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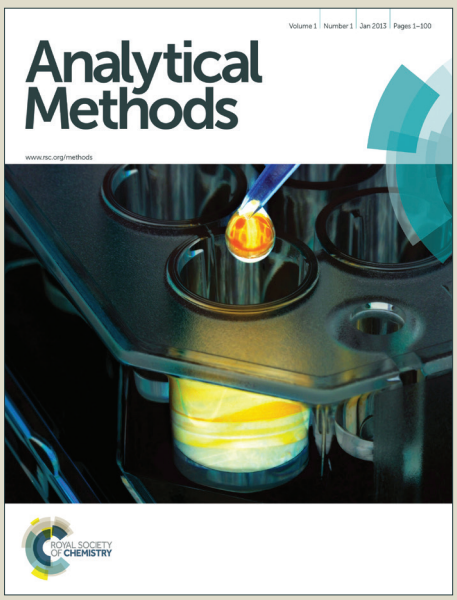

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Development of a 15-classes multiresidue method for analyzing 78 hydrophilic and hydrophobic veterinary drugs in milk, egg and meat by liquid chromatography-tandem mass spectrometry

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Abstract

Development of a multiclass, multiresidue method for analyzing veterinary drugs in food is recent trend of research for regulatory monitoring laboratories. This work reports a multiclass method for the determination of both hydrophilic and hydrophobic veterinary drug residues, including anthelmintics, avermectins, benzimidazoles, β-agonists, β-lactames, coccidiostats, corticosteroids/steroids, lincosamides, macrolides, non-steroidal anti-inflammatory drugs, phenicoles, quinolones, tranquillisers, antiviral drugs and some other veterinary drugs, in milk, egg and meat. By using liquid-liquid extraction low temperature partition with centrifugation for acetonitrile (MeCN)/aqueous phase separation, hydrophilic and hydrophobic drugs were recovered separately. MeCN phase was cleaned up with dispersive solid phase extraction. To achieve retention and separation of veterinary drugs with wide range of polarity, ultra performance hydrophilic interaction liquid chromatography (HILIC) and reversed phase liquid chromatography (RPLC) coupled to tandem mass spectrometer (MS/MS) were employed. The method was successfully validated. Method recoveries were in general ranged from 70-120% with precision RSD ≤ 20%. Method limits of quantification were ranged from 0.1 to 10 μg/kg for targeted veterinary drugs.

Keywords:

Multiclass method; Veterinary drugs; LLE; LTPc; HILIC; RPLC; MS/MS

Introduction

Veterinary drugs are widely used in the animal husbandry for treating and preventing diseases or as growth promoters. Despite obvious benefits, extensive or improper use of these drugs can lead to residues in animal products such as meat, milk and eggs. In order to protect public health and safety with minimum resource and maximum benefit, multiclass multiresidue methods have recently become the focus of public analysts.

QuEChERS, which stands for quick, easy, cheap, effective, rugged and safe, has been well established on multiresidue pesticides determination in food over a decade.[1] Pesticides in high moisture foods are extracted by acetonitrile (MeCN), followed by phase separation and dispersive solid phase extraction (dSPE) cleanup and then determined by gas or liquid chromatography coupled with mass spectrometry. It was further developed and validated as AOAC Official Method 2007.01 (acetate buffered) and CEN Standard Method EN 15662 (citrate buffered). QuEChERS based methods with modification were also applied to veterinary drug residues analyses. Kinsella et al. [2] used octadecyl bonded silica (C18) for cleanup in the analysis of anthelmintic drug residues. Whelan et al.[3] introduced a dimethyl sulphoxide pre-concentration step for QuEChERS extract and achieved a lower detection limits for macrocyclic lactones. Clarke et al. [4] excluded the dSPE step to recover ionophore residues of coccidiostats. Stubbings and Bigwood [5] reported a multiresidue/multiclass method by using 1% acetic acid in MeCN and sodium sulfate (Na_2SO_4) for extraction, followed by dSPE cleanup with amino bonded silica (NH_2). Pang et al. [6] used 5% acetic acid in MeCN, sodium chloride (NaCl) and Na_2SO_4 for extraction without a phase separation step. Different modifications were successfully applied to different targeted group(s) of veterinary drug. Basically, these methods only applied to common veterinary drug residues including, tetracyclines, sulfonamides, quinolones, macrolides, β-lactams, etc. However, hydrophilic drugs were rarely included in QuEChERS preparation and likely lost in the discarded aqueous phase.

Test method CLG-MRM 1.04 of United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS), which used aqueous MeCN for extraction, followed by dSPE cleanup and solvent exchange to 0.1% formic acid. It was successfully applied to some polar veterinary drugs determination. Geis-Asteggiante et al. [7] extended its application to over 100 veterinary drugs of different classes.

Veterinary drugs, regulated by different food safety authorities in terms of maximum residue limits (MRLs), broadly cover different classes of chemicals. Even within a veterinary functional class, several subclasses of chemicals could be involved. Besides, residue definitions of some veterinary drugs include their polar metabolites, such as amino- or hydroxyl- derivatives, as well as their parent drugs. As such, there is a need of a simple and fast sample preparation that does not capable of analyzing multiclasses of veterinary drugs

but also applicable to wide range of hydrophilic/hydrophobic compounds.

The objective of this work is to develop a multiclass, multiresidue method for determination of polar and non-polar veterinary drugs with one single sample preparation for a background survey of non-regulated veterinary drugs in Hong Kong. Liquid-liquid extraction (LLE) low temperature partition with centrifugation (LTPc) is employed for phase separation of different drugs in an aqueous MeCN solution. The MeCN phase was cleaned up by dSPE with C18 and magnesium sulfate ($MgSO_4$), followed by a pre-concentration step when deemed necessary. The nonpolar and moderate polar drugs in MeCN phase were determined by a tandem mass spectrometer (MS/MS) coupled to a liquid chromatograph worked in reversed phase liquid chromatography (RPLC) while polar drugs enriched in the aqueous phase were analyzed by a MS/MS coupled to a liquid chromatograph worked in hydrophilic interaction liquid chromatography (HILIC). The method was validated on the accuracy, repeatability, reproducibility and matrix effect. Finally, this method was successfully applied to different classes of veterinary drugs including anthelmintics, avermectins, benzimidazoles, β-agonists, β-lactames, coccidiostats, corticosteroids/steroids, lincosamides, macrolides, nonsteroidal anti-inflammatory drugs (NSAIDs), phenicoles, quinolones, tranquillisers, antiviral drugs and some other veterinary drugs.

Materials and methods

Reference materials and reagents

Reference materials and internal standards were obtained from US Pharmacopeia (Rockville, MD, USA), Sigma-Aldrich Co. (St. Louis, MO, USA), Dr. Ehrenstorfer GmbH (Augsburg, Germany), Witega Laboratorien Berlin-Adlershof GmbH (Berlin, Germany), Toronto Research Chemicals Inc. (Toronto, Ontario, Canada), Wako Pure Chemical Industries, Ltd (Osaka, Japan), BioAustralis (Smithfield, Australia) and C/D/N Isotopes Inc. (Quebec, Canada). Detail information is incorporated in Table S1.

LCMS grade MeCN and methanol (MeOH) were purchased from Anaqua Chemicals Supply (Houston, TX, USA) and Fisher Scientific (Waltham, MA, USA) respectively. Water was purified through a Milli-Q synthesis system integral with LC-Pak polisher from Millipore (Billerica, MA, USA). Puriss p.a. grade ammonium formate and formic acid and $MgSO_4$ were purchased from Sigma–Aldrich Co. Endcapped C18 was obtained from Agilent (Santa Clara, CA, USA).

