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Analysis of tetracycline residues in royal jelly by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A confirmatory method coupling liquid chromatography with tandem mass spectrometry (LC/MS/MS) was developed to determine the concentration of oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC), which make up the tetracycline (TC) groups present in royal jelly. Sample preparation included deproteination, control of pH, extraction and clean-up on a solid-phase extraction (SPE) cartridge. The analyses were achieved by LC/MS/MS in selected reaction monitoring mode (SRM). The overall recovery of fortified royal jelly at the levels of 5.0, 10.0 and 40.0 μg/kg ranged from 62% to 115%, and the coefficients of variation ranged from 3.4% to 16.3% ($n = 6$). The detection limits for TCs were under 1.0 μg/kg. The transformation between the TCs and its epimers (EpiTCs) was studied in standard solution and during the sample preparation process. This method can be used for the detection of tetracycline residues in royal jelly.

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1. Introduction

Tetracyclines (TCs) produced by *Streptomyces* spp. are broad-spectrum antibiotics, which are active against most Gram-positive and Gram-negative bacteria. Members of the TC group include oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC). The TC group is commonly added to the diets of animals used to produce human food, especially honeybees. Because of the broad-spectrum activity and low production costs [1], the TC group accounts for over 60% of all antibiotics used in veterinary medicine in Japan [2].

There is a possibility that TC residues may remain in animal products and will therefore be consumed by humans. The U.S. Food and Drug Administration (FDA) set the tolerance for the combined residues of TC, OTC and CTC at 2 mg/kg in meat [3]. The European Community (EC) proposed a maximum residue limit (MRL) of 10 μg/kg for honey and 100 μg/kg for meat and milk to protect consumers from exposure to TCs in original animal products [4].

The main components of royal jelly are carbohydrates, proteins, and lipids (approximately 11%, 13%, and 5% respectively). In royal jelly, the proteins are rich in natural hormones such as pheromones, different types of amino acids including essential amino acids, B group vitamins, and various other vitamins. The sugars, such as fructose and glucose, are similar to those found in honey. The lipids consist of medium-chain hydroxy fatty acids, such as *trans*-10-hydroxy-2-decenoic acid. They are presumed to be the active components which contribute to the nutritional and therapeutic properties of royal jelly [5]. There have been few published reports concerning drug residues in royal jelly [6,7]. Many liquid chromatography methods have been published for the determination of TCs in various food matrices [8–15]. Ishii et al. reported a method to determine the TCs in royal jelly by using 2% metaphosphoric acid as the deproteinizing reagent. The quantification limits of TC, OTC, and CTC using this method were 25, 25 and 50 μg/kg, respectively, which are 20-fold higher than those in our study. Additionally, we found that a C18 SPE column was not suitable due to the deposits of silanol on the column padding [7].

In this work, we developed a rapid analytical LC–MS/MS method for the detection and confirmation of TCs in royal jelly using a positive ESI mode. The epimerization of TCs was investigated in the standard solution and during the sample preparation process. Various techniques for the quantification of TCs are discussed in this paper.

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2. Experimental

2.1. Chemicals and materials

The reference standards of tetracycline and chlortetracycline were purchased from Sigma–Aldrich (Steinheim, Germany). Oxytetracycline and doxycycline were purchased from Dr. Ehrenstorfer (Augsburg, Germany). 4-Epi-oxytetracycline (4-EpiOTC), hydrochlorides of 4-epichlortetracycline (4-EpiCTC) and 4-epitetracycline (4-EpiTC) were purchased from Acros Organics (Pittsburgh, PA, USA). All the reference standards were stored at −4 °C.

Standards were dissolved in methanol up to 1.0 mg/mL and gradually diluted to 1.0 μg/mL (stock solutions). The stock solutions were renewed monthly because of their limited stability. The purity of the stock solutions was checked before each analytical run by LC–MS/MS. All stock solutions were stored in the dark at −20 °C.

