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Simultaneous Determination of Tetracycline Antibiotics in Beehives by Liquid Chromatography–Triple Quadrupole Mass Spectrometry

Mei Bie[a,b,c], Rong Li[a], Tingting Chai[a,b,c], Shouhui Dai[b,c], Hualin Zhao[b,c], Shuming Yang[b,c], Jing Qiu[b,c *]

[a] *Department of Food Science, School of Biotechnology and Food Science, Key Laboratory of Food and Biotechnology, Tianjin University of Commerce, Tianjin, China*
[b] *Institute of Quality Standards & Testing Technology for Agro-Products, Key Laboratory of Agro-product Quality and Safety, Chinese Academy of Agricultural Sciences, Beijing, China*
[c] *Key Laboratory of Agro-product Quality and Safety, Ministry of Agriculture, Beijing, China*

ABSTRACT

A simple and effective method was developed for the simultaneous determination of chlortetracycline (CTC), oxytetracycline (OTC), tetracycline (TC) and doxycycline (DC) in beehives using liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS). Analytes were extracted in EDTA-McIlvaine buffer from beehives by ultrasonication and shaking, purified using hexane distribution and HLB cartridges, quantified using a matrix-matched standard calibration curve, and validated according to Commission Decision 2002/657/EC and SANCO/10684/2009. The responses were linear in the range of 1 to 500 μg L^{-1} amount to concentration of 0.2 to 100 μg kg^{-1} based on 5 g of sample with correlation coefficients (r^2)>0.99. The overall recoveries were in the range of 73.8 to 106.7% with RSD of 1.19 to 9.8%. The limits of quantification (LOQ) were 1 μg kg^{-1} for CTC and DC and 0.2 μg kg^{-1} for OTC and TC. The decision limits (CCα) ranged from 0.041 to 0.31 μg kg^{-1}, and the detection capabilities (CCβ) ranged from 0.064 to 0.43 μg kg^{-1}. The application of this method revealed low concentrations of OTC and TC in some real beehive samples. The results showed that the developed method is sensitive and accurate and can be used to determine the levels of tetracycline antibiotics in beehives.

Keywords: Tetracycline; antibiotic; beehive; LC-MS/MS; 2002/657/EC Decision.

INTRODUCTION

Tetracycline antibiotics are widely used in veterinary medicine because of their broad-spectrum activity against most gram-positive and gram-negative bacteria. The protein inhibiting properties of these antibiotics make them effective in the prevention and treatment of several infectious diseases [1]. Chlortetracycline (CTC), oxytetracycline (OTC), tetracycline (TC) and doxycycline (DC) (Fig. 1) are four members of this antibiotic group that are commonly used in the treatment of American and European foulbrood disease because of their high activity and low production costs [2].

Beehives, habitats in which honeybees reproduce and store food, contain all of the elements of bee culture, including larvae, honey, pollen, propolis and wax [3, 4]. TCs can be delivered to hives by the use of antibiotics in apiculture and by the introduction of polluted pollen [5]. Liquid chromatography (LC) methods with ultraviolet detection (UV), diode array detection (DAD), fluorescence detection (FLD) and tandem mass spectrometric (MS/MS) detection are universally used for the screening of TC antibiotics in food material [6]. Honey can be analyzed with LC-ESI-MS/MS and LC-APCI-MS/MS methods [7-10], propolis with LC-UV and royal jelly with LC-MS/MS [11,

12]. However, as far as we know, no method has been reported for beehives.

Antibiotics can accumulate in beehives and migrate from the hives to honey, propolis, royal jelly and wax, resulting in contamination of these bee products. Therefore, the objective of this study was to develop a simple and sensitive method to simultaneously determine the amounts of CTC, OTC, TC, and DC residues in beehives by LC-MS/MS. Then, the method was applied to screen beehive samples for TCs

