 μg kg^{-1}	4 μg kg^{-1}	20 μg kg^{-1}	1 μg kg^{-1}	4 μg kg^{-1}	20 μg kg^{-1}
FQs	Difluoxacin	0.9979	0.1	1.5	62.4	61.8	63.5	12	11	8.0
	Sarafloxacin	0.9955	0.1	1.5	61.6	74.5	88.0	8.6	9.2	7.1
	Enrofloxacin	0.9938	0.05	0.4	65.2	70.6	68.3	6.0	7.4	5.5
	Ciprofloxacin	0.9981	0.2	0.5	61.7	77.5	78.9	9.5	9.0	7.2
	Enoxacin	0.9965	0.1	0.5	59.2	63.5	63.4	11	10	8.5
	Norfloxacin	0.9968	0.1	0.5	57.9	66.5	70.5	12	11	7.6
TCs	Chlortetracycline	0.9961	0.2	1.0	60.0	60.8	66.7	13	8.3	6.7
	Oxytetracycline	0.9974	0.2	1.0	70.4	68.0	71.4	14	12	4.4
	Doxycycline	0.9952	0.2	1.0	65.2	66.5	71.0	14	13	5.3

	Tetracycline	0.9978	0.5	1.5	53.8	60.1	67.4	9.5	3.4	8.0
SAs	Sulfaquinoxaline	0.9948	0.3	1.0	60.5	63.7	75.0	12	9.0	6.6
	Sulfaclozine	0.9972	1.0	2.0	55.4	68.1	60.0	10	5.9	7.0
	Sulfamethoxydiazine	0.9954	0.2	1.0	64.4	63.8	72.9	3.8	2.8	3.0
	Sulfamonomethoxine	0.9980	0.2	1.0	60.0	73.9	86.0	5.0	3.9	2.7
	Sulfadimidine	0.9959	0.5	1.0	60.8	61.9	63.4	5.4	6.7	6.0
	Sulfamethoxazole	0.9985	0.5	1.0	65.5	72.0	71.9	6.4	5.3	3.8
MLs	Tylosin	0.9958	0.05	0.2	72.3	90.0	83.3	6.8	3.4	2.7
	Roxithromycin	0.9988	0.05	0.2	83.0	79.8	80.6	4.9	5.0	5.0
	Kitasamycin	0.9970	1.0	2.5	79.5	75.0	79.8	6.2	3.5	2.4
	Erythromycin	0.9974	2.0	5.0	95.8	96.3	107	10	5.5	4.7
	Tilmicosin	0.9984	0.04	0.1	85.7	84.8	70.4	9.5	6.7	5.3
LAs	Clindamycin	0.9968	0.01	0.04	80.6	84.0	93.3	8.0	4.4	3.0

PMs	Valnemulin	0.9974	0.05	0.3	60.3	61.2	61.5	8.1	7.8	6.5
	Tiamulin	0.9956	0.05	0.2	70.8	78.5	75.0	6.7	6.0	3.3

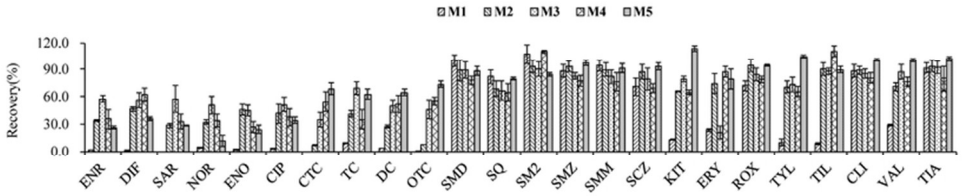
Continue table 3

Group	Analyte	Inter-day recovery, (%, n = 18)			Inter-day RSD, (%, n = 18)			ME (± SD) (%, n = 3)
		1 μg kg^{-1}	4 μg kg^{-1}	20 μg kg^{-1}	1 μg kg^{-1}	4 μg kg^{-1}	20 μg kg^{-1}	
FQs	Difluoxacin	61.3	61.7	63.6	14	12	12	75.2 ± 7.9
	Sarafloxacin	61.9	75.3	87.8	8.8	8.7	10	62.6 ± 4.9
	Enrofloxacin	64.8	69.9	66.0	6.1	7.4	13	73.4 ±13
	Ciprofloxacin	62.9	79.9	76.4	9.5	8.8	7.8	69.0 ± 7.2
	Enoxacin	58.8	62.2	62.9	12	15	10	68.1 ± 1.5
	Norfloxacin	56.5	65.1	70.7	10	9.5	6.7	74.9 ± 10
TCs	Chlortetracycline	59.9	58.2	65.2	11	8.8	7.3	71.3 ± 11
	Oxytetracycline	71.5	68.3	71.6	14	10	3.9	86.9 ± 4.8
	Doxycycline	64.5	65.4	70.4	12	11	4.4	76.1 ± 2.0
	Tetracycline	53.2	58.6	66.0	10	2.9	8.8	68.6 ± 4.2