Standard solutions and calibration

Stock standard solutions were prepared by dissolving accurately weighted neat reference materials in MeOH, MeCN or water to produce a concentration at 1000 mg/L. Mixed intermediate standard solutions of hydrophilic and hydrophobic drugs were prepared by mixing and diluting appropriate amount of stock solutions in MeCN and water respectively. Individual internal standard (IS) stock solutions, including 49 labelled compounds, diclazuril-methyl and selamectin, were prepared separately with same approach as the standards and then the mixed intermediate standard solutions. Stock and intermediate standard solutions in MeOH/MeCN and in water were stored under -20℃ in a freezer and 4℃ in a refrigerator respectively. Working standard solutions were prepared freshly with appropriate mixing and dilution of intermediate standard and ISs solutions in either MeCN with 0.5 mM formic acid and ammonium formate for RPLC or water with 50 mM formic acid and ammonium formate for HILIC.

Internal standardization was used for quantification. 7 points calibration curves were established from 1/2 MLOQ, MLOQ (0.1, 1, 5 or 10 μg/L) and 5 concentration levels 10-50 μg/L for quantification, which was equivalent to a working range from 0.05/0.5 to 50 μg/kg in sample.

Sample preparation

4 g of homogenized sample was weighed into a 50 mL polypropylene (PP) tube (Sarstedt, Nümbrecht, Germany). Mixed ISs working solutions were added. Suitable amount of water (1.6, 1.0 and 0.6 mL for pork, egg and milk respectively) was added according to

moisture content estimated for different sample matrices. 16 mL MeCN were then added. The mixture was shaken for 5 min by a vertical shaker (ShaQer, SPEX SamplePrep, Metuchen, NJ, USA) and was frozen in a -80 ℃ freezer (Panasonic Biomedical, Netherlands) by storing for around 30 min. The mixture was thawed and centrifuged at cfg > 3000 g for 5 min at room temperature by a centrifuge (Falcon 6/300, MSE, London, UK). The supernatant was transferred to a new 50 mL PP tube. Phase separation was induced by centrifuging 30 min by a high-speed refrigerate centrifuge (CR21G, Hitachi-Koki, Tokyo, Japan) with setting cfg at 8000 g and temperature at -20 ℃. Lower aqueous layer was pipetted out and was filtered through 0.2 μm regenerated cellulose (RC) syringe filters (Sartorius AG, Goettingen, Germany) into vial (Waters, USA) for HILIC-MS/MS analysis. Formic acid and ammonium formate solution was added to achieve 50 mM formic acid/formate buffering. MeCN upper layer left in the 50 mL PP tube was added with 1 g of C18 and 4 g of $MgSO_4$. MeCN extract was shaken with cleanup materials for 1 min and then centrifuged at cfg > 3000 g for 5 min at room temperature. For milk, MeCN supernatant was further evaporated to 2 mL under a slow stream of nitrogen at 35 ℃ in a water bath (N-EVAP 112, Organomation, Berlin, MA, USA) and then filtered through 0.2 μm RC syringe filters. Formic acid and ammonium formate solution was also added to achieve 0.5 mM formic acid/formate buffering before RPLC-MS/MS determination. Figure 1 summarized workflow of the sample preparation.

RPLC-MS/MS and HILIC-MS/MS

The chromatographic separation was carried out using a Waters Acquity UPLC system, which consisted of a sample manager, a column manager and a binary solvent manager (Milford, MA, USA). The UPLC system was coupled to a Qtrap 5500 triple quadrupole mass spectrometer (AB Sciex, Framingham, USA) equipped with a TurboV ion source for analysis. Software Acquity UPLC Console (Waters) and Analyst (AB Sciex) were used to operate the UPLC and MS respectively. Software MultiQuant (AB Sciex) was used for data processing.

Moderate and non-polar veterinary drugs were determined by RPLC-MS/MS with electrospray ionization (ESI) source in either positive or negative mode. Analytical column was a Waters UPLC column, Acquity CSH C18, 2.1 x 150 mm, 1.7 μm, in connection with corresponding pre-column. Column temperature was set at 40℃. Gradient elution was made with MeOH/MeCN mixture with ratio 3:1(v/v) (mobile phase A) and water (mobile phase B). Both mobile phases were buffered with 0.5 mM ammonium formate and 0.5 mM formic acid. Flow rate was set at 0.3 mL/min Initial mobile phase composition of 5 % of organic solvent was hold for 1 min. Organic solvent composition was linearly increased to 95 % at 7.5 min and then hold for 2.5 min. Organic solvent composition was reduced back to 5 % in 1 min and hold for 9 min for re-conditioning of column. The total run time was 20 min. Injection volumes of 1 μL and 4 μL were used in positive and negative ionization respectively. Weak and strong wash solvents were 900 μL water and 300 μL MeCN respectively. Ionspray

voltage was set at +/- 4000V. Source temperature (TEM) was set at 350 ℃ and 400 ℃ for positive and negative ionization respectively. Nitrogen was used as collision gas (CAD) and set at medium. Curtain gas (CUR), GS1 and GS2 were set at 20, 50 and 50 respectively. Both quadrupole 1 (Q1) and quadrupole 3 (Q3) resolution were set as unit. The entrance potential (EP) and cell exit potential (CXP) were set at +/-10 V and +/-15 V respectively. The declustering potential (DP) was set at +/-100 V. Scheduled multiple reaction monitoring algorithm mode was used with target scan time (TST) set as 0.5 s and MS detection window set as 60 s.

Polar veterinary drugs were determined by HILIC-MS/MS with ESI in positive mode. Waters UPLC column, Acquity BEH HILIC, 2.1 x 100 mm, 1.7 μm with corresponding pre-column was used. Column temperature was maintained at 40 ℃. Gradient elution was made with MeCN with 50 mM formic acid and water with 50 mM ammonium formate and 50 mM formic acid. Flow rate was set at 0.4 mL/min. Aqueous mobile phase 5 % was hold for 1 min. and was linearly increased to 50 % at 8.5 min and then hold for 1.5 min. Aqueous mobile phase composition was reduced back to 5 % in 1 min and hold for 9 min for re-conditioning of column. The total run time was 20 min. Injection volume was 1 μL. Weak and strong wash solvents were 900 μL MeCN and 300 μL water respectively. Ionspray volatage was set at +4000 V. TEM was set at 450 ℃. All other settings including CAD, CUR, GS1, GS2, Q1/Q3 resolution, EP, CXP, TST and DP were same as RPLC-MS/MS determination.

Regardless of types of chromatographic separation as well as MS/MS detection polarity, optimized values for collision energy were tested by flow injection analysis and summarized in Table 1, as well as the indicative retention times on the column.

Validation

The validation of this method was made on accuracy, repeatability and reproducibility. Since different analyte has different sensitivity and maximum residue levels (MRLs) and this method was developed for analyzing different types of matrices, spike recovery experiments were carried out at a reasonably and achievable low level, method limit of quantification (MLOQ), instead of at specified MRLs. MLOQ for non-polar drugs and moderate drugs recovered in MeCN phase were set at 5 μg/kg. Lower MLOQs were set for corticosteriods and steriods at 0.5 μg/kg. For milk, the MLOQs were lowered 5-folded as the final MeCN phase was pre-concentrated before LCMS analysis. MLOQs for polar drugs recovered in aqueous phase were set as 10 μg/kg for all 3 food types. Recovery and precision were evaluated by 6 replicates of sample spikes at 1 x, 1.5 x and 2 x MLOQ in blank samples of pork, egg and milk. Linearity was checked by calculating residuals. 5 concentrations evenly spaced across the calibration range and weighted linear regression was used to establish calibration curves. Matrix-matched and reagent-only calibration standards were prepared at 1

x, 1.5 x and 2 x MLOQ for assessing the matrix effect.

