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Analytical Methods

Development and validation of a multiclass method for the quantification of veterinary drug residues in honey and royal jelly by liquid chromatography–tandem mass spectrometry

Yue Jin [a,b,c], Jinzhen Zhang [a,b,c], Wen Zhao [a,b,c], Wenwen Zhang [a], Lin Wang [a,b,c], Jinhui Zhou [a,b,c,1,*], Yi Li [a,b,c,1,*]

[a] Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing 100093, PR China
[b] Bee Product Quality Supervision and Testing Center, Ministry of Agriculture, Beijing 100093, PRChina
[c] Laboratory of Risk Assessment for Quality and Safety of Bee Products, Ministry of Agriculture, Beijing 100093, PRChina

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ABSTRACT

The aim of this study was to develop an analytical method for the analysis of a wide range of veterinary drugs in honey and royal jelly. A modified sample preparation procedure based on the quick, easy, cheap, effective, rugged and safe (QuEChERS) method was developed, followed by liquid chromatography tandem mass spectrometry determination. Use of the single sample preparation method for analysis of 42 veterinary drugs becomes more valuable because honey and royal jelly belong to completely different complex matrices. Another main advantage of the proposed method is its ability to identify and quantify 42 veterinary drugs with higher sensitivity than reference methods of China. This work has shown that the reported method was demonstrated to be convenient and reliable for the quick monitoring of veterinary drugs in honey and royal jelly samples.

1. Introduction

The honey bee is the principal species responsible for global plant pollination by transferring pollen grains to create next generation plant offspring and enhance the fruit of crops (Klein et al., 2007). Another major contribution of the honey bee is production of useful products including honey, royal jelly, propolis, pollen and so on. To this end, honey is produced and processed by honey bees from the nectar and honeydew of plants and contains natural sugars such as glucose and fructose, amino acids, enzymes, minerals, antioxidants (Zhou et al., 2014). Royal jelly, secreted from the hypopharyngeal, mandibular, and postcerebral glands of young worker bees, is a nutritional bee product that is valuable as a health-beneficial food and a pharmaceutical product (Takenaka & Echigo, 1980). However, the honey bee is easily attacked by bacteria, viruses, fungi and exotic parasitic mites under inappropriate environmental conditions which can result in the occurrence of disease such as American foulbrood (AFB), European foulbrood (EFB), chalkbrood, nosema, cripaviridae, varroa mites (Delaplane, 2010). Hence, various veterinary drugs have been employed to control and treat these diseases by adding them to feed or through spraying into hives. However, the misuse and illegal use of veterinary drugs may lead to the presence of drug residues in honey and royal jelly, posing potential hazards to consumers.

These drugs found to be used belong to nitroimidazoles, sulfonamides, fluoroquinolones, macrolide, lincomycin and tetracyclines in beekeeping. Nitroimidazoles have been commonly used to prevent and control *Nosema apis* in hives in China. Fumagillin is also used to treat *Nosema* diseases in some countries. However, most beekeepers prefer to replace fumagillin with 5-nitroimidazoles due to high cost of fumagillin. Nitroimidazoles, however, have been suspected of being human carcinogens and mutagens and authorized as the banned drugs by Ministry of Agriculture in China during routine beekeeping practices (Malone, Gatehouse, & Tregidga, 2001; Zhou et al., 2007). The Ministry of Health, Labor and Welfare in Japan have set "not detected" as the standard for metronidazole, dimetronidazole and ronidazole in honey and royal jelly (Dobiáš, Černá, Rössner, & Šrám, 1994). The sulfonamides are wide spectrum of synthetic bacteriostatics used to control most gram-positive and many gram-negative microorganisms and protozoa. They have been shown to be synergistic and enhanced activity by inhibiting bacterial dihydropteroate synthase and dihydrofolate reductase when used in combination with dapsone

* Corresponding authors at: Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing 100093, PR China.
E-mail address: zhoujinhui@caas.cn (J. Zhou).
[1] Jinhui Zhou and Yi Li contributed equally to this work.

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or trimethoprim (Piddock, Garvey, Rahman, & Gibbons, 2010). In bees, sulfonamides are used to prevent and treat severe bacterial brood disease such as AFB and EFB (Bogdanov, 2006). However, the presence of sulfonamide residue contamination in honey and royal jelly poses risks to human health that include an increased resistance of bacteria to antimicrobial agents, allergic reactions, and possible carcinogenicity (Chang, Hu, Asami, & Kunikane, 2008). Until now, there are no maximum residue limits (MRLs) for honey and royal jelly established by the European Union, the United States and Japan for sulfonamides (Dubreil-Chéneau, Pirotais, Verdon, & Hurtaud-Pessel, 2014). However, the MRL value of 50 ng/g for the sum of all sulfonamides has been set by Chinese ministry of agriculture as a regulation of pollution-free honey (NY5134-2008). Fluoroquinolones are usually used for the prevention and treatment of AFB and have been known to cause a decrease of bee population and honey production in apiculture (Blasco, Picó, & Torres, 2007). They have been banned for the use in American and Chinese beekeeping (Zhou et al., 2009). Macrolides and beta-lactam can potentially be used in the prevention and treatment AFB disease but pose a potential risk to consumers due to their ability to produce allergic reactions (Peng et al., 1996). However, macrolides are not allowed to be present in honey under the Canadian Food and Drugs Act and Regulations (Wang, 2004). The working residue level for tylosin in honey recommended by Canada has been set at 60 ng/g but no MRL has been established for any macrolides and lincomycin drugs in the honey matrix (Wang & Leung, 2007). Tetracyclines are also widely used to treat bacterial brood diseases such as AFB and EFB in beekeeping (Reybroeck, Ooghe, Brabander, & Daeseleire, 2007). Their residues can lead to increased drug-resistance of microbial strains in consumers and can cause allergic or toxic reactions in some hypersensitive individuals (Wangfuengkanagul, Siangproh, & Chailapakul, 2004). European countries do not have fixed MRLs for honey because tetracyclines are illegal for use to control honeybee diseases. But MRLs have been set at 300 ng/g and 500 ng/g in honey in Japan and China (Wang et al., 2014).