SAs	Sulfaquinoxaline	60.9	59.4	75.1	12	10	5.6	56.8 ± 3.5
	Sulfaclozine	54.4	67.0	60.2	9.3	5.4	8.2	78.0 ± 2.4
	Sulfamethoxydiazine	62.6	64.5	73.6	4.8	4.0	3.2	81.2 ± 6.7
	Sulfamonomethoxine	60.0	74.8	84.7	6.5	5.6	6.3	84.6 ± 4.8
	Sulfadimidine	58.9	61.1	64.3	7.9	9.4	5.1	62.3 ± 4.8
	Sulfamethoxazole	64.0	69.2	70.8	8.3	8.1	4.2	82.1 ± 2.2
MLs	Tylosin	71.8	89.4	79.4	6.7	2.6	6.3	90.8 ± 3.0
	Roxithromycin	82.4	81.0	79.3	4.9	4.9	5.2	93.4 ± 6.0
	Kitasamycin	75.9	75.6	77.0	5.5	3.5	5.7	90.4 ± 2.3
	Erythromycin	98.6	98.9	104	13	7.3	10	83.1 ± 2.7
	Tilmicosin	86.9	85.8	69.1	9.6	7.0	6.5	80.9 ± 5.7
LAs	Clindamycin	81.5	85.5	92.9	9.3	5.7	3.3	97.3 ± 3.4
PMs	Valnemulin	58.5	60.9	61.7	8.8	8.6	10	80.8 ± 6.1

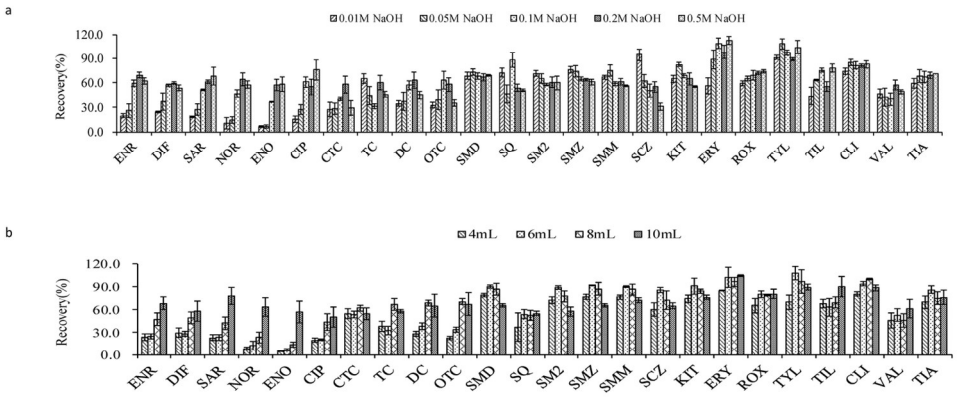
Tiamulin	72.6	77.3	74.1	3.7	7.2	2.9	79.9 ± 2.1

LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation; RSD, relative standard deviation; ME, matrix effect.