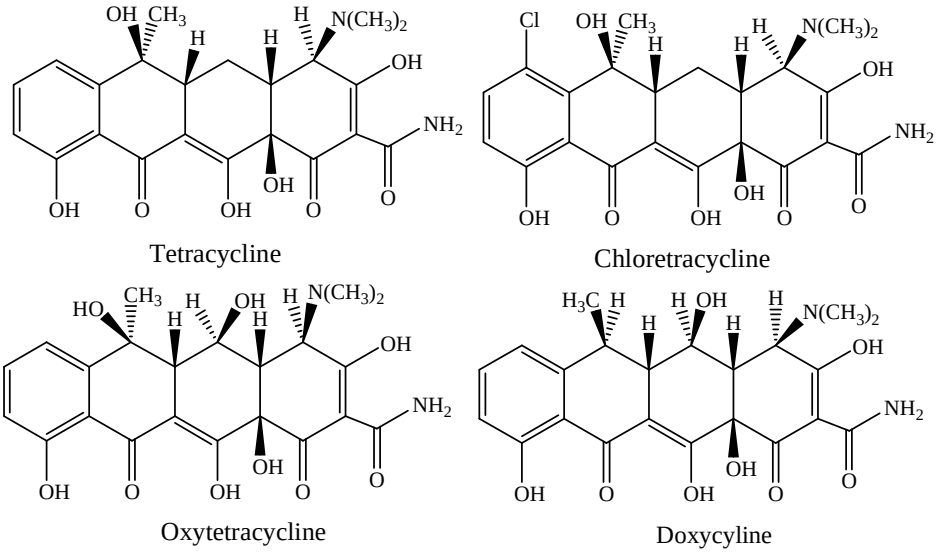

Fig. 1. Chemical structures of TC antibiotics

MATERIALS AND METHODS

Chemicals and reagents

Methanol and hexane were of HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ). Formic acid was of HPLC grade and was obtained from Dima Technology (Richmond Hill, USA). Citric acid monohydrate, disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) and Titriplex III ($Na_2EDTA \cdot 2H_2O$) were of analytical grade and were obtained from Beijing Chemical Plant (Beijing, China). Water was purified using a Milli-Q purification apparatus (Millipore S.A.S, France).

CTC, OTC, TC and DC standards were purchased from Dr. Ehrenstorfer (Germany). Stock solutions of 2000 μg mL^{-1} were prepared in methanol. Mixed standard solutions at concentrations of 0.1, 1, 10 and 100 μg mL^{-1} were gradually diluted with methanol and 4 individual stock solutions, and the calibration working solutions were prepared by diluting an appropriate volume of the mixed standard solutions in water containing 40% methanol (v/v). All standard solutions were stored in a refrigerator at 4°C. OASIS hydrophobic lipophilic balanced sorbent (HLB) SPE columns (200 mg, 6 mL) were purchased from Waters Corporation (Milford, MA, USA).

EDTA-McIlvaine buffer (0.1 mol L^{-1}, pH=4.0) was prepared by dissolving 21.01 g of citric acid monohydrate, 44.78 g of $Na_2HPO_4 \cdot 12H_2O$ and 60.5 g of $Na_2EDTA \cdot 2H_2O$ in 1.625 L of water. Beehive samples were collected from apiaries in Beijing suburbs.

LC-MS/MS conditions

An API 5000 (Applied Biosystems /MDS Sciex, CA, USA) triple quadrupole mass spectrometer with a TurboIonSpray® source was connected to an Agilent 1200 HPLC system (Agilent, CA, USA) equipped with a G1312B binary gradient pump, a G1322A vacuum degasser and an G1367C auto-sampler. LC-MS/MS system control, data acquisition and data processing were performed with Analyst 1.4.2 software.

Methanol (A) and water containing 0.1% formic acid (v/v) (B) were selected as the mobile phase. An Agilent

XDB-C18 (4.6×50 mm, 1.8 μm) column was used for separation. A gradient elution was performed with a flow rate of 0.3 mL min^{-1}: methanol decreased from 60% (0 min) to 5% (1 min), held for 2 min (1-3 min), returned to 60% (3.0-3.1 min) and held for 5.9 min (3.1-9 min) to equilibrate the column for the next injection. The injection volume was 10 μL. Positive electric spray ionization (ESI+) with the multiple reaction monitoring (MRM) mode was used for LC-MS/MS analysis.