In China, each national standard method has been developed for determination of a single class antibiotic due to their similar sample preparation performance resulting from their similar chemical structures. In other words, seven national standard methods in China are conducted to separately detect sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim. Similar situation about standard method also exists in testing lab of other countries. Based on the experience from our lab, seven classes of antibiotics will cost an analyst about two weeks in the procedure of sample preparation and instrumental analysis for each batch sample. Moreover, all standard methods require a solid phase extraction (SPE) cartridge to clean up the target analytes from complex matrices and the total cost of SPE cartridge used in each method is about 7–15 dollars. The standard analysis of each batch sample will require significant cost in terms of time and consumables. There are several methods available to simultaneously detect multi-veterinary drug residues in honey (Cronly et al., 2010; Lopez, Pettis, Smith, & Chu, 2008; Vidal, Aguilera-Luiz, Romero-González, & Frenich, 2009; Wang & Leung, 2012) and royal jelly (Zhou et al., 2010). The multi-residue method has been subjected to the longer running time in chromatography system and tedious sample preparation treatment due to the different physicochemical properties of the antibiotics. Therefore, it is imperative to develop a quick and economical method to simultaneously detect the high usage of multi-class veterinary drugs (sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim) employed in beekeeping.

Over the past few years, a sample preparation method named QuEChERS (quick, easy, cheap, effective, rugged and safe) has enjoyed a rapid development in the extraction and clean-up the analytes of interest from complex matrices. This technique involves an acetonitrile extraction followed by a salting out step, and subsequent subjection of the sample to a dispersive solid-phase extraction (d-SPE) cleanup procedure with absorbents (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003). The QuEChERS approach requires minimum operational steps and less solvent than a standard liquid-liquid extraction and conventional SPE extraction procedures. It was initially developed for the analysis of pesticide residues in fruits and vegetables and was extended to the analysis of veterinary drugs and environmental pollutants residues (Albinet, Tomaz, & Lestremau, 2013; Hu et al., 2014; Liu et al., 2014). In addition, liquid chromatography tandem mass spectrometry (LC–MS/MS) has become valuable technique for the analysis of antibiotics in food matrices because it allows simultaneous qualification and quantification of the analytes of interest as well as high selectivity and sensitivity.

The objective of this work was to develop a rapid multi-residue method for the simultaneous trace level analysis of forty-two frequently used antibiotics in beekeeping including sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim, based on QuEChERS-based sample preparation and LC–MS/MS approaches. This method has been successfully used to simultaneously analyze veterinary drug residues in honey and royal jelly samples for screening or confirmation of risk evaluation analysis.

2. Materials and methods

2.1. Reagents and chemicals

The sulfonamides standards (sulphacetamide, sulfapyridine, sulfadiazine, sulfamethoxazole, sulfathiazole, sulfamerazine, sulfisoxazole, sulfamethizole, sulfadimidine, sulfamonomethoxine, sulfamethoxypyridazine, sulfameter, sulfachloropyridazine, sulfadoxine, sulfadimethoxine), fluoroquinolones standards (flumequine, oxolinic acid, norfloxacin, enoxacin, ciprofloxacin, danofloxacin, enrofloxacin, ofloxacin, marbofloxacin, fleroxacin, sarafloxacin, sparfloxacin, orbifloxacin, difloxacin), macrolides standards (erythromycin, spiramycin, tilmicosin, tylosin), nitroimidazoles standards (dimetridazole, metronidazole, ronidazole), tetracyclines standards (doxycycline, tetracycline, oxytetracycline, chlortetracycline), dapsone and trimethoprim were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All the standards were high purity grade (>95.0%). Acetonitrile, methanol and glacial acetic acid were the HPLC grade reagents (DIMA Technology Inc, Richmond, VA., U.S.A.). Ethylene diaminetetraacetic acid disodium salt, citric acid, sodium chloride and anhydrous sodium sulfate were analytical reagent grade and were obtained from Beijing Reagent Company (Beijing, China). Anhydrous Na_2SO_4 was heated at 600 °C for 6 h, cooled in a desiccator, and then stored in sealed containers. Primary secondary amine (PSA, 40 μm) and Endcapped octadecyl (C18-EC, 40 μm) sorbents were purchased from Agela Technologies, Inc. (Beijing, China). Pure water was prepared from a Milli-Q system (MilliPore, Bedford, MA, USA).

2.2. Standards solutions

Individual standard stock solutions (1.0 mg/mL) were prepared by dissolving the appropriate amount of each standard compound in MeCN and MeOH or water with different ratios depending on their solubility. A mixed standard working solution (1.0 μg/mL) used for spiking the control samples was prepared by appropriate dilution of an intermediate standard solution (10.0 μg/mL per analyte) with MeCN. All stock and working solutions were stored in dark at 4 °C away from light for three months.

2.3. Sample collection and preparation

A total of 300 honey samples were collected from beekeepers and supermarkets and stored at 4 °C in darkness. Prior to analysis, honey samples were allowed to liquefy at room temperature for 1 h, and mixed by inversion. If the honey was crystallized, it was gently heated in a thermostatic bath at a temperature not exceeding 50 °C. One hundred fifty royal jelly samples were obtained from apiaries and supermarkets and stored at −18 °C. Cold samples were equilibrated at 25 °C for 1 h and then homogenized before analysis.

Each homogeneous sample (2 g of honey; 1 g of royal jelly) was weighed in a 50 mL polypropylene centrifuge tube (samples for method validation were fortified with the predesignated appropriate volume of working mixed standard solutions and allowed to sit for ten minutes). Two hundred mg of Na_2EDTA, 100 mg of citric acid and 5.0 mL of pure water were added, and the tube was shaken vigorously for 5 min. Next, 10 mL acetonitrile containing 1% acetic acid, 4 g of anhydrous Na_2SO_4 and 1 g of NaCl were added sequentially, and the tube was shaken immediately for 5 min and then centrifuged at 8000 rpm for 5 min. Four mL of the supernatant was transferred to a 15 mL polypropylene centrifuge tube containing 50 mg of PSA, 150 mg C18-EC and 900 mg anhydrous Na_2SO_4 sorbents. After being vortexed for 2 min, the mixture was centrifuged at 8000 rpm for 5 min. Then, 300 μL extract filtered through a 0.22 μm nylon membrane filter was transferred to a vial containing 700 μL mobile phase with initial proportion prior to LC–MS/MS analysis. The sample preparation procedure involves the extraction of 42 veterinary drugs in honey and royal jelly with acidified acetonitrile followed by salting-out extraction/partitioning step. Subsequently, the clean-up is performed by the addition of the sorbent material into the extractant to remove the matrix interferences and residual water (Fontana & Bottini, 2014).