Results and discussion

Difficulties in sample extraction

The method development was initiated for analysis of certain classes of veterinary drugs with MRL set by the CODEX and other veterinary drugs of public health concerned, but their MRLs were not set in Hong Kong. Some most commonly used veterinary drugs, such as sulfonamides, tetracyclines, etc., were not covered herewith as there were included in the scope of analysis of a number of different published multiclass multiresidue methods. In view of the wide variety of target veterinary drugs, QuEChERS with RPLC-MS/MS was initially adopted as it was well known to be suitable for multiclass multiresidue analysis. Original unbuffered, citrate buffered and acetate buffered version of QuEChERS were tested and were found to be applicable to many moderate polar and nonpolar veterinary drugs with some differences in recoveries between the 3 versions. However, poor or no recovery was noted for some water soluble drugs, such as piperazine, cefalexin and diminazene, in the MeCN extract. Increasing the amount of NaCl and $MgSO_4$ were tested to improve their recoveries but failed. Limited 'salting out' effect was observed for hydrophilic drugs. We believed that those water soluble drugs were partitioned in the aqueous phase, but no attempt was made to analyze it by LC-MS owing to its high salt content.

After that, we tested our scope of analytes by a method, CLG-MRM 1.04, without phase separation. Hydrophilic drugs were retained and recovered in the aqueous MeCN extract. However, keeping the water content in raw MeCN extract led to some drawbacks. Although polar drugs had been recovered, difficulties were still encountered on chromatographic separation and quantification. Strong ion suppression caused by co-elution of matrices was noted for early eluting analytes in RPLC, including florfenicol-amine. Geis-Asteggiante et al. [7] reported similar matrix effect could not be cleaned up by Z-sep$^+$/C18/n-hexane. Even a 15cm C18 UPLC column was used, sufficient retention for separating polar analytes from matrices could not be achieved and some analytes were lost in the preparation step too. Besides, evaporation of MeCN with ~20% of water during solvent exchange was time consuming and heat sensitive analytes may degrade under higher temperature or prolong heating. Similar finding was reported by Piatkowska et al. [8] that the presence of water lengthened the time of evaporation and caused loss of fluoroquinolones, β-lactams and other veterinary drugs. Clarke et al. [4] mentioned that water in QuEChERS-style extraction can be removed by adding NaCl and $MgSO_4$ so as to reduce evaporation time of raw extract.

As such, we decided to develop a method with phase separation step, but no salt was added. Hence, hydrophilic analytes partitioned in the aqueous phase could be amenable by LC-MS.

Low temperature partition

To induce phase separation of MeCN/water mixture without adding salts, LTP was used.[9] Typical LTP was carried out by overnight equilibration of MeCN/water mixture by storing in a freezer at temperature below -1.3℃.[10] To keep the sample preparation 'quick', we introduced centrifugation to LTP (LTPc). Phase separation could be completed in a faster way. In addition, a clearer meniscus between phases was obtained. Since temperature of centrifuge would affect partition of analytes and separation of MeCN/water, best separation was achieved when the temperature was set at -20 ^{o}C. In general, 30 minutes was required to cool the solution from room temperature to set temperature inside centrifuge. Discrete phase separation was achieved as depicted in Figure 2.

The LTPc experiment was initially tried with 10 mL of MeCN and 10 mL of water, which assumed that 10 g of high moisture content sample extracted by 10 mL of MeCN in QuEChERS. It resulted in ~15 mL of aqueous phase and ~5 mL of MeCN phase. Polar and non-polar drugs were recovered in aqueous and MeCN phase respectively as expected. Similar enrichment had been reported by Lopes et al.[11,12] using LLE-FPLTP for veterinary drugs determination. However, the recoveries of some moderate polar veterinary drugs in MeCN phase were found to be low as significant amount of them partitioned into the aqueous phase. As such, MeCN to water ratio 4:1 [13] was used. LTP of 20 mL of solvent mixture produced ~16-17 mL of MeCN phase and ~3-4 mL of aqueous phase. Improved recoveries of moderate polar drugs could then be obtained in MeCN phase. Moreover, higher MeCN to sample ratio favoured protein precipitation too.

Although using lyophilized samples could better control the overall solvents ratio, we did not freeze-dry the samples because it was time consuming. The MeCN/water ratio was maintained at about 4:1 by taking the water content of targeted samples into account and add suitable amount of water into the extraction system instead. Furthermore, internal standards were added so that the influence on quantification by small change of solvent ratio could be corrected.

The collection of the two separated phases should be done immediately after the sub-zero temperature centrifugation. We pipetted out the lower aqueous phase with a glass dropper because the meniscus could be observed easily at the conical bottom of the centrifuge tube. It must be paid attention that the phase separation would become blurred after prolong leaving solutions in room temperature.

Repeatability of the phase separation were evaluated by replicate LTPc experiments (n=8) of 20 mL of MeCN/water (4:1 v/v). Average MeCN and aqueous phase volumes were found to be 16.8 and 3.1 mL (RSD of 1 and 3%) respectively. The average MeCN/water ratio was found to be 5.4 with RSD of 3 %. Temperature measured immediately after LTPc for the MeCN layer was -16 ±1 ℃ with RSD of 6%.

Furthermore, freezing out co-extracted matrices and removing them together with solid residues by room temperature centrifugation before LTPc was essential. Otherwise, cloudy

suspension would appear between phases after LTPc in meats.

Selection of procedures for cleanup

After phase separation, MeCN phase was cleaned up by dSPE with C18 which is a fast step and has been widely employed in veterinary drugs analysis for removing fatty co-extracts. Among different types of commercial available C18 for dSPE cleanup, we had tested for our target veterinary drugs and endcapped C18 gave the best recoveries. n-Hexane defatting was not attempted because Zhan et al. [14] reported that ionophores and other non-polar veterinary drugs could be lost.

For aqueous phase, cleanup was also attempted. Commercial available dSPE materials, including PSA, GCB and C18, were tested. When compared to extraction with and without cleanup, there was no reduction of matrix suppression after 0.1 g PSA was added. Figure 2 showed that yellow pigment in egg yolk was the only extracted colouring matter in MeCN phase while milk and meat provided a colourless solution. Thus, GCB was excluded. C18 repels water and is not applicable to aqueous solution. Other SPE cartridges, strong anion exchange (SAX) and strong cation exchange (SCX), which are commonly used for trapping interference in aqueous solution, were tested but acidic drugs (e.g. cefalexin) and basic drugs (e.g. florfenicol amine) were lost, respectively.

Separating sample extract into aqueous phase and MeCN phase

Separating sample extracts into aqueous and MeCN phase instead of keeping an aqueous MeCN mixture for LCMS analysis gave the advantage of enrichment too.

LLE-LTPc separated ~3 mL of water from ~20 mL of raw extract with polar drugs partitioned in. It resulted in strong enrichments of ~ 4 to 6-folded of the polar drugs in the aqueous phase such that a subsequent concentration step was not required. In fact, concentration of analytes in aqueous solution is complicate and time consuming. Enrichment factors were calculated from response ratio of 10 ng/mL standards in 20 mL MeCN/water (4:1 v/v) after/before phase separation for 4 replicates. Slight enrichment < 20% was noted for non-polar drugs in MeCN phase. Moderate polar drugs, which partitioned in both phases, showed insignificant enrichment in either phase.

A concentration step of MeCN extract for milk samples was added to achieve sufficient low reporting limits for corticosteroids. $MgSO_4$ was first added to remove residual water in MeCN phase. Without the influence of water, evaporation of MeCN was much faster than evaporating an aqueous MeCN mixture and it also minimized any potential degradation of veterinary drugs. Moreover, the concentration step could apply to other matrices when lower detection limits are required.

Chromatographic separation of veterinary drugs by HILIC and RPLC

Although the sample preparation recovers drugs with wide range of polarity, neither a single LC run of HILIC nor RPLC could give sufficient retention for all analytes. Benefited from the drugs separated in 2 phases based on their polarities, a HILIC and a RPLC run was established for each phase such that chromatographic limitations could be overcome.