The cartridges used for solid-phase extraction were 3 mL disposable Oasis HLB (60 mg, Waters Corp., Milford, MA). The SPE instrument was made of Supelco Corp. Trichloroacetic acid and K_2HPO_4 were analytical grade and purchased from Nanjing chemical reagent factory (Nanjing, China). All the solvents used were of analytical grade. Methanol and formic acid for HPLC analysis were from Merck (Darmstadt, Germany). HPLC grade Milli-Q water was obtained with a MILLI-RO PLUS 90 apparatus (Millipore, Molshelm, France).

1% trichloroacetic acid solution was prepared by dissolving 5.0 g trichloroacetic acid in 100 mL water and diluted to 500 mL. 1.0 mol/L K_2HPO_4 solution was prepared by dissolving 87.0 g K_2HPO_4 in water and diluted to 500 mL.

2.2. Sample preparation

2.2.1. Sample extraction

2.0 g royal jelly was transferred to a 50 mL polypropylene centrifuge tube and 20 mL 1% trichloroacetic acid was also added into the tube. The mixture was vortexed for 2 min. After centrifugation at 6500 rpm for 5 min, the supernatant was transferred to a new 50 mL polypropylene centrifuge tube. Then 1.5 mL 1.0 mol/L K_2HPO_4 solution was added into the supernatant to adjust pH to 6.0–7.0. The tube was centrifuged at 6500 rpm for another 5 min. The supernatant after the second centrifugation was filtered through a filter paper (quick, 90 mm, Hangzhou Xinhua Paper Corp., China) and set aside for further solid-phase clean-up.

2.2.2. Solid-phase extraction and clean-up

The Oasis HLB cartridges were treated successively with 5 mL methanol and 5 mL Milli-Q water. Then, the supernatant of the centrifuged extract was passed through the cartridges under vacuum, at a flow rate of 1–2 mL/min. After washing with 5 mL Milli-Q water and 5 mL methanol:water (2:8, v/v), by applying a slight vacuum to remove traces of water, TCs were eluted with methanol (5 mL) and collected into a 10 mL tube. The extracts were evaporated to dryness under a stream of nitrogen at 45 °C (Organomation Associates Inc., Berlin, MA) and reconstituted in 1.00 mL methanol:water (3:7, v/v) solution. Finally, the solution was filtered through a 0.45 μm nylon filter directly into HPLC vials for LC–MS/MS analysis.

2.3. Liquid chromatography tandem mass spectrometry (LC–MS/MS)

2.3.1. Liquid chromatographic separation

A Sunfire C18 column (150 mm × 2.1 mm i.d., 5.0 μm particle size) (Waters, Milford, MA, USA) was used for the chromatographic separation. Eluent A was formic acid 0.1% in Milli-Q water, and eluent B was methanol. The mobile phase gradient was 0.0–8.0 min 10–90% B, 8.01–10.0 min 90% B, and 10.1–12.0 min 10% B. The flow rate was 250 μL/min, and the column was kept at room temperature. The injection volume was 25 μL each time. The injection mode was full-loop, and the rinsing solution for the autosampler was methanol:water (1:1, v/v).

Table 1
Precursor ions, product ions and corresponding collision energies for the tetracycline residues

	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
TC	445.1	410.1[a]	18
		427.2	16
OTC	461.1	337.0	25
		426.3[a]	18
		443.2	14
CTC	479.2	444.1[a]	20
		462.2	18
DC	445.1	410.1	27
		428.2[a]	15

[a] The product ion used for quantification.

2.3.2. Mass spectrometry (MS–MS)

The instrument used was a Thermo Quantum Ultra AM tandem mass spectrometer coupled with a modular Spectra system LC system, which included an SCM1000 degasser, P4000 MS pump and AS3000 autosampler (Thermo Fisher Scientific, San Jose, CA, USA). The software was Xcalibur Version 1.4. The instrument calibration for the mass spectrometer was performed according to the instrument manufacturer's specifications.