2.4. HPLC conditions

Chromatographic analyses were performed using an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA) consisting of a vacuum degasser (G1322A), a binary pump (G1312B), an autosampler (G1367D) and a thermostated column oven (G1316B). The analytes were separated on an Agilent Poroshell 120, EC-C18, (2.7 μm, 100 mm × 2.1 mm). The injection volume was 5.0 μL, and the temperature of the column oven was maintained at 30 °C. The mobile phases were (A) 2 mM ammonium formate + 0.1% formic acid in Milli Q water and (B) methanol with the following linear gradient flow program: 0 min, 5% B; 3.0 min, 20% B; 10 min, 30% B; 16 min, 98% B; 18 min, 98% B; 20 min, 5% B. The total chromatography run time was 25 min to allow the conditioning of the column prior to next injection with a constant flow rate of 0.2 mL/min.

2.5. MS/MS conditions

An Agilent 6460 triple quadruple tandem MS coupled to electrospray ionization (ESI) interface and Agilent Jet Stream Ion Focusing (Agilent Technologies, Palo Alto, USA) was used for mass analysis and quantification of target analytes. The MS was operated in the positive ion mode. The tuning parameters were optimized for the target analytes: gas temperature 320 °C, drying gas flow 6 L/min, nebulizer pressure 35 psi, Vcap voltage: 3500 V, sheath gas temperature: 350 °C, sheath gas flow: 12 L/min, Nozzle voltage: 0 V. The system operation, data acquisition and analysis are controlled and processed by the MassHunter software.

2.6. The Chinese national standard method

For the purposes of comparison, the national standard method of China was carried out to analyze the control sample with the same spiked concentrations as a procedure for method validation. The national standard code is listed as GB/T 18932.17-2003 for honey (sulfonamides), GB/T 22947-2008 for royal jelly (sulfonamides); GB/T 23412-2009 for honey (fluoroquinolones), GB/T 23411-2009 for royal jelly (fluoroquinolones); GB/T 23408-2009 for honey macrolides; GB/T 23410-2009 for honey (nitroimidazoles), GB/T 23407-2009 for royal jelly (nitroimidazoles); GB/T18932.23-2003 for honey (tetracyclines), GB/T 23409-2009 for royal jelly (tetracyclines); GB/T 22940-2008 for honey (dapsone) and GB/T 22943-2008 for honey (trimethoprim), GB/T 22948-2008 for royal jelly (trimethoprim).

2.7. Method validation

In this study, the parameters including selectivity, linearity, recovery (accuracy), repeatability (precision), instrumental detection limit (IDL), limit of detection (LOD), limit of quantification (LOQ), matrix effects and carry-over were validated to evaluate the performance of developed method. Control samples of honey and royal jelly were collected from our own apiary and tested to confirm their veterinary drug-free via the national standard method of China.

3. Results and discussion

In this study, modified QuEChERS method was selected in order to achieve a quick, economic and high throughout sample preparation method compared with all standard methods of China. We tried to use a single method to simultaneously analyze sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim from honey and royal jelly to exhibit its advantages to the extreme.

3.1. Optimization of extraction conditions

Water was used as the most appropriate solvent to disperse honey sample because organic solvents such as methanol, acetonitrile and ethyl acetate are not miscible with honey. For the QuEChERS method, organic solvents including methanol, acetonitrile, ethyl acetate and dichloromethane were initially added to the homogenous aqueous solutions of honey and royal jelly followed by the addition of salts, then investigated to test the recoveries and matrix effects of sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim. Ethyl acetate and dichloromethane exhibited a significant matrix effect enhancement and the extracted solutions present the obvious yellow and cloudy attributes of honey and royal jelly. It was shown that some veterinary drugs including sulfonamides and fluoroquinolones had low recoveries when methanol was used as the extraction solvent due to wide range of the polarity of these analytes. Consistent recovery results were obtained for most of the compounds with the exception of tetracyclines and fluoroquinolones extracted with acetonitrile, even though acetonitrile was found to extract most analytes of interest with a wider range of polarity. The results are shown in Supplementary Table S1.

Acidified acetonitrile was used as extraction solvent in QuEChERS methods to extract the target analytes from aqueous honey and royal jelly solutions. Different extraction solvents (0.1% acetic acid-acetonitrile, 0.5% acetic acid-acetonitrile, 1% acetic acid-acetonitrile, 2% acetic acid-acetonitrile and 5% acetic acid-acetonitrile) were examined to compare their recoveries.

Moreover, in this study we found that the addition of acetic acid in acetonitrile can effectively increase the recoveries of sulfonamides due to the acid hydrolysis step to liberate the sugar-bound sulfonamides which is in agreement with the description of another study (Sheridan, Policastro, Thomas, & Rice, 2008). Higher acid concentrations (exceeded to 2%) were adverse to the recovery of some sulfonamides and macrolides, but also decreased the cleanup efficiency because the PSA particle can easily combine with acetic acid (Hu et al., 2014). The results indicated in Supplementary Table S2 that better recoveries were obtained for all target analytes as a compromise when the concentration of acetic acid in acetonitrile was 1%.

The addition of inorganic salts including Na_2SO_4, $MgSO_4$ and NaCl should also benefit the partitioning of the target analytes between organic and aqueous phases. Satisfactory recoveries of all analytes were achieved by adding anhydrous Na_2SO_4 (4.0 g) and NaCl (1.0 g) except tetracyclines because anhydrous Na_2SO_4 and NaCl can effectively absorb the excess water and increase the ionic strength which increased the partition of the analytes from aqueous phase to the acetonitrile phase. To increase the recoveries of tetracyclines in honey and royal jelly, Na_2EDTA as dissociation agent and citric acid as deproteinizing agent were added to the sample to sequester the divalent metal ions with tetracyclines and precipitate the protein (Peysson & Vulliet, 2013). Increasing the amount of Na_2EDTA and citric acid and adjusting their proportion had a significant influence on the recoveries of the tetracyclines. It can be seen that the recoveries of TCs increases with the amount of EDTA from 50 to 500 mg and with the amount of citric acid from 20 to 300 mg, and then finally level off at 200 mg of EDTA and 100 mg of citric acid. The optimization details are illustrated in Supplementary Table S3.