Chiaochan et al. [15] reported the effectiveness of HILIC-MS/MS on the determination of certain polar veterinary drugs, including aminoglycosides, β-lactams, lincosamides, macrolides, quinolones, sulfonamides, tetracyclines, and amprolium. As such, hydrophilic drugs partitioned in aqueous phase were separated by the HILIC system and eluted out within 7 min as depicted in Figure 3 (a). Retentions achieved by HILIC gave much better quality on analyte identification in terms of area ratio of two MRM transitions. On the contrary, most hydrophilic drugs got limited retention in RPLC system and eluted closely with the solvent and non-retained polar matrices. For example, piperizine has a RT of 6.1 min in our HILIC system but only 0.8 min in our RPLC system. Moreover, HILIC separation avoided strong ion suppression or false negative occurred in RPLC which caused by co-elution of non-retained polar analytes and matrix compounds.

On the other hand, chromatographic separation of moderate polar and non-polar drugs recovered in MeCN layer was effectively carried out by RPLC with C18 column. For better sensitivity, analytes were monitored either in positive (+ve) or negative (-ve) ESI mode depending on its ionization efficiencies and responses, Figure 3 (b) and (c) depicted chromatographs of RPLC with +ve and -ve ionization, respectively. Target analytes were eluted within 11 min. Owing to polarity switching time of the mass spectrometer was 50 msec, separate LC runs were required. For the latest state-of-the-art instrument, single LC run is possible.

Method validation

In order to evaluate the trueness of the proposed method, six sets of fortified samples were prepared in egg, pork and milk samples at three levels, low, medium, and high spiking (1x, 1.5x, 2x MLOQ, respectively) in different days and recoveries were summarized in Table 2. Average recoveries were within 70-120% with RSD ≤ 20%. One exception case was found, cefquinome in egg, in which matrix enhancement could not be corrected by using D_4-cephapirin as internal standard. Specificity and selectivity were achieved by monitoring two MRM transitions for each drug. All target analytes in spike recovery study fulfilled the identification points and ion ratio requirement. Performance criteria for mass spectrometric detection and chromatographic separation were set as the maximum permitted tolerances on MRM ratio (± 20-50% depends on relative intensity) and relative retention time (RRT) deviation tolerances (2.5%). MRMs interfered by co-eluting matrices or drugs were identified and another MRM was re-selected at the early stage of development. Linearity was verified and the correlation coefficients were found to be > 0.995. Random distributions of residuals

were noted with percentage deviation of observed response ratio to calculated response ratio < 20%. The matrix effect was determined as the percentage difference between matrix-matched and reagent-only calibration standards and summarized in Table S2.

Simplifying internal standardization

Internal standardization with multiple internal standards was employed for the following reasons. The method involved a phase separation and analytes partitioned in different phases. Hence, internal standardization is partially needed for some moderate polar drugs that partitioned in both phases. Besides, analytes could be lose in sample extraction step and absorbed on dSPE materials. Moreover, LCMS determination with ESI was known to be strongly affected by matrix effects. As such, each analyte was paired-up with a representative internal standard, either structurally related or closely eluted. Two native ISs were used since structurally related labelled standard was not commercially available.

In order to check whether lower cost could be achieved by using less ISs, we re-calculated recoveries and precisions with only one IS for each phase. The spiked recoveries were only corrected for the changes in solvent volume. D_5-dexamethasone was selected for RPLC because of its good spiked recovery, insensitivity to matrix effects and capability to be monitored in both +/-ve ESI ionization. For HILIC, D_4-cephapirin was selected. Spiked recovery performance obtained by single IS correction was summarized as Figure 4.

Good robustness was observed for analytes in RPLC. Among 65 veterinary drugs determined by RPLC, 57 for pork, 54 for egg and 53 for milk still felt within 70-120 % and ≤ 20 % RSD respectively when corrected by D_5-dexamethasone. Amongst 13 HILIC drugs, 9 drugs for pork, 4 drugs for egg and 7 drugs for milk still gave satisfactory performances. Hence, using 2 ISs could provide satisfactory performance for over 70% of targeted analytes. As such, the use of costly isotopically labelled ISs could be largely reduced. Unsatisfactory performances were summarized in Table 3. Matrix effect is the most likely reason for unsatisfactory performance. For example, the spiked recovery of closantel (RT 8.9 min) and rafoxanide (RT 9.5 min) were exceptionally low in milk (4%), whereas their recoveries were > 90% in pork, it strongly indicated there was co-elution of milk matrix at ~ 9 min suppressed their ionization. Another possible reason for unsatisfactory performances was partition and procedural lost. The recoveries of amantadine, memantine and rimantadine (adamantanes with an amino function group) were similarly low in all three matrices which suggested analytes' properties play a more significant role than matrix effect.

Conclusion

A multiclass method for 13 hydrophilic and 65 hydrophobic veterinary drugs extracted into aqueous and MeCN phases respectively by low temperature partition with centrifugation was developed and validated. Equipment, apparatuses and reagents were similar to QuEChERS. The simple sample preparation, which could be completed within a working day, covered 15 classes of veterinary drugs. Ultra performance HILIC/RPLC provided sufficient retention to different classes of analytes with short chromatographic separation time. Tandem mass spectrometry supported sensitivity and selectivity for trace level multiresidues analyses. Method was successfully validated with targeted matrices. We believe this procedure could be applied to other water soluble polar compounds determination in other chemical residue areas.

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Table 1. MS/MS parameters and retention times of the veterinary drugs and internal standards

RPLC-MS/MS +ve							
Veterinary drugs and Internal standards	**(Quasi)-molecular ion**	**Q1 mass (*m/z*)**	**Q3 mass (*m/z*)**	**CE (V)**	**RT (min.)**	**Internal standard**	**Replace by D_5-Dexa**
Avermectins							
Avermectin B1a	$[M+NH_4]^+$	890.5	305.1/567.1	37/19	9.4	Selamectin	N
Doramectin	$[M+NH_4]^+$	916.5	331.2/593.3	37/19	9.7	Selamectin	Y
Eprinomectin B1a	$[M+H]^+$	914.5	112/186	88/26	9.3	Selamectin	Y
Moxidectin	$[M+H]^+$	640.4	528.3/498.4	14/18	9.7	Selamectin	Y
Selamectin	$[M+H]^+$	770.3	608.4	30	10.2		
Benzimidazoles and Azoles							
Albendazole	$[M+H]^+$	266.1	234.1/191	27/46	7.6	D_3-Albendazole	Y
Albendazole sulfoxide	$[M+H]^+$	282.1	240/208	19/35	5.8	D_3-Albendazole sulfoxide	Y
Albendazole sulfone	$[M+H]^+$	298.1	159/224	53/35	6	D_3-Albendazole sulfone	Y
Albendazole-2-aminosulfone	$[M+H]^+$	240.1	133/198	35/29	4	D_3-Albendazole-2-aminosulfone	Y
Fenbendazole	$[M+H]^+$	300.1	268/159	30/45	7.9	D_3-Fenbendazole	Y
Oxfendazole	$[M+H]^+$	316.1	159/191	47/30	6.3	D_3-Oxfendazole	Y
Oxfendazole sulfone	$[M+H]^+$	332.1	300/159	32/54	6.5	D_3-Oxfendazole sulfone	Y
Febantel	$[M+H]^+$	447.1	415.2/383.1	19/30	8.1	D_6-Febantel	Y
Flubendazole	$[M+H]^+$	314.1	282/123	31/49	7.2	D_3-Flubendazole	Y
2-Aminoflubendazole	$[M+H]^+$	256.1	123/95	36/57	5.7	D_3-Albendazole-2-aminosulfone	Y
Mebendazole	$[M+H]^+$	296.1	264.1/105	32/45	7.1	D_3-Mebendazole	Y
Mebendazole-amine	$[M+H]^+$	238.1	105/77	35/47	5.5	D_3-Albendazole-2-aminosulfone	Y
5-Hydroxymebendazole	$[M+H]^+$	298.1	266.2/77	32/72	6.4	D_3-5-Hydroxy mebendazole	Y
Oxibendazole	$[M+H]^+$	250.1	218/176	25/38	6.9	D_7-Oxibendazole	Y
Thiabendazole	$[M+H]^+$	202	175/131	39/46	5.8	$^{13}C_6$-Thiabendazole	Y
5-Hydroxythiabendazole	$[M+H]^+$	218	191/147	39/43	4.6	$^{13}C_2$,^{15}N-5-Hydroxy thiabendazole	Y
Triclabendazole	$[M+H]^+$	359	274/344	52/38	8.7	D_3-Triclabendazole	Y
Triclabendazole sulfoxide	$[M+H]^+$	375	360/258	32/51	8.4	D_3-Oxfendazole	Y
Triclabendazole sulfone	$[M+H]^+$	390.9	242.1/312	55/39	8.3	D_3-Oxfendazole sulfone	Y
Hydroxytriclabendazole	$[M+H]^+$	375	290/360	50/37	8	D_3-5-Hydroxy mebendazole	Y
Levamisole	$[M+H]^+$	205.1	178/91	30/50	3.6	D_5-Levamisole	Y