TCs were detected in positive electrospray ionization (ESI) in selected reaction monitoring (SRM) acquisition mode. The spray voltage was set at 4.5 kV. The sheath and auxiliary gas pressures (N_2) in the system were 30 and 5 (arbitrary units), respectively. The capillary temperature was 350 °C. The results of the precursor ion, product ions and the collision energy are listed in Table 1.

3. Results and discussion

3.1. Sample preparation

3.1.1. Extraction of TCs from royal jelly

There are many proteins in royal jelly which could interact with the TCs leading to low recovery and inaccurate detection of these antibiotic residues. In previous studies McIlvaine/ethylenediaminetetraacetic acid (EDTA) (pH 4) solution was selected as the extraction solution, however, the EDTA solution was not able to eliminate the interaction with proteins. Therefore, it was not an appropriate extraction solution for royal jelly. There have been reports of the use of metaphosphoric acid–methanol (6:4) to deproteinate the samples [7], but the process is quite time-consuming.

Some inorganic acids can denature proteins. It has been reported that trichloroacetic acid can be used to extract TCs [17]; however, we have been unable to find any reports relating to the use of trichloroacetic acid to extract TCs in royal jelly. In our research, trichloroacetic acid was used to deproteinate and extract the TC residues from royal jelly. In further studies, it was found that optimization of the pH was essential for maximum recovery of TCs. A pH of 6–7 was found to be the optimum value for recovery of TCs. The effect of different concentrations of trichloroacetic acid was also studied, and the results showed that 1% (m/m) was the most effective concentration.