Sample preparation

Kibbled beehive samples (5 g) were weighed into 100 mL polypropylene centrifuge tubes. Spiked samples were prepared by fortifying blank samples with the appropriate volumes of the mixed standard solutions. After incubation of the samples for 30 min, 20 mL of hexane and 20 mL of EDTA-McIlvaine buffer were added to each tube. The tubes were capped, vortex-mixed for 30 s, and then shaken for 20 min after ultrasonic extraction for 10 min at a frequency of 40 kHz. Samples were divided into three layers after being centrifuged at 4000 r min^{-1} for 3 min. The supernatant hexane layer and the buffer layer were separated by a solid phase layer. The bottom liquid layers were transferred to 50 mL polypropylene tubes. Subsequently, another 10 mL of hexane was added to each tube and then removed after the tubes were vortex-mixed for 1 min.

OASIS HLB SPE columns preconditioned consecutively with 5 mL of methanol and 5 mL of water were used for the clean-up procedure. The above sample solutions were allowed to pass through the columns at a rate of approximately 3 mL min^{-1}. HLB columns were washed with 5 mL of water containing 5% methanol (v/v). The SPE cartridges were vacuum-dried for 5 min, and the analytes were eluted with 10 mL of methanol. The eluate was evaporated to dryness with a rotary evaporator, and the residue was redissolved in 1 mL of water containing 40% methanol (v/v). The reconstituted samples were filtered into auto-sampler vials using 0.22 μm syringe filters for LC-MS/MS analysis.

RESULTS AND DISCUSSION

Table 1. MRM ions with working parameters for TCs

Analyte	Precursor ion(m/z)	Product ion(m/z)	DP(V)	EP(V)	CE(eV)	CXP(V)
CTC	479.2	154.2[a]	30	12	40	15
	479.2	444.3	40	10	30	10
	479.2	462.3	30	5	30	15
OTC	461.2	426.2[a]	30	5	25	15
	461.2	444.4	30	12	20	15
	461.2	337.1	40	5	40	15
TC	445.3	410.3[a]	50	5	25	15
	445.3	154.3	35	5	40	15
	445.3	427.2	35	5	15	15
DC	445.2	428.3[a]	30	12	25	15
	445.2	339.2	25	5	40	15
	445.2	410.2	35	12	25	15

[a]*indicates MRM transitions for quantification*

Optimization of instrumental parameters

As is well known, LC-MS/MS in MRM mode is considered to be sensitive and selective for simultaneous trace-level determination. The MS parameters option was performed by a Q1 scan and a product ion scan in positive and negative modes for all compounds using a syringe pump injection. The ionization of all compounds was more efficient in the positive mode. The transition ions with the most intense signals were used for quantification, and the remaining two transition ions were used for qualitative confirmation of the TCs. The optimized source and gas parameters were as follows: curtain gas (CUR), 15; collision gas (CAD), 7; ion source temperature (TEM), 450 °C; ion source gas 1 (GS1), 45; ion source gas 2 (GS2), 60; and ion spray voltage, +5500 V. Compound-dependent parameters including the declustering potential (DP), the collision energy (CE), the entrance potential (EP) and the collision exit potential (CXP) are shown in Table 1. Methanol and water were used as the mobile phase, and 0.1% formic acid (v/v) was added to water to improve the protonation of the target analytes [13, 14].

Assessment of matrix effects

The causes of matrix effects were various and included the chemical properties of the target components, the co-eluting species that interfere in the determination of the analyte concentration ratio, the extraction procedure and the ionization conditions. Two methods were used to evaluate the matrix effects. One was comparing the response (peak area) obtained for each analyte in an extract of a blank sample and that in the reconstituted solution; the other was comparing the slopes of the standard calibration curves in solvent and the matrix-matched standard calibration curves. There was no significant matrix effect if the ratios are between 80% and 120%. A lower slope for the matrix-matched standard calibration curves indicates the suppression of the signal, whereas a greater slope indicates signal enhancement [15-18].

In this study, the comparison of slopes was performed. The slope ratios displayed in Table 2 reveal three different types of matrix effects: significant signal enhancement for TC and OTC, insignificant variation for CTC and significant signal suppression for DC. To overcome the matrix effects, a matrix-matched standard calibration curve was chosen for quantification.