3.2. Optimization of cleanup conditions

To obtain the high recoveries and less matrix effect, it is imperative to select the effective sorbent to absorb the sugars, organic acids and pigments from extractant of the honey and royal jelly samples. The recovery and cleanup effect of analytes was evaluated via the combination use of different sorbents: C18 + Na_2SO4 (150 mg + 900 mg); C18 + PSA + Na_2SO4 (150 mg + 50 mg + 900 mg); C18 + PSA + Na_2SO4 (150 mg + 100 mg + 900 mg); C18 + PSA + Na_2SO4 (100 mg + 50 mg + 900 mg); C18 + PSA + Na_2SO4 (200 mg + 20 mg + 900 mg); C18 + PSA + GCB + Na_2SO4 (150 mg + 50 mg + 100 mg + 900 mg); C18 + PSA + MWNT + Na_2SO4 (150 mg + 50 mg + 100 mg + 900 mg) (PSA = primary secondary amine, GCB = graphitized carbon and MWNT = multi-walled carbon nanotubes). In fact, PSA can remove various polar organic acids, polar pigments, some sugars and fatty acids; GCB can remove sterols and pigments such as chlorophyll; C18 can remove non-polar interfering substances like lipids; MWNT can remove trace chemical compounds in the environment, such as dioxins, polycyclic aromatic hydrocarbons and pesticides from water (Hou et al., 2014; Wilkowska & Biziuk, 2011). The sorbent combination containing WMNT was first not adopted because it can easily lead to the presence of a layer of black contamination on the ion source of the mass spectrometer. The lowest recoveries were obtained using the sorbent combination containing GCB. An increase in amount of PSA ranging from 10 mg to 50 mg leads to an increase in the peak areas of all analytes. But the peak areas of fluoroquinolones were drastically decreased when the amount of PSA was greater than 50 mg. Therefore, the optimum amount of PSA particles used was 50 mg. The effect of C18-EC particles was evaluated by performing assays in the 50 and 500 mg range. The results shown in Supplementary Table S4 indicate that the peak areas of analytes were the highest when the amount of C18-EC was 150 mg. And the peak areas of nitroimidazoles and macrolides decreased drastically when the amount of C18-EC further increased. Therefore, 150 mg of C18-EC was selected. The most plausible reason for this was that parts of these analytes were absorbed in the C18-EC particles. In the end, it was found that the best recoveries ranged from 80.4% to 118.4%, which was therefore selected when the C18EC + PSA + Na_2SO4 (150 mg + 50 mg + 900 mg) sorbent was used in the procedure for cleanup of honey and royal jelly samples.

3.3. Chromatographic separation and optimization of MS/MS conditions

Different mobile phases and additives were optimized to achieve satisfactory chromatographic separation and high sensitivity for the 42 veterinary drugs. An aqueous mobile phase consisting of ammonium formate (1 mM, 2 mM, 3 mM and 5 mM), formic acid and acetic acid were evaluated, with acetonitrile and methanol being examined as organic solvents to increase the sensitivity. High response of target analytes was obtained using water containing formic acid instead of acetic acid. This improvement may be due to the fact that formic acid can provide more protons than acetic acid which improves the ionization of analytes in ion source section. Formic acid (0.1%) as modifier in mobile phase was finally applied for the rest of the optimization procedure due to incomplete ionization (<0.1% formic acid) and ionization suppression (>0.1% formic acid). To achieve appropriate separation among the 42 veterinary drugs with short running time, different percentages of ammonium formate were added to the aqueous phase to enhance the separation of these analytes of interest. The total ion chromatogram (TIC) of 42 veterinary drugs showed the 1 mM of ammonium formate in mobile phase gave rise to the poor peak separation and unsymmetrical peak shape (Fig. 1A). The results also suggested that 2 mM of ammonium formate in mobile phase is sufficient to provide satisfactory separation and sharper peaks with the running time of 17.5 min (Fig. 1B). However, destroyed peak shapes and low response (lower ten folds than that of 2 mM ammonium formate) in TIC were obtained when 3 mM ammonium formate was added in the mobile phase (Fig. 1C). The mobile phase containing 5 mM ammonium formate caused the late eluting of target analytes with consequent poor separation (Fig. 1D). Ammonium formate with high content in mobile phase for LC–MS/MS system also easily leads to the blockage of chromatographic column due to the presence of salt crystallization in organic solvent. On the other hand, the organic modifier in the mobile phase greatly affected both resolution and retention time. Methanol and acetonitrile as the most frequently used organic solvents in LC–MS/MS mobile phase were evaluated by fixing other chromatographic conditions. Results showed no significant change in response of most analytes using either acetonitrile or methanol as the modifier in the mobile phase. But methanol can provide better separation between the 42 veterinary drugs due to its relatively weak elution strength. Therefore, in our studies methanol was preferred as the organic modifier for the mobile phase.

Four different chromatographic columns were studied, including Zorbax SB-C18 (50 mm × 2.1 mm, 1.8 μm), Zorbax XDB-C18 (50 mm × 2.1 mm, 1.8 μm), Poroshell 120, SB-C18, (2.7 μm, 100 mm × 2.1 mm) and Poroshell 120, EC-C18, (2.7 μm, 100 mm × 2.1 mm). XDB-C18 column provided the poor retention of metronidazole within the dead time. SB-C18 column presented poor separation for sulfonamides and fluoroquinolones due to the presence of contiguous cluster of peaks. Satisfactory separation and sensitivity were obtained using Poroshell 120 column. In the end, the Poroshell 120, EC-C18 was selected for use for the rest of experimental analysis. Poroshell EC-C18 with endcapped character, relative to Poroshell SB-C18, can diminish the tailing peak for tetracyclines and provids better peak shape. Based on the above