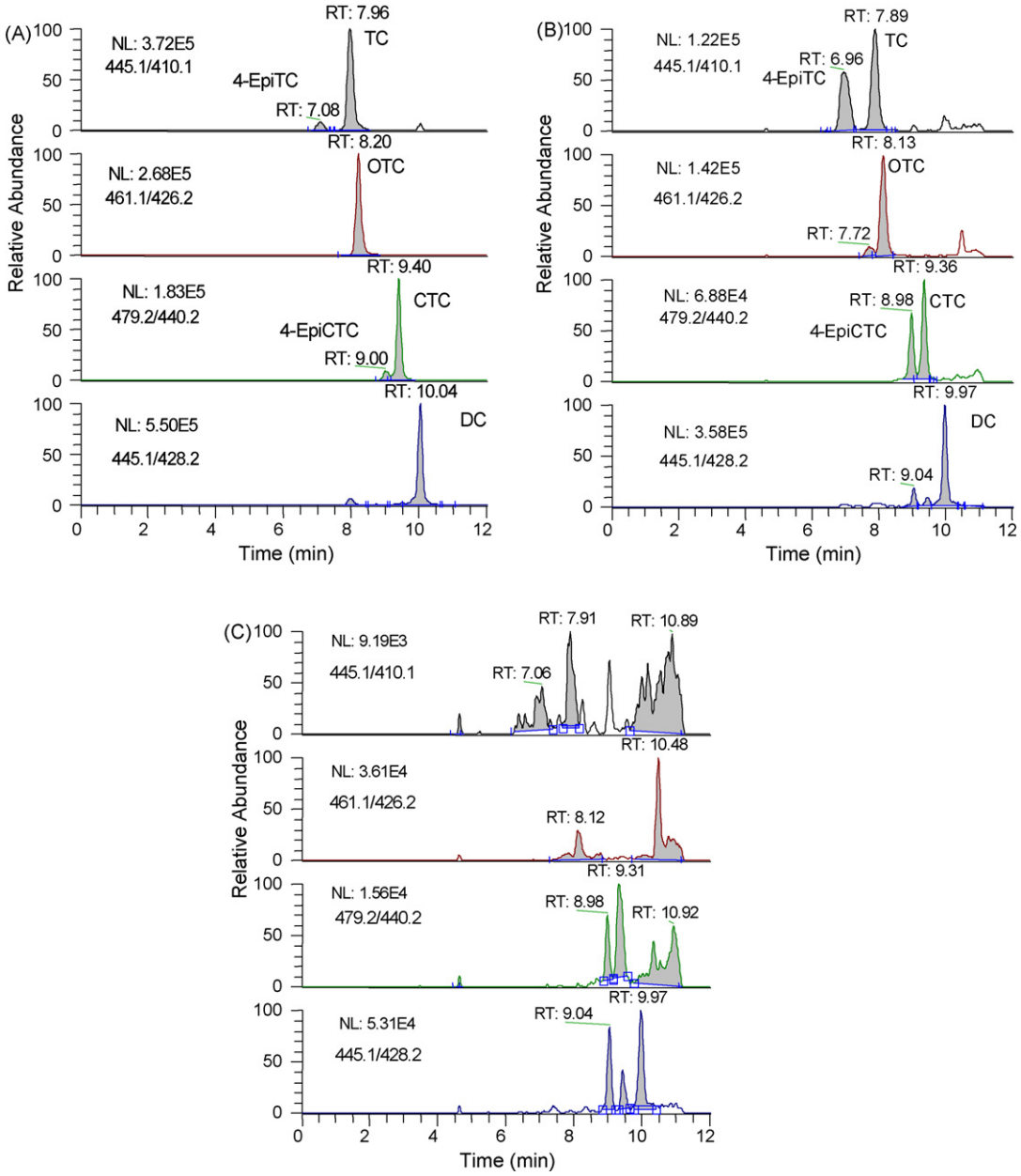

Fig. 1. Selected reaction monitoring mode (SRM) chromatograms of (A) a standard solution of tetracyclines (TCs) containing 10 ng/mL; (B) samples of royal jelly spiked with tetracycline residues at a concentration of 10 μg/kg; and (C) negative royal jelly samples.

3.1.2. Extraction and clean-up using a solid-phase extraction method

Because TCs have good water solubility, solid-phase extraction is the preferred method for their extraction. The SPE column was the key for the extraction recovery. The Oasis HLB was selected as the reverse phase column, and it was discovered that because the column is made of polymer there were no Ca^{2+} residues and no silanol effect [8]. Recoveries were proven to be good by comparing the analyte concentration in extracted standard solutions with that in non-processed standard solutions [9]. In order to reduce the matrix effect, 20% methanol solution was used to wash the column in this experiment.

Table 2
Maximum permitted tolerances of relative ion intensities for confirmation

Relative ion intensity (%)	Permitted tolerances (%)
>50	±20
>20–50	±25
>10–20	±30
≤10	±50

3.2. Chromatography conditions and separation

TCs are able to interact with the cation Ca^{2+} and silanol on the chromatographic columns to produce peak tailing. The polar end-capped column and EDTA in the mobile phase are usually used to maximize peak shape and separation; however, EDTA is not a volatile salt. One disadvantage was that the addition of EDTA to the mobile phase for LC–MS/MS was found to block the ion transfer capillary. After comparing different brands of chromatography

columns, the Sunfire C18 was selected as the analytical column. The column is made of high purity organic silane, which maintains the peak shape of the TCs without the need for the chelating reagent EDTA.

The matrix effect was reported in the detection of TCs in different samples. It was suggested that the natural organic matter was responsible for the matrix effects [17]. The natural organic matter has surfactant properties that could enhance the electrospray signals by promoting ionization in positive electrospray mode. The matrix effect can be reduced by changing the chromatographic column and mobile phase gradient to separate out the organic matter. In our experiments, the separation conditions were optimized to reduce the matrix effect and analysis time. The analysis time for the TCs was less than 12 min. The gradient used is described in Section 2.3.1. The chromatograms of the standard solution, negative royal jelly sample, and fortified royal jelly samples are shown in Fig. 1.