Table 2. Method linearity and matrix effects

Analyte	Calibration curves in solvent		Matrix-matched calibration curves		Ratio of the slopes (%)
	Linear regression equation	Correlation coefficients (r^2)	Linear regression equation	Correlation coefficients (r^2)	
CTC	y=10400x-635	0.9999	y=11200x-6600	0.9927	107.7
OTC	y=46200x-4000	0.9999	y=63600x+29100	0.9960	137.7
TC	y=59900x-6790	0.9988	y=101400x+61500	0.9986	169.3
DC	y=97200x-8160	0.9998	y=42000x-37100	0.9907	43.2

Method validation

Method validation was performed in accordance with Commission Decision 2002/657/EC and SANCO/10684/2009 [19, 20]. Parameters including the limit of quantification (LOQ), the decision limit (CCα) and detection capability (CCβ), the trueness, the precision and the selectivity were measured.

Trueness, precision and LOQ

In the present study, three spiked concentration levels of 1, 10, 100 μg kg^{-1}, with 6 replicates for each concentration, were used to evaluate the precision and accuracy of the TCs analyses. For OTC and TC, 0.2 μg kg^{-1} spiked samples were additionally prepared because this level still met the requirements of SANCO/10684/2009. The same experiment was repeated 3 times on different days. The recovery was the response ratio of the fortified sample and standard in the extract of the blank one. A summary of the results of the recovery and precision experiments are shown in Table 3. The average recoveries at the investigated levels were all in the range of 73 to 107%, indicating good trueness. Both the intra-day and inter-day relative standard deviations (RSDs) were below 9.8%, revealing that the precision and accuracy of the method were acceptable.

The LOQ is defined as the lowest spiked level meeting the method performance acceptability criteria (mean recoveries in the range of 70 to 120% with an RSD ≤20%) [19]. The LOQs were determined to be 1 μg kg^{-1} for CTC and DC and 0.2 μg kg^{-1} for OTC and TC, with average recoveries of 84.4 to 100.6% and RSDs below 9.19%. These LOQs for TCs were considered reasonable and were much lower than the maximum residue limit (MRL, 50 μg kg^{-1}) of the total amount of TC antibiotics in honey set by China [21].

Selectivity

The selectivity of the developed method was demonstrated by analyzing blank matrix samples and samples spiked with the standard solutions. No significant interference was found at the corresponding retention time, as determined by comparison of the extracted ion chromatograms of the blank samples and the spiked samples (Fig. 2-3). The identifications of the TCs were performed in accordance with the ratio of intensities of the MRM transitions each (Table 1). Additionally, the identity of the analytes could be confirmed using the relative retention times of the TC standards [22].

Table 3. Method trueness and precision

Analyte	Spiked level (μg kg^{-1})	Intra-day		Inter-day	
		Recovery (%, n=6)	RSD (%, n=6)	Recovery (%, n=3)	RSD (%, n=3)
CTC	1	84.4	1.57	84.8	1.35
	10	73.8	3.29	76.6	4.85
	100	76.9	2.65	77.5	2.97
OTC	0.2	93.3	4.91	100.5	7.89
	1	102.3	2.60	99.5	3.24
	10	103. 7	2.49	97.0	9.80
	100	91.3	1.74	92.3	4.28
TC	0.2	100.6	6.85	97.3	9.19
	1	98.0	3.73	94.	3.87
	10	106.7	1.93	97.0	9.70
	100	90.6	1.57	93.9	3.18
DC	1	88.7	2.04	88.9	1.19
	10	86.9	2.56	85.6	3.54
	100	87.	2.33	86.7	2.12