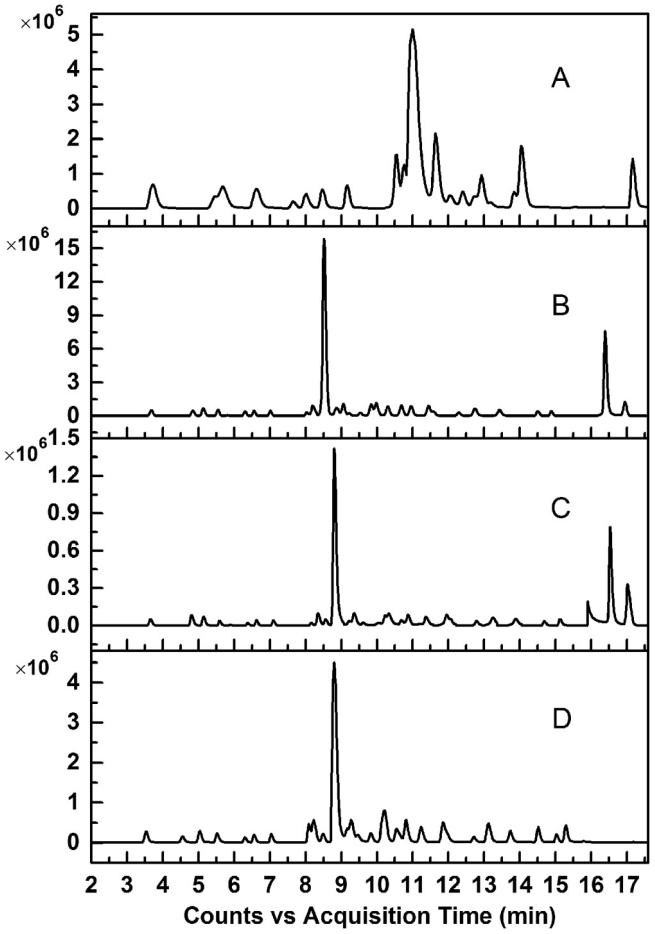

Fig. 1. Influence of ammonium formate in the aqueous mobile phase on the separation of 42 veterinary drugs in the chromatogram. (A: the TIC of 42 antibiotics using 1 mM ammonium formate in mobile phase, B: the TIC of 42 antibiotics using 2 mM ammonium formate in mobile phase, C: the TIC of 42 antibiotics using 3 mM ammonium formate in mobile phase, D: the TIC of 42 antibiotics using 5 mM ammonium formate in mobile phase).

optimization procedure, the use of a Poroshell EC-C18 column, with methanol as organic phase and HPLC grade water containing 2 mM ammonium formate and 0.1% of formic acid as the aqueous phase were the conditions chosen to provide better resolution, peak shapes and responses.

For each individual veterinary drug analyzed in this study, the mass spectrometer conditions were optimized to provide the best responses for quantification. Standard solutions (1.0 μg/mL) of each analyte were individually injected directly into the mass spectrometer to obtain their transition including precursor ion and relevant product ions. In the injection procedure, the parameters such as fragmentor, dwell time and collision energy were optimized for each veterinary drug in order to obtain the maximum sensitivity. Each analyte of interest was characterized by its retention time and by two precursor-product ion transitions. The most intense product ion was used for quantification, whereas the second one was used for the identification. The MS parameters applied for sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim with regard to the transitions from precursor to product ions are shown in Table 1.

In this LC–MS/MS method, it is difficult to set a short dwell time to allow simultaneous evaluation of 42 veterinary drugs in a targeted approach unless dynamic multiple reaction monitoring (d-MRM) is employed. The merit of d-MRM is that it allows the MS/MS system to be focused directly on the expected analyte retention time in a defined window range. In fact, it is difficult for users to define non-overlapping time segments to capture the close groups of eluting compounds. The d-MRM procedure has ability to improve peak symmetry and guarantee sensitivity of target analytes. Furthermore, sensitivity can be enhanced since optimal dwell times can be automatically achieved under d-MRM by reducing the number of concurrent ion transitions (Strassburg et al., 2012).

3.4. Method validation

The selectivity of the method was assessed separately by analyzing 20 blank honey and royal jelly samples using the developed method and observing the existence of the matrix interferences at the retention time of each analyte above a signal to-noise ratio of 3. The detection of sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim by MRM in the honey and royal jelly samples was highly selective with no interference.

Mixed calibration standards of 0.1, 0.5, 1, 5, 10, 50, and 100 ng/ml concentrations for sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim were prepared by further diluting the individual working solutions with pure water, respectively. Calibration curves (1) were constructed separately by plotting the peak area of the 42 veterinary drugs versus their concentrations in pure water using a least squares linear regression with a weighting factor ($1/x^2$). Another set of calibration curves (2) was prepared by adding working solutions of sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim into the QC samples (honey and royal jelly) that were first subjected to the extraction procedure to yield a serial of the same concentrations as calibration standards (1). Five replicates of each concentration were performed in one day. Linear calibrations were obtained for all analytes with correlation coefficients in the range of 0.9927–0.9984 for calibration (1) and 0.9989–0.9999 for calibration (2) in honey and royal jelly. Typical chromatograms of MRM transitions for sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim at the concentration of 10 ng/mL are shown in Fig. 2.

The instrumental detection limits (IDL) was defined as the concentration of target compounds producing a peak with a signal-to-noise ratio of 3 by directly injecting the standards into the instrument. Under the optimized LC–MS/MS parameters, the mean IDLs for the 42 veterinary drugs in honey and royal jelly extract ranged from 0.01 to 0.71 ng/mL. Based on the quantification ion response, Limit of detection (LOD) was determined as the concentration at which the signal-to-noise ratio was greater than 3 and limit of quantification (LOQ) was set as the concentration with a signal-to-noise of 10 and less than 20% coefficient of variation (CV) for precision. The obtained values of LOD and LOQ ranged from 0.14 to 2.91 ng/g for LOD, 0.50–9.70 ng/g for LOQ in honey; 0.17–3.81 ng/g for LOD, 0.58–12.68 ng/g for LOQ in royal jelly, respectively and are listed in Table 2. The results indicated that the similar sensitivity for 42 veterinary drugs in honey and royal jelly was present using the developed QuEChERS and LC–MS/MS methodology.

The recovery, matrix effect and precision were calculated by analyzing spiked honey and royal jelly samples at three concentration levels (LOQ, 3 × LOQ and 10 × LOQ). Precision was determined by analyzing QC honey and royal jelly samples at three different spiked concentration levels in quintuplicate per batch. Precisions given as relative standard deviation (%) were less than or equal to 15.9% and 17.1% for intra-batch precision and inter-batch precision (three batches) and provided in Table 3. The matrix effect was evaluated by comparing the MS/MS responses of known

Table 1
The mass parameters of sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim.