D_3-Albendazole	$[M+H]^+$	269.1	191	45	7.6		
D_3-Albendazole sulfoxide	$[M+H]^+$	285.1	208	34	5.8		
D_3-Albendazole sulfone	$[M+H]^+$	301.1	159	47	6		
D_3-Albendazole-2-amino sulfone	$[M+H]^+$	243.1	133	45	4		
D_3-Fenbendazole	$[M+H]^+$	303.1	268	29	7.9		
D_3-Oxfendazole	$[M+H]^+$	319.1	194	31	6.3		
D_3-Oxfendazole sulfone	$[M+H]^+$	335.1	300	30	6.5		
D_6-Febantel	$[M+H]^+$	453.2	418.2	18	8.1		
D_3-Flubendazole	$[M+H]^+$	317.1	123	48	7.2		
D_3-Mebendazole	$[M+H]^+$	299.1	77	75	7.1		
D_3-5-Hydroxymebendazole	$[M+H]^+$	301.1	266.2	32	6.4		
D_7-Oxibendazole	$[M+H]^+$	257.2	225	27	6.9		
$^{13}C_6$-Thiabendazole	$[M+H]^+$	208.1	181	34	5.8		
$^{13}C_2$,^{15}N-5-Hydroxy thiabendazole	$[M+H]^+$	221	81	60	4.6		
D_3-Triclabendazole	$[M+H]^+$	362	344	39	8.7		
D_5-Levamisole	$[M+H]^+$	210.1	183	31	3.6		
β-Agonists							
Ractopamine	$[M+H]^+$	302.2	284.2/164	18/24	4.5	D_6-Ractopamine	Y
D_6-Ractopamine	$[M+H]^+$	308.2	168	22	4.5		
Corticosteriods and Steriods							
Flugestone acetate	$[M+H]^+$	407.2	267.2/225.1	32/39	7.6	D_5-Dexamethasone	NA
Melengestrol acetate	$[M+H]^+$	397.2	337.1/279.2	21/29	8.4	D_3-Melengestrol acetate	Y
Trenbolone	$[M+H]^+$	271.2	253.2/199.2	30/33	7.5	D_5-Dexamethasone	NA
D_3-Melengestrol acetate	$[M+H]^+$	400.3	279.2	29	8.4		
D_5-Dexamethasone	$[M+H]^+$	398.2	378.2	13	7.2		
Coccidiostats							
Clopidol	$[M+H]^+$	192	101/87	38/43	4.5	D_5-Levamisole	Y
Decoquinate	$[M+H]^+$	418.3	121.1/390.2	99/32	9.3	D_5-Decoquinate	Y
Halofuginone	$[M+H]^+$	416	100/120	42/28	6.1	$^{13}C_6$-Halofuginone	Y
Lasalocid A	$[M+Na]^+$	613.4	377.3/577.3	55/45	9.2	D_5-Decoquinate	N
Maduramicin	$[M+Na]^+$	939.5	877.6/719.4	57/90	10.0	D_5-Decoquinate	Y
Monensin A	$[M+Na]^+$	693.4	675.4/461.3	56/73	9.7	D_5-Decoquinate	Y
Narasin A	$[M+Na]^+$	787.5	431.2/531.4	70/65	10.4	$^{13}C_6$-Halofuginone	N
Robenidine	$[M+H]^+$	334.1	111/155	68/30	8.0	D_8-Robenidine	Y
Salinomycin	$[M+Na]^+$	773.5	431.5/531.3	70/62	10.0	D_5-Decoquinate	N
D_5-Decoquinate	$[M+H]^+$	423.3	377.2	35	9.3		
$^{13}C_6$-Halofuginone	$[M+H]^+$	422	100	34	6.1		

D_8-Robenidine	$[M+H]^+$	342.1	142	37	8.0		
Lincosamides							
Pirlimycin	$[M+H]^+$	411.2	112/363.1	39/24	5.8	D_{12}-Pirlimycin	N
D_{12}-Pirlimycin	$[M+H]^+$	423.2	122	33	4.2		
Macrolides							
Acetylisovaleryltylosin	$[M+H]^+$	1042.6	109/174	90/54	7.9	D_5-Dexamethasone	NA
3-O-acetyltylosin	$[M+H]^+$	958.5	174/109	47/115	7.1	D_5-Dexamethasone	NA
Tilmicosin	$[M+H]^+$	869.6	696.4/88	56/91	6	D_{12}-Pirlimycin	N
NSAIDs							
4-Methylaminoantipyrin	$[M+H]^+$	218.1	56/97	52/20	4.6	D_3-4-Methylamino antipyrine	Y
D_3-4-Methylaminoantipyrine	$[M+H]^+$	221.1	100	18	4.6		
Quinolones							
Difloxacin	$[M+H]^+$	400.1	356.1/382.1	28/35	5	D_3-Difloxacin	N
D_3-Difloxacin	$[M+H]^+$	403.2	359.2	28	5		
Tranquillisers							
Azaperone	$[M+H]^+$	328.2	165/123	30/58	5.5	D_4-Azaperone	Y
Azaperol	$[M+H]^+$	330.2	121/312.2	33/23	5.2	D_4-Azaperol	Y
Carazolol	$[M+H]^+$	299.2	116/222.1	29/29	5.5	D_7-Carazolol	Y
D_4-Azaperone	$[M+H]^+$	332.2	127	53	5.5		
D_4-Azaperol	$[M+H]^+$	334.2	121	33	5.2		
D_7-Carazolol	$[M+H]^+$	306.2	123	30	5.5		
Others							
Dicyclanil	$[M+H]^+$	191.1	150/109	29/34	4	D_5-Levamisole	Y
Isometamidium	$[M+H]^{2+}$	230.6	135/120	17/19	5.4	D_5-Isometamidium	Y
D_5-Isometamidium	$[M+H]^{2+}$	233.1	135	15	5.4		
Antiviral drugs							
Amantadine	$[M+H]^+$	152.1	135/77	25/51	4.4	D_{15}-Amantadine	N
Memantine	$[M+H]^+$	180.2	163/107	22/32	6.2	D_6-Memantine	N
Rimantadine	$[M+H]^+$	180.2	163/107	22/35	6	D_4-Rimantadine	N
D_{15}-Amantadine	$[M+H]^+$	167.2	150	24	4.4		
D_6-Memantine	$[M+H]^+$	186.2	110	35	6.2		
D_4-Rimantadine	$[M+H]^+$	184.2	167	24	6		