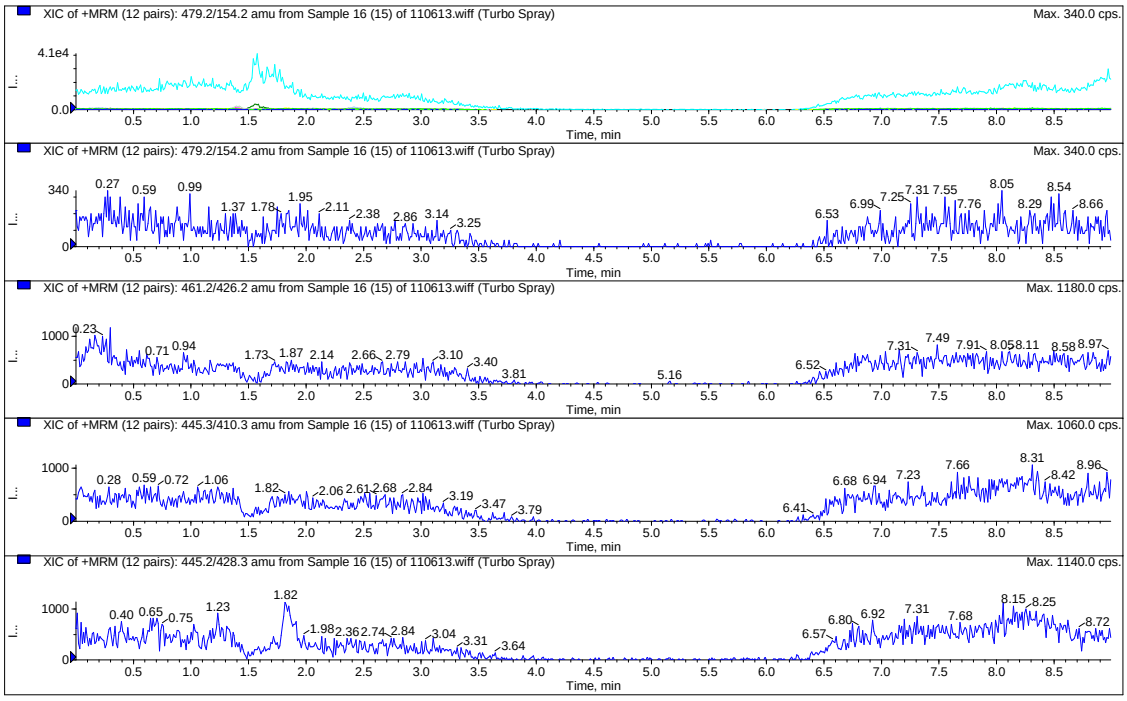

Fig. 2: Total product ion and extracted ion chromatograms of a blank hive sample

Linearity

Calibration curves were generated by plotting the instrumental response (peak area) of each analyte versus its concentration. In plotting the calibration curves, "linear" regression and "1/x" weighting were used by the Analyst software. The method was demonstrated to have good linearity for TCs in the interval of 1.0 to 500.0 μg L^{-1} with correlation coefficients (r^2)>0.99. The linear regression equations are given in Table 2.

Decision limit and detection capability

To calculate CCα and CCβ, a set of samples fortified with TC standard solutions to yield concentrations equivalent to 1, 1.5 and 2 times the minimum required performance limits were used. The LOQs were selected as the minimum

required performance limits because of the absence of MRLs in the beehives. Calibration curves were generated by plotting the peak area against each spiked concentration. The CCα equals the corresponding concentration at the intercept plus 2.33 times the standard deviation of the intra-laboratory reproducibility of the intercept. The CCβ equals the corresponding concentration at CCα plus 1.64 times the standard deviation of the intra-laboratory reproducibility of the mean measured content at the decision limit [20]. The values of CCα and CCβ for each TC, summarized in Table 4, were low and sufficient for detection and quantification.