Compound Name	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (eV)	Retention Time (min)
Dimetridazole	142.2	96.1	90	14	5.79
		81.1	90	30	
Metronidazole	172.3	128.1	100	12	4.79
		82.1	100	26	
Ronidazole	201.2	140.1	80	4	5.07
		55.2	80	18	
Sulphacetamide	215	156	80	5	3.60
		92	80	20	
Dapsone	249.1	156	130	9	8.17
		92	130	23	
Sulfapyridine	250.1	184	100	15	6.55
		156	100	10	
Sulfadiazine	251.1	156	100	10	5.50
		108	100	22	
Sulfamethoxazole	254.1	156	100	10	9.53
		92	100	26	
Sulfathiazole	256	156	100	10	6.26
		108	100	21	
Flumequine	262	244	120	15	16.41
		202	120	30	
Oxolinic acid	262.1	244	100	15	16.41
		216	100	30	
Sulfamerazine	265.1	172	100	15	7.01
		92	100	30	
Sulfisoxazole	268.1	156	100	10	10.71
		113	100	10	
Sulfamethizole	271	156	100	10	8.25
		108	100	22	
Sulfadimidine	279.1	186	100	15	8.45
		156	100	16	
Sulfamonomethoxine	281	156	100	15	9.91
		108	100	26	
Sulfamethoxypyridazine	281.1	156	100	15	8.03
		108	100	25	
Sulfameter	281.2	156	100	15	8.85
		108	100	25	
Sulfachloropyridazine	285	156	100	10	9.21
		108	100	25	
Trimethoprim	291.2	230.1	100	25	8.56
		123	100	25	
Sulfadoxine	311.1	156	110	15	10.73
		92	110	30	
Sulfadimethoxine	311.2	156	115	20	14.49
		108	115	26	
Norfloxacin	320	302.1	140	20	10.41
		276.1	140	15	
Enoxacin	321	303.1	130	18	10.10
		232	130	38	
Ciprofloxacin	332.1	314.1	130	20	11.08
		231	130	42	
Danofloxacin	358.2	340.1	140	25	11.71
		255	140	46	
Enrofloxacin	360	342.1	130	20	11.59
		316.2	130	20	
Ofloxacin	362	318.1	130	15	9.95
		261.1	130	26	
Marbofloxacin	363	345.1	120	20	8.98
		320.1	120	10	
Fleroxacin	370.1	326	130	18	9.13
		269	130	28	
Sarafloxacin	386.1	368.1	130	20	13.54
		342.1	130	15	
Sparfloxacin	393.1	349	130	21	14.94
		292	130	36	
Orbifloxacin	396.2	352.1	150	15	12.44
		295.1	150	22	
Difloxacin	400	382.1	140	20	12.86
		356.1	140	20	
Doxycycline	445.1	428	130	15	15.73
		321	130	33	
Tetracycline	445.2	427.1	130	8	9.51
		410	130	16	
Oxytetracycline	461.2	426	130	16	9.82
		443.1	130	8	

(*continued on next page*)

Table 1 (*continued*)

Compound Name	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (eV)	Retention Time (min)
Chlortetracycline	479.1	462	130	16	14.04
		444	130	19	
Erythromycin	734.5	576.3	170	15	17.05
		158.1	170	30	
Spiramycin	843.5	174	200	42	15.65
		101	200	46	
Tilmicosin	869.6	696.4	210	45	16.35
		174	210	50	
Tylosin	916.5	174	220	35	17.00
		132	220	40	

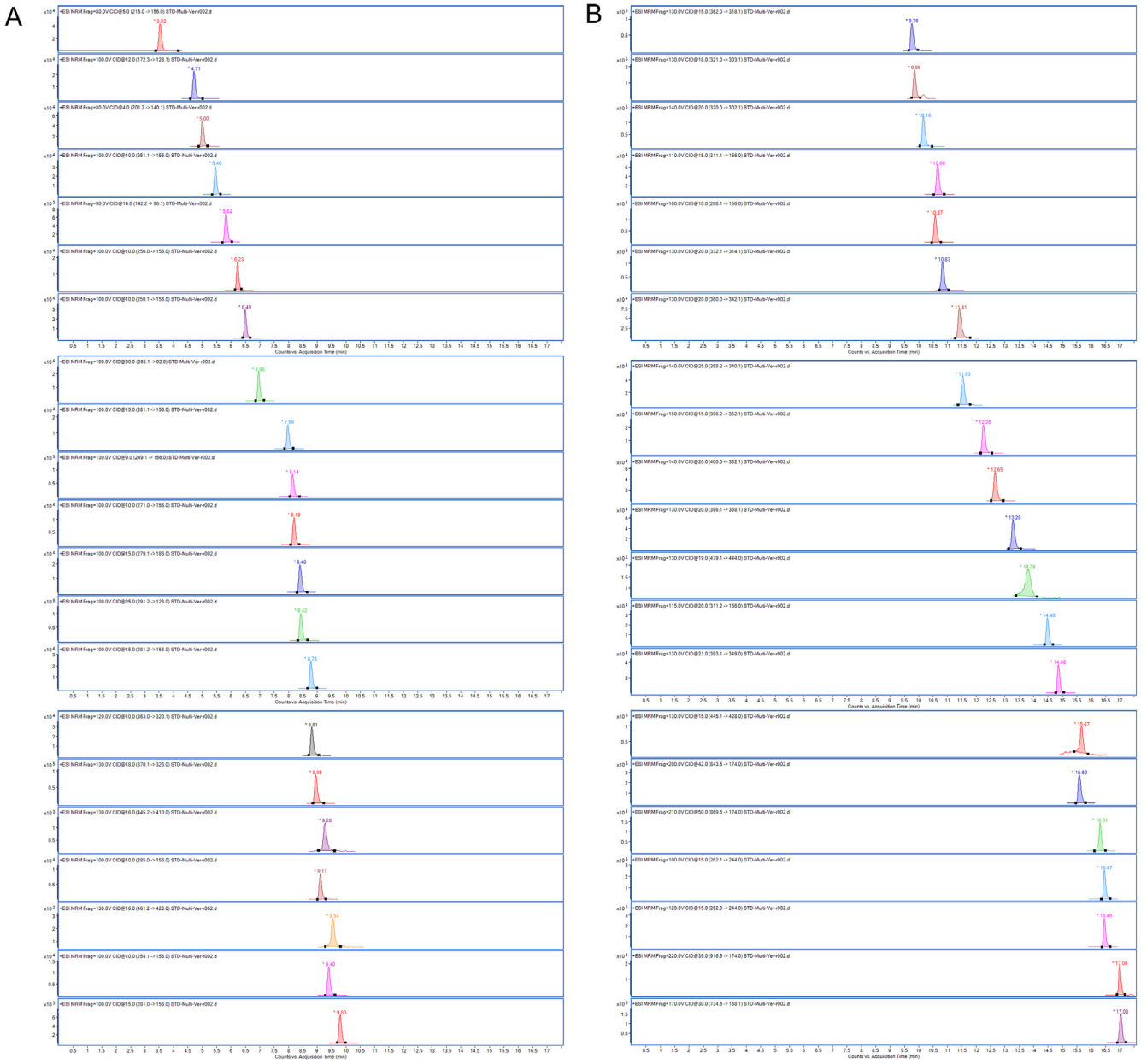

Fig. 2. Typical chromatograms of MRM transitions for sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim at the concentration of 10 ng/mL.