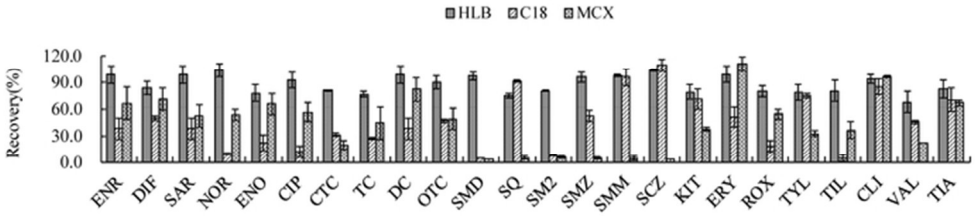
Influence of the extraction solvents on the recoveries of the target compounds / DIF, difluoxacin; SAR, sarafloxacin; ENR, enrofloxacin; CIP, ciprofloxacin; ENO, enoxacin; NOR, norfloxacin; CTC, chlortetracycline; OTC, oxytetracycline; DC, doxycycline; TC, tetracycline; SQ, sulfaquinoxaline; SCZ, sulfaclozine; SMD, sulfamethoxydiazine; SMM sulfamonomethoxine; SM2, sulfadimidine; SMZ, sulfamethoxazole; TYL, tylosin; ROX, roxithromycin; KIT, kitasamycin; ERY, erythromycin; TIL, tilmicosin; CLI, clindamycin; VAL; valnemulin; TIA, tiamulin. M1, ACN/MeOH (1:1, v/v); M2, ACN/acetate buffer (1:1, v/v, pH 4.0); M3, 0.5 g Na2EDTA and ACN/acetate buffer (1:1, v/v, pH 4.0); M4, 0.5 g Na2EDTA and ACN/citrate buffer (1:1, v/v, pH 4.0); M5, ACN : Na2EDTA-McIlvaine buffer (5:5, v/v, pH 4.0). Error bars represent standard deviation of the individual compound spiked at 10 μg kg-1 (n = 3)

71x20mm (300 x 300 DPI)



Influence of the concentration (a) and amount (b) of NaOH on the recoveries of 24 antimicrobials at the spiked 4 μg kg-1 / The abbreviations are the same as Fig. 1.

128x63mm (300 x 300 DPI)



Influence of the different types of SPE columns on extraction efficiency for 24 antimicrobials at the spiked 4 μg kg-1 / The abbreviations are the same as Fig. 1.

59x15mm (300 x 300 DPI)

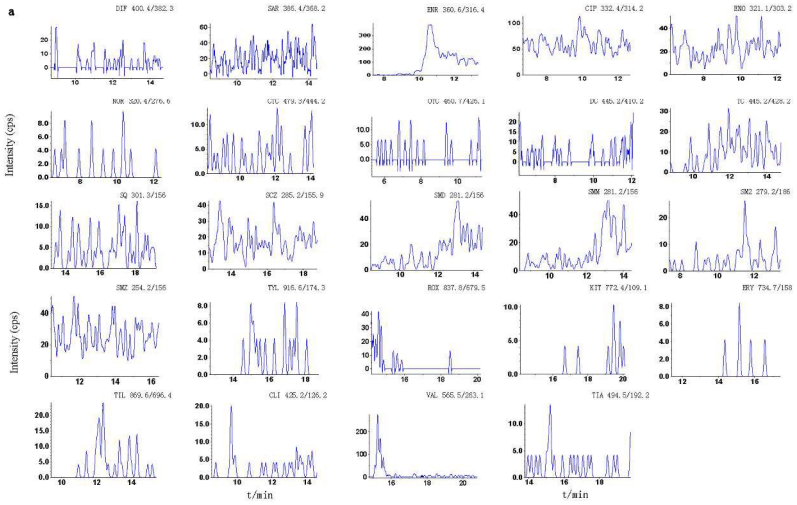
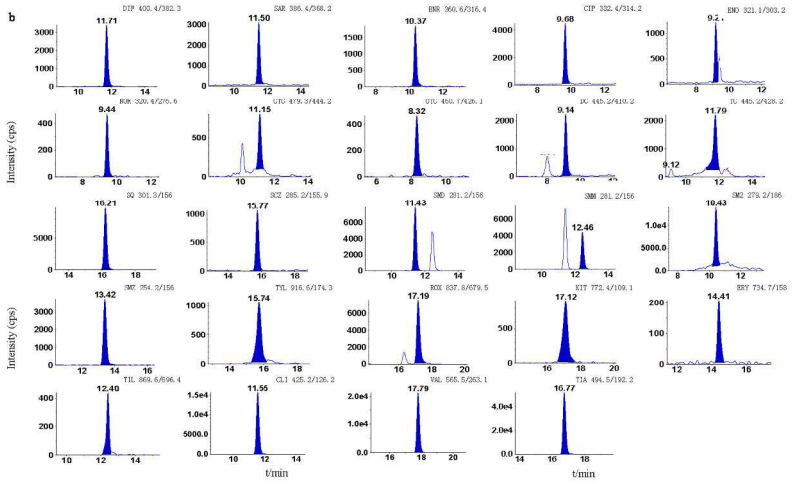


Figure 4
297x210mm (200 x 200 DPI)



Typical MRM chromatograms obtained from the blank soil extracts (a) and blank soil extracts spiked at 4 μg kg-1 (b)
/ The abbreviations are the same as Fig. 1.
297x210mm (200 x 200 DPI)