3.3. ESI-MS/MS and confirmation

MS/MS analysis is usually carried out to qualify and quantify the presence of protonated molecules. Several papers have been published concerning the analysis of TCs by MS/MS which detail the parent ion selection and fragmentation mechanism [13–16]. The fragmentation pathways of TCs have been described by Kamel et al. [16], and were mainly explained by the losses of H_2O (18 u), NH_3 (17 u), CO (28 u) and $NH(CH_3)_2$ (45 u). Thus, the most abundant product ions chosen as the quantification ions corresponded to a loss of water and ammonia in TC, OTC and CTC, and a loss of ammonia in DC. The SRM spectra of the TCs studied in our experiments are shown in Fig. 2.

The test samples and standard working solutions were tested under the same conditions as those described above. The variation in the retention time of the analyte peak in both the unknown sample and standard working solution should be within the range of ±0.25 min. The qualification ions for every compound must be found, and must include at least one parent ion and two daughter ions. For the same analysis batch and the same compound, the variation in the ion ratio between the two product ions for the unknown sample and the standard working solution at similar concentrations

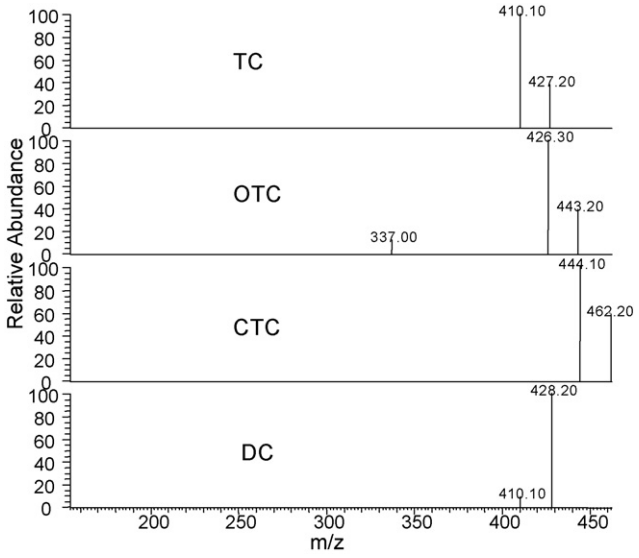

Fig. 2. The selected reaction monitoring mode (SRM) mass spectra of a standard solution of tetracyclines. Argon was used as the collision gas at a pressure of 1.5 mTorr.

cannot be out of the range suggested in the Commission Decision 2002/657/EC as shown in Table 2.

3.4. Calibration and quantification

It has been reported that the TCs can be converted into their epimers in an acid environment. During the extraction of royal jelly, trichloroacetic acid was used as the extraction solvent and protein denaturant. Comparing Fig. 1A with Fig. 1B, the epimer area peaks shown in Fig. 1B were obviously larger than the peak areas in Fig. 1A. Conversion to the epimers would create difficulties for the accurate quantification of TCs. European regulations have established a maximum residue limit (MRL) for TC, OTC, CTC and DC in various food matrices taking into account both the marker residue of the parent drug and its epimer (except for DC). In this