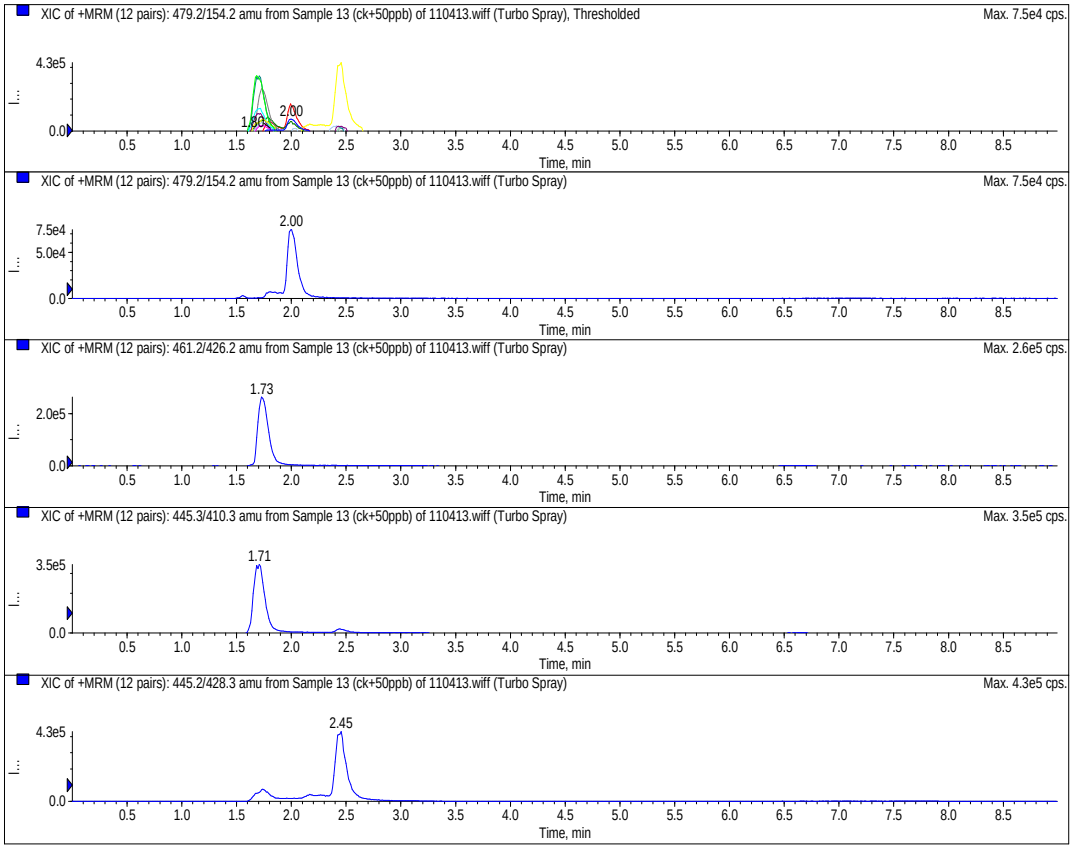

Fig. 3: Total product ion and extracted ion chromatograms of a spiked beehive sample at 10 μg kg^{-1}

Table 4. Method decision limit and detection capability

Analyte	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
CTC	0.25	0.32
OTC	0.041	0.064
TC	0.060	0.11
DC	0.31	0.43

Application

The developed method was applied to determine the concentration of TCs in beehive samples collected from different apiaries in Beijing suburbs. CTC and DC were not detected in the 15 samples, but detectable residues of OTC and TC were found in 7 samples. OTC and TC were both found in 2 samples, and only OTC was found in the remaining 5 samples. TC at concentrations of 1.08 μg kg^{-1} and 0.27 μg kg^{-1} and OTC at concentrations of 0.39 μg kg^{-1} and 2.06 μg kg^{-1} were respectively detected in two samples. OTC at concentrations of 0.66, 0.92, 1.95, 3.26 and 52.2 μg kg^{-1} were found in the 5 remaining samples. These concentrations were lower than the MRLs (100 or 300 μg kg^{-1}) in honey set by many regulations [23-24], but higher than the MRL (50 μg kg^{-1}) of TC residues in honey set by China [21].

CONCLUSION

In this study, a method was developed and validated for the simultaneous identification and quantification of CTC, OTC, TC, and DC in beehives. Good linearities for the four TCs were obtained in the concentration range of 1.0 to 500.0 μg L^{-1} with correlation coefficients (r^2)>0.99. The LOQs were 1 μg kg^{-1} for CTC and DC and 0.2 μg kg^{-1} for OTC and TC. The CCα and CCβ values also were determined according to Commission Decision 2002/657/EC. The mean recoveries and RSD fulfilled the requirement of SANCO/10684/2009. Other validation parameters also met the European Union method performance criteria. The application of the developed method for determining TC concentrations in real beehive samples showed that lower concentrations of OTC and TC were found in some samples.

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