Table 2
The obtained values of LOQ of sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim.

Analytes	Honey (ng/g)	Royal jelly (ng/g)
Dimetridazole	0.46	4.27
Metronidazole	0.50	2.24
Ronidazole	2.42	2.45
Sulfaguanidine	1.10	1.32
Dapsone	0.58	1.34
Sulfapyridine	1.51	3.04
Sulfamethoxazole	1.40	2.14
Oxolinic acid	3.46	3.18
Flumequin	5.95	10.54
Sulfisoxazole	1.37	1.71
Sulfamethizole	1.29	1.66
Sulfamethazine	1.89	1.71
Sulfamonomethoxine	0.73	1.03
Sulfachloropyridazine	1.19	1.37
Trimethoprim	1.26	1.85
Sulfadoxine	1.12	1.19
Sulfadimethoxine	1.02	1.23
Sulfaphenazole	0.55	0.58
Norfloxacin	1.78	3.51
Enoxacin	8.59	10.75
Ciprofloxacin	2.93	3.44
Danofloxacin	3.64	3.86
Enrofloxacin	2.63	3.10
Ofloxacin	2.90	4.01
Marbofloxacin	4.30	4.61
Fleroxacin	3.41	3.99
Sarafloxacin	1.59	1.69
Sparfloxacin	1.51	2.00
Orbifloxacin	1.84	1.83
Difloxacin	1.85	2.48
Lincomycin	9.70	12.68
Doxycycline	4.95	8.63
Tetracycline	5.05	11.92
Oxytetracycline	5.55	7.93
Chlortetracycline	9.42	10.31
Erythromycin	6.53	7.05
Spiramycin	1.85	1.97
Tilmicosin	1.11	1.60
Tylosin	2.06	3.55
Sulfamerazine	1.23	1.33
Sulfamethoxypyridazine	1.47	1.97
Sulfameter	1.95	2.53

concentrations of sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim standard solution in pure water (B) and those of matrix matched solution with the same concentration (A). Matrix effects through the method were performed by spiking QC honey and royal jelly samples with known amounts of standard solutions at three concentration levels. For the matrix effect (A-B)/B, a negative value (<0%) indicates ionization suppression; whereas a positive value (>0%) indicates an ionization enhancement effect. Finally, 10 of different honey and royal jelly samples were analyzed to show that the mean matrix effects for sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim were between -58.5% and 57.3%. Most of them have a reasonable matrix vibration ranges (<±20%); but some of them exhibited the significant matrix enhancement (doxycycline in honey, sulfapyridine, sulfamethoxazole, sulfamethazine, sulfadoxine, marbofloxacin, orbifloxacin, doxycycline in royal jelly) and matrix suppression (marbofloxacin, oxolinic acid, flumequin, sulfisoxazole, sulfamethizole, lincomycin and macrolides in honey, oxolinic acid, flumequin, sulfamethizole and macrolides in royal jelly). Indeed, the use of isotopically labeled internal standards can effectively compensate for matrix effects, but it is difficult to acquire internal standards for each analyte in the practical analysis. Therefore, matrix-matched calibrations were used to qualify and quantify sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim in honey and royal jelly samples. Similarly, the recovery is given as the MRM response of honey and royal jelly that was spiked with a fixed concentration of the 42 veterinary drugs standard solution before extraction (C) relative to the response of the honey and royal jelly blank samples first subjected to the extraction procedure and then spiked with the same amount of 42 veterinary drugs (A); thus, the recovery is equal to $[(C/A) \times 100]$. The main purpose of the recovery calculation method described above was free from the contribution of the matrix effect and mutual interference among the 42 veterinary drugs. Recoveries ranged from 80.4% to 118.4% for sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim in honey and royal jelly samples (Table 3).

Carry-over effect was assessed by alternatively testing a high level of concentration of the 42 veterinary drug standard solutions (1000 ng/mL) and pure water. This step was reproduced five times. The result showed that the signal response of pure water in the retention time of target analyte was lower than that of three times of LOD value. So, it was concluded that no carry-over effect was present in this study based on the developed method.

3.5. Comparison of the present method with the national standard method of China

For comparison, the modified QuEChERS method developed in this work was referred to the national standard method of China. The developed method in this study showed compared with national standard method of China, similar sensitivity for honey and high sensitivity for royal jelly were achieved. The results of the comparison are listed in Table 4 and indicate that compared with the national standard method of China, the present method has other advantages in terms of sample preparation time per batch and cost-saving per sample in a practical analysis. The present QuEChERS method provided a minimum of sample preparation steps which allowed for time saving. The sample preparation time was only 0.5 h for this method, far below 49–58 h required for the national standard method of China, if conducted by one analyst. A cost per sample extraction can be estimated about 4.5–7.5 Dollars in comparison to about 60–90 Dollars using national standard methods of China. These results indicate that the present method is a useful and promising approach for the simultaneous determination of sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim in honey and royal jelly samples.

3.6. Method application

In order to demonstrate the practicality of the developed method in this study, this method was applied to real samples. Honey and royal jelly samples collected in 2014 from apiaries and supermarkets were subjected to the sample preparation method described in Section 2.3 and analyzed by HPLC–MS/MS. A few antibiotics were detected at nanogram per gram levels. Among them, the largest concentrations of norfloxacin, ciprofloxacin, ofloxacin, enrofloxacin, metronidazole, sulfamethoxazole and oxytetracycline were 397.1 ng/g, 74.2 ng/g, 29.7 ng/g, 281.4 ng/g, 580.6 ng/g, 61.2 ng/g and 545.1 ng/g in honey and royal jelly samples. The high detection rates were presented for norfloxacin (5.4%) and metronidazole (8.2%) which is in similar agreement with that of Chinese standard methods used.