RPLC-MS/MS -ve							
Veterinary drugs and Internal standards	**(Quasi)-molecular ion**	**Q1 mass (Da)**	**Q3 mass (Da)**	**CE (V)**	**RT (min.)**	**Internal standard**	**Replace by D_5-Dexa**
Anthelmintics							
Closantel	$[M-H]^-$	660.8	127/345	-106/-49	8.9	$^{13}C_6$-Closantel	N
Nitroxinil	$[M-H]^-$	288.9	127/89	-37/-62	6.7	$^{13}C_6$-Nitroxinil	Y
Rafoxanide	$[M-H]^-$	623.8	127/344.6	-102/-48	9.5	$^{13}C_6$-Rafoxanide	N
$^{13}C_6$-Closantel	$[M-H]^-$	666.9	351	-50	8.9		
$^{13}C_6$-Nitroxinil	$[M-H]^-$	294.9	127	-36	6.7		
$^{13}C_6$-Rafoxanide	$[M-H]^-$	629.8	350.7	-50	9.5		
β- Lactams							
Nafcillin	$[M-H]^-$	413.1	272.1/242.9	-20/-33	7.1	D_5-Nafcillin	N
Oxacillin	$[M-H]^-$	400.1	259/356	-19/-12	6.7	D_5-Nafcillin	N
D_5-Nafcillin	$[M-H]^-$	418.1	277	-20	7.1		
Coccidiostats							
Diclazuril	$[M-H]^-$	405/407	334/336	-30/-30	8.2	Diclazuril-methyl	Y
Nicarbazin	$[M-H]^-$	301.1	137/107	-28/-50	7.8	D_8-Nicarbazin	Y
Clorsulon	$[M-H]^-$	378/380	342/344	-29/-20	5.6	D_6-Clorsulon	Y
Diclazuril-methyl	$[M-H]^-$	419	321	-42	8.2		
D_8-Nicarbazin	$[M-H]^-$	309.1	141	-26	7.8		
D_6-Clorsulon	$[M-H]^-$	384	348	-21	5.6		
Corticosteriods and Steriods							
Dexamethasone	$[M+HCOO]^-$	437.2	361.1/307.1	-29/-45	7.2	D_5-Dexamethasone	NA
Zeranol	$[M-H]^-$	321.2	277.2/303.2	-32/-32	7.7	D_5-Dexamethasone	NA
D_5-Dexamethasone	$[M+HCOO]^-$	442.2	364.1	-27	7.2		
Phenicoles							
Florfenicol	$[M-H]^-$	356	185/119	-28/-46	5.4	D_3-Florfenicol	Y
Thiamphenicol	$[M-H]^-$	354	185/79	-30/-46	4.7	D_3-Thiamphenicol	N
D_3-Florfenicol	$[M-H]^-$	359	188	-31	5.4		
D_3-Thiamphenicol	$[M-H]^-$	357	230	-19	4.7		
Others							
Fluazuron	$[M-H]^-$	504	304.9/262	-20/-40	8.9	$^{13}C_6$-Diflubenzuron	N
$^{13}C_6$-Diflubenzuron	$[M-H]^-$	315	295	-15	8.1		

HILIC-MS/MS +ve							
Veterinary drugs and Internal standards	**(Quasi)-molecular ion**	**Q1 mass (Da)**	**Q3 mass (Da)**	**CE (V)**	**RT (min.)**	**Internal standard**	**Replace by D_4-Ceph**
Anthelmintics							
Piperazine	$[M+H]^+$	87.1	44.1/70.1	23/21	6.1	D_8-Piperazine	N
D_8-Piperazine	$[M+H]^+$	95	48	30	6.1		
β- Agonists							
Zilpaterol	$[M+H]^+$	262.2	244.1/185	20/38	3.9	D_7-Zilpaterol	N
D_7-Zilpaterol	$[M+H]^+$	269.2	251.1	21	3.9		
β- Lactams							
Cefalexin	$[M+H]^+$	348.1	158/106	14/41	4.4	D_4-Cephapirin	NA
Cefquinome	$[M+H]^+$	529.1	134/396	26/21	4.8	D_4-Cephapirin	NA
Cefazolin	$[M+H]^+$	455	323/156	17/22	2.9	D_4-Cephapirin	NA
Cephapirin	$[M+H]^+$	424.1	292/152	20/35	3.4	D_4-Cephapirin	NA
Desacetylcephapirin	$[M+H]^+$	382.1	124/152	58/35	4.0	D_6-Desacetyl cephapirin	N
D_4-Cephapirin	$[M+H]^+$	428.1	296.1	22	3.4		
D_6-Desacetylcephapirin	$[M+H]^+$	388.1	115	66	4.0		
Coccidiostats							
Amprolium	$[M]^+$	243.2	150/94	20/25	4.8	D_4-Cyromazine	N
Phenicoles							
Florfenicol-amine	$[M+H]^+$	248.1	230/130	19/40	3.0	D_3-Florfenicol- amine	N
D_3-Florfenicol-amine	$[M+H]^+$	251.1	233	17	3.0		
Others							
Cyromazine	$[M+H]^+$	167.1	68/125	50/25	2.3	D_4-Cyromazine	N
Diminazene	$[M+H]^+$	282.1	119/103	25/57	5.2	$^{13}C_2$,$^{15}N_4$- Diminazene	N
Imidocarb	$[M+H]^+$	349.2	188/162	40/34	5.4	D_8-Imidocarb	N
Methyl-3-quinoxaline-2 carboxylic acid	$[M+H]^+$	189.1	145/143	23/24	2.8	D_4-Cephapirin	NA
D_4-Cyromazine	$[M+H]^+$	171.1	86	30	2.3		
$^{13}C_2$,$^{15}N_4$-Diminazene	$[M+H]^+$	288.1	260.1	13	5.2		
D_8-Imidocarb	[M+H]+	357.2	192	42	5.4		

Remarks: 'N' and 'Y' denotes 'no' and 'yes', which indicates unsatisfactory and satisfactory performances when replacing internal standard (IS) by D_5-dexamethasone/ D_4-cephapirin respectively. NA denotes 'not applicable', which the original IS is D_5-dexamethasone/ D_4-cephapirin.

Table 2. Average recoveries and precision on sample spikes at 1x, 1.5x and 2x MLOQ (n=6) in different foods.

Drug class (no. of analyte)	MLOQ*	Pork		Egg		Milk	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
RPLC-MS/MS +ve							
Avermectins (4)							
Avermectin B1a	5	102	9	82	12	113	12
Doramectin	5	101	7	110	12	114	11
Eprinomectin B1a	5	120	7	117	11	116	14
Moxidectin	5	117	7	112	13	91	13
Benzimidazoles and Azoles (21)							
Albendazole	5	103	7	105	7	100	7
Albendazole sulfoxide	5	104	9	100	10	100	7
Albendazole sulfone	5	99	10	103	11	104	10
Albendazole-2-aminosulfone	5	103	6	103	5	98	5
Fenbendazole	5	101	7	105	5	103	5
Oxfendazole	5	101	7	102	7	103	7
Oxfendazole sulfone	5	104	5	103	6	99	6
Febantel	5	100	4	101	4	100	4
Flubendazole	5	103	6	103	6	101	5
2-Aminoflubendazole	5	104	8	102	6	101	7
Mebendazole	5	101	7	103	5	100	5
Mebendazole-amine	5	99	13	102	10	99	6
5-Hydroxymebendazole	5	101	8	103	6	102	5
Oxibendazole	5	104	5	103	5	104	5
Thiabendazole	5	98	8	100	8	101	6
5-Hydroxythiabendazole	5	102	7	103	6	99	4
Triclabendazole	5	104	4	103	4	100	3
Triclabendazole sulfoxide	5	99	8	97	7	103	6
Triclabendazole sulfone	5	102	6	95	8	96	6
Hydroxytriclabendazole	5	95	6	93	5	88	6
Levamisole	5	105	5	102	3	101	3
beta-Agonists (1)							
Ractopamine	5	105	7	101	6	98	5
Corticosteriods and Steriods (3)							
Flugestone acetate	0.5	93	13	108	15	97	10
Melengestrol acetate	0.5	93	10	100	12	95	8
Trenbolone	0.5	98	9	107	8	86	8
Coccidiostats (9)							
Clopidol	5	96	6	88	6	97	4
Decoquinate	5	100	5	104	4	104	5
Halofuginone	5	113	20	102	16	105	9
Lasalocid A	5	106	5	72	10	85	5
Maduramicin	5	100	7	107	4	98	4
Monensin A	5	100	7	114	7	115	5
Narasin A	5	82	15	76	13	83	6
Robenidine	5	99	6	103	5	104	4
Salinomycin	5	78	6	92	9	83	5
Lincosamides (1)							
Pirlimycin	5	97	13	102	14	106	10
Macrolides (3)							
Acetylisovaleryltylosin	5	102	7	100	6	99	6
3-O-acetyltylosin	5	98	10	94	7	89	7
Tilmicosin	5	109	10	115	9	115	11
NSAIDs (1)							
4-Methylaminoantipyrin	5	96	7	101	10	108	9
Quinolones (1)							