4. Conclusions

To the best of our knowledge, this is the first LC–MS/MS assay to be validated for quantifying sulfonamides, fluoroquinolones,

Table 3
The recoveries, precisions and mean matrix effects of sulfonamides, fluoroquinolones, macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim.

	Honey				Royal jelly			
	Mean recovery (%)	Intra-batch precision (%)	Inter-batch precision (%)	Mean matrix effect (%)	Mean recovery (%)	Intra-batch precision (%)	Inter-batch precision (%)	Mean matrix effect (%)
Dimetridazole	85.3	8.5	10.6	0.2	94.8	9.4	10.8	14.5
Metronidazole	95.9	4.9	6.4	−16.4	93.7	6.7	7.1	−12
Ronidazole	93.0	6.1	9.4	−15.1	96.8	4.6	6.7	−16
Sulfaguanidine	101.6	10.2	9.9	−20.1	88.6	8.7	8.4	12.5
Dapsone	97.4	2.8	5.7	−16	86.3	5.1	6.9	2.2
Sulfapyridine	93.7	5.4	7.1	−3.3	94.8	5.9	7.9	46.6
Sulfamethoxazole	92.5	3.4	6.4	−3.2	102.2	4.7	8.2	33.7
Oxolinic acid	92.8	5.7	6.9	−42.6	103.3	9.8	8.4	−75
Flumequin	108.3	5.3	7.3	−38	110.2	7.8	9.4	−35.1
Sulfisoxazole	97.5	2.6	4.9	−22	102.0	6.7	10.6	11.2
Sulfamethizole	101.3	6.4	8.1	−58.5	102.3	9.4	8.3	−38.7
Sulfamethazine	87.4	9.1	11.5	−10.3	99.5	10.4	16.9	28.1
Sulfamonomethoxine	110.2	7.5	9.4	−22.7	94.8	11.7	15.3	18
Sulfachloropyridazine	88.1	4.4	6.4	−5.9	91.7	12.9	17.1	4.2
Trimethoprim	103.6	5.1	6.9	−3.2	83.6	9.8	13.4	16.7
Sulfadoxine	103.5	6.1	9.5	−16.9	95.1	7.6	10.6	26.9
Sulfadimethoxine	100.7	5.8	8.4	−10.2	100.6	8.5	12.6	−6.7
Sulfaphenazole	106.7	9.8	11.9	4.7	99.0	15.9	17.4	−4.2
Norfloxacin	90.2	4.2	6.8	−1.8	80.4	6.9	12.6	−3.8
Enoxacin	84.2	5.9	8.7	−5.9	82.6	7.7	15.1	11
Ciprofloxacin	81.2	7.1	9.9	−19	87.1	10.8	16.3	4.9
Danofloxacin	85.3	8.4	11.4	−9.8	99.2	8.4	10.5	11.9
Enrofloxacin	85.8	6.4	9.4	−9.8	99.5	6.9	11.7	10.2
Ofloxacin	88.4	6.4	10.7	−20.3	81.6	8.4	13.6	3
Marbofloxacin	93.3	5.3	11.8	−52.1	92.5	6.3	15.7	25
Fleroxacin	85.4	6.4	15.9	2.7	90.2	8.9	9.5	8.2
Sarafloxacin	87.6	3.8	7.8	−6.7	88.9	9.7	7.9	9.2
Sparfloxacin	97.2	6.4	9.8	−3.4	108.7	10.5	15.8	−1
Orbifloxacin	92.4	4.9	5.8	11	96.9	11.8	9.8	29.2
Difloxacin	101.6	5.6	7.4	−5.3	102.6	12.4	11.3	−4
Lincomycin	88.5	11.7	17.2	−53.1	89.7	13.7	12.7	−7.8
Doxycycline	89.5	8.4	10.8	19.4	80.9	9.4	10.8	57.3
Tetracycline	87.4	7.9	14.8	11.5	89.8	10.4	9.5	17.9
Oxytetracycline	84.2	10.4	15.4	3	85.5	14.7	12.6	2.3
Chlortetracycline	84.7	11.6	14.2	6.3	87.2	14.3	11.9	6.5
Erythromycin	84.6	8.9	16.1	−34.6	81.0	10.9	13.8	−30.6
Spiramycin	81.8	5.7	15.4	−40	103.6	6.8	9.7	−39
Tilmicosin	85.0	5.8	8.4	−48.5	118.4	9.8	14.1	−44.1
Tylosin	83.9	6.9	9.4	−27.5	105.3	10.7	13.7	−21.7
Sulfamerazine	97.7	9.7	5.9	−17.1	86.3	12.5	13.6	−6.1
Sulfamethoxypyridazine	97.0	9.1	10.8	−17.9	83.6	14.6	10.8	−7
Sulfameter	84.2	6.4	8.2	1.7	86.2	11.7	12.7	−4.4

Table 4
Comparison of the present method with the national standard method.

			Nitroimidazoles	Fluoroquinolones	Sulfonamides	Tetracyclines	Macrolides	Dapsone	Trimethoprim
National standard method	Honey	Sample preparation time (hour/per batch)	7–8	7–8	8–10	12–14	7–8	4–5	4–5
		Cost (Dollar/per sample)	7–12	7–12	10–15	10–15	7–12	7–12	7–12
	Royal jelly	Sample preparation time (hour/per batch)	7–8	7–8	8–10	12–14	7–8	4–5	4–5
		Cost (Dollar/per sample)	7–12	7–12	10–15	10–15	7–12	7–12	7–12
Present method	Honey	Sample preparation time (hour/per batch)				0.5			
		Cost (Dollar/per sample)				4.5–7.5			
	Royal jelly	Sample preparation time (hour/per batch)				0.5			
		Cost (Dollar/per sample)				4.5–7.5			

macrolides, nitroimidazoles, tetracyclines, dapsone and trimethoprim in honey and royal jelly. Strengths of the assay include significantly reduced sample preparation time and cost-saving relative to national standard methods of China. Another merit is the simultaneous determination of 42 veterinary drugs based on d-MRM of LC–MS/MS with satisfactory sensitivity, recovery and precision. This method has been successfully applied in the work aiming to monitor contamination information of veterinary drugs in beekeeping industry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.11.026.

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