Difloxacin	5	103	11	104	8	102	6
Tranquillisers (3)							
Azaperone	5	105	11	99	9	101	5
Azaperol	5	102	7	102	7	103	5
Carazolol	5	101	8	104	10	99	7
Others (2)							
Dicyclanil	5	108	6	95	5	76	6
Isometamidium	5	120	5	90	5	83	11
Antiviral drugs (3)							
Amantadine	5	105	7	103	7	100	6
Memantine	5	106	9	105	8	101	8
Rimantadine	5	103	8	110	9	106	8
RPLC-MS/MS -ve							
Anthelmintics (3)							
Closantel	5	106	5	100	3	95	9
Nitroxinil	5	105	6	102	3	104	3
Rafoxanide	5	109	7	102	3	97	9
beta-Lactams (2)							
Nafcillin	5	98	5	102	4	99	3
Oxacillin	5	93	5	94	4	90	3
Coccidiostats (3)							
Diclazuril	5	97	4	101	4	99	3
Nicarbazin	5	103	6	105	2	110	2
Clorsulon	5	101	5	102	3	99	3
Corticosteriods and Steriods (2)							
Dexamethasone	5	108	5	102	3	104	5
Zeranol	5	109	10	95	7	96	5
Phenicoles (2)							
Florfenicol	5	104	7	101	2	98	3
Thiamphenicol	5	104	5	100	3	89	3
Others (1)							
Fluazuron	5	93	6	83	10	92	6
HILIC-MS/MS +ve							
Anthelmintics (1)							
Piperazine	10	113	17	108	4	119	10
beta-Agonists (1)							
Zilpaterol	10	94	8	90	16	106	6
beta-Lactams (5)							
Cefalexin	10	74	10	119	6	70	10
Cefquinome	10	104	19	**181**	7	93	10
Cefazolin	10	70	11	109	7	102	5
Cephapirin	10	101	6	102	6	99	6
Desacetyl Cephapirin	10	85	11	92	8	88	8
Coccidiostats (1)							
Amprolium	10	90	16	104	5	117	6
Phenicoles (1)							
Florfenicol-amine	10	104	8	93	9	100	8
Others (4)							
Cyromazine	10	98	2	100	2	103	3
Diminazene	10	120	6	115	9	113	13
Imidocarb	10	115	11	120	5	114	7
Methyl-3-quinoxaline-2 carboxylic acid	10	100	10	107	6	111	6

Note: * MLOQ refers to method limit of quantification of the analyte in meat and egg. The MLOQ for analytes milk is one fifth of the specified value, except for analytes analyzed by HILIC-MS/MS.

Table 3. Unsatisfactory performance (underlined) when corrected with single internal standard.

	Pork		Egg		Milk	
	Recovery (%)	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**
Avermectin B1a	84	10	66	12	105	11
Lasalocid A	92	10	59	10	72	6
Narasin A	53	11	64	16	63	4
Salinomycin	69	7	75	10	69	5
Pirlimycin	35	12	70	14	55	10
Tilmicosin	40	13	81	9	67	12
Difloxacin	33	37	29	14	76	7
Isometamidium	88	13	109	20	146	12
Amantadine	33	25	30	7	41	15
Memantine	52	17	42	8	50	14
Rimantadine	39	16	29	7	35	19
Closantel	102	15	75	16	4	43
Rafoxanide	91	18	67	20	4	44
Nafcillin	85	15	68	12	75	7
Oxacillin	81	15	63	11	68	6
Thiamphenicol	103	9	98	12	34	11
Fluazuron	104	4	127	12	115	11
Piperazine	13	43	57	15	25	16
Zilpaterol	67	6	24	15	29	25
Desacetyl Cephapirin	34	16	45	13	36	14
Amprolium	72	15	64	6	71	6
Florfenicol-amine	46	8	26	8	35	7
Cyromazine	74	9	54	7	57	5
Diminazene	76	8	35	15	52	8
Imidocarb	89	11	52	17	77	15

Figure 1. Workflow of the method.

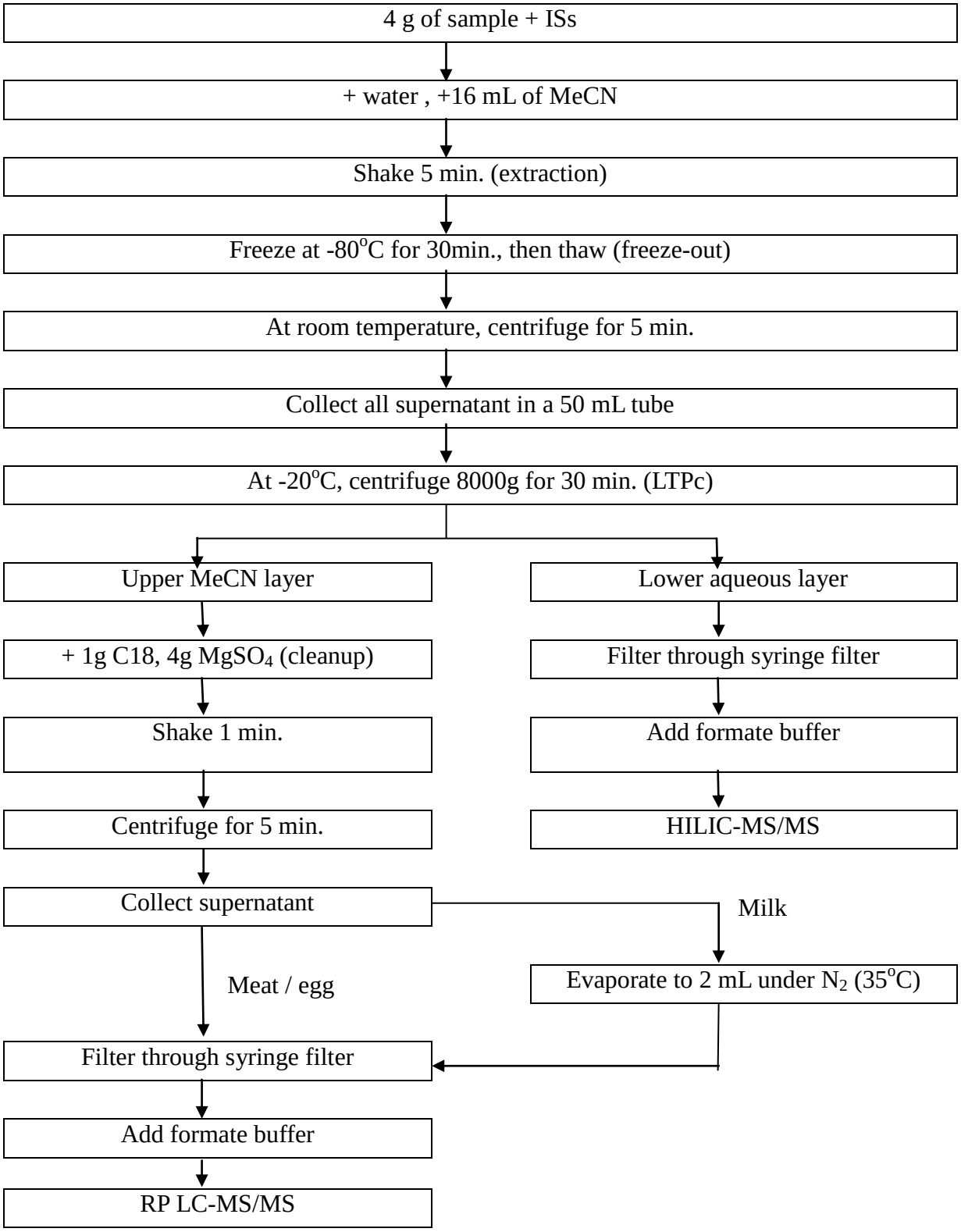

Figure 2. Extract of an egg sample (left) before LTPc and (right) after LTPc with discrete separated upper MeCN phase and lower aqueous phase.

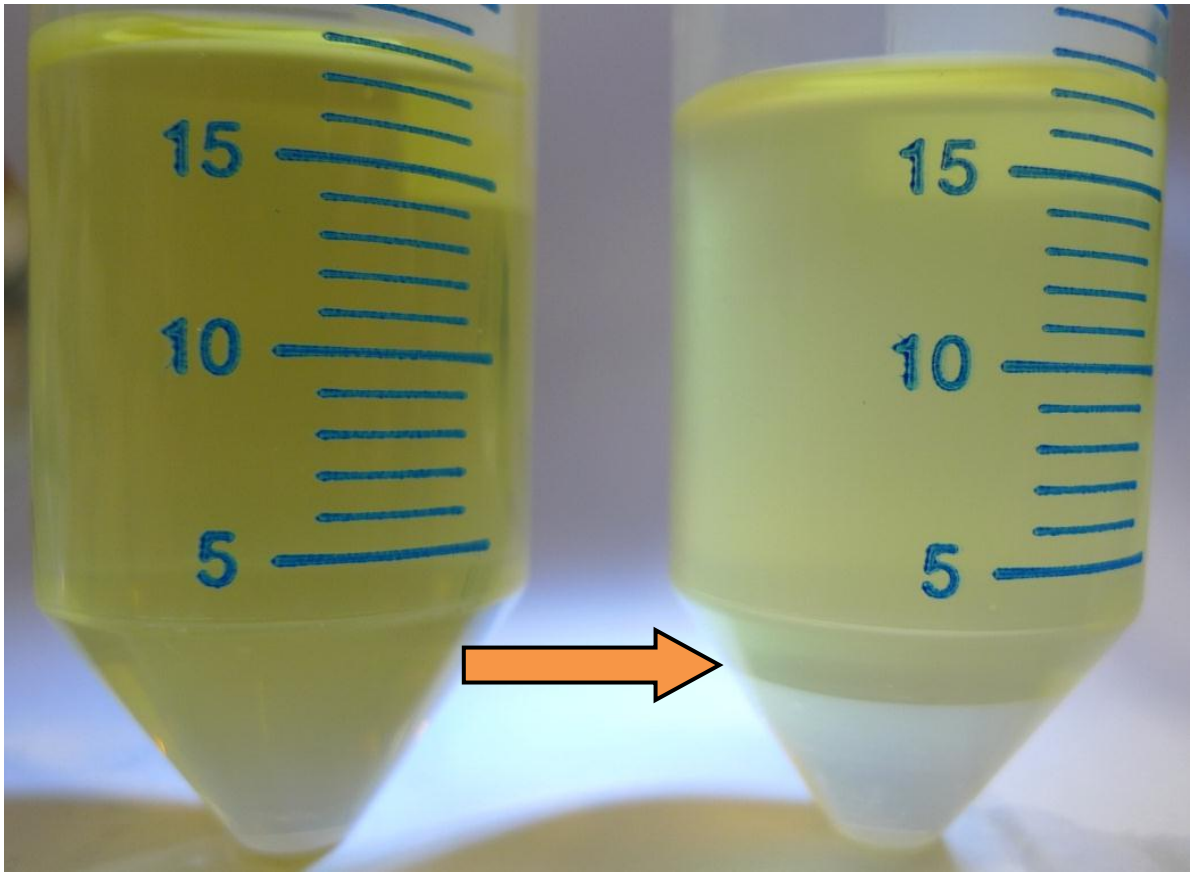

Figure 3. LC-MS/MS chromatograms of quantification transitions of veterinary drugs at MLOQ concentrations in (a) HILIC; (b) RPLC +ve; and (c) RPLC –ve ionization.

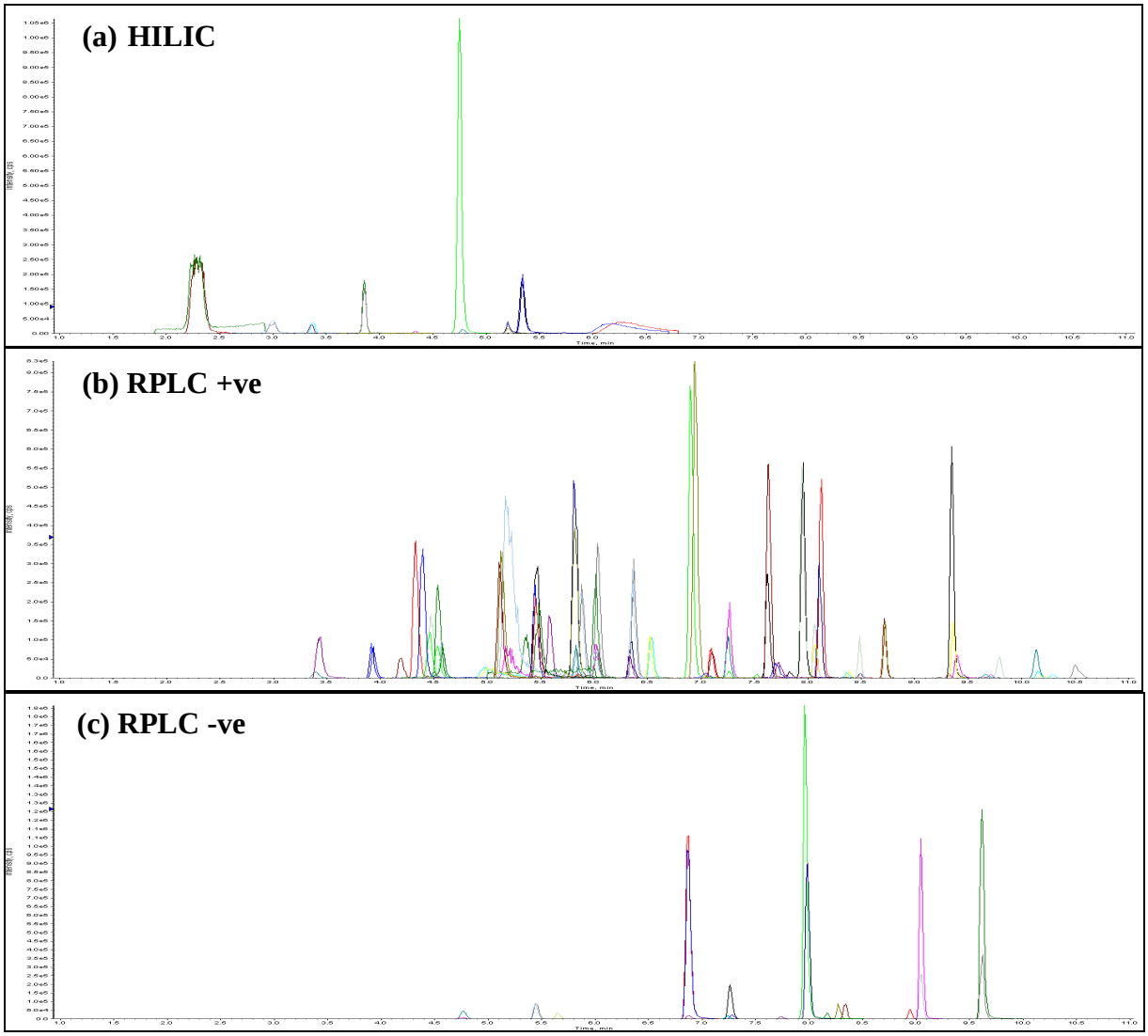

Figure 4. Spiked recovery performance obtained by single IS correction for each phase.