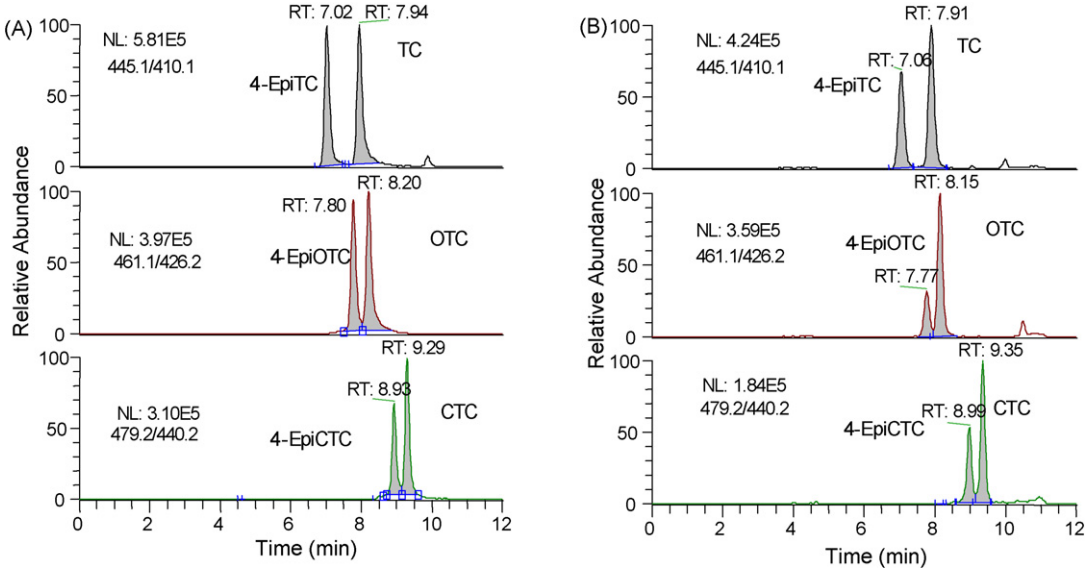

Fig. 3. Selected reaction monitoring mode (SRM) chromatograms for (A) tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and their epimers in a standard solution having a total concentration of tetracyclines of 10 ng/mL; (B) sample of royal jelly spiked with 10 μg/kg of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and their epimers.

Table 3
The ratio of the peak areas of the standard solution and royal jelly sample spiked with only epimers of TCs, TCs and mixture of TCs and EpiTCs at a concentration of 10 μg/kg

	Ratio of peak area		
	EpiTC/TC	EpiOTC/OTC	EpiCTC/CTC
100 ng/mL	0.864	0.925	0.768
10 ng/mL	0.723	0.808	0.765
200 ng/mL	0.948	0.973	0.806
20 ng/mL	0.748	0.844	0.766
50 ng/mL	0.868	0.919	0.905
5 ng/mL	0.784	0.906	0.787
Average	0.822	0.896	0.800
Spike level 10 μg/kg			
EpiTCs	0.826	0.778	0.569
	0.683	0.964	0.562
	0.746	0.843	0.573
TCs	0.797	0.097	0.615
	0.719	0.104	0.552
	0.762	0.098	0.603
TCs and EpiTCs	0.698	0.543	0.605
	0.826	0.409	0.620
	0.813	0.465	0.624

way, TC epimers should be taken into account when quantifying TCs.

The response relationship between the TCs and their epimers has been studied and reported by Khong et al. [9]. TCs and their epimers have the same product ions with similar abundances under lower collision energies. The relative response factors (RFs) were detected and calculated for each epimer after normalizing their respective tetracyclines to 1 in this experiment. The results are shown in Table 3. The RFs were between 0.80 and 0.90, which differed from a previous report [9].

The transformation from tetracycline to its epimer was observed in the standard solution and during the sample preparation process. The TCs and their EpiTCs are stable at −20 °C for at least 2 months. At +4 °C and +20 °C, TC and CTC were easily converted to their epimers, and all epimers were also easily converted to their parent TCs. The sample solution after extraction was stable during the 24-h injection process at +20 °C. The ratio of the peak areas of EpiTC/TC and EpiCTC/CTC increased by about 20% and the TC and CTC concentrations decreased by about 20% after 2 weeks at +4 °C. The OTC in the sample solutions was stable for at least 2 weeks at +4 °C.

In order to study the transformation in the sample preparation process, TCs, EpiTCs, and a mixture of EpiTCs and TCs were spiked into royal jelly samples. The results of the recovery were calibrated using the standard solution and its epimer. The ratio of the peak areas of EpiTC/TC, EpiOTC/OTC and EpiCTC/CTC are given in Table 3. The results showed that the transformation between TC, CTC, and their respective epimers was simple to balance in the sample preparation process, no matter what kind of single parent drug, epimer or mixture of TCs–EpiTCs was spiked into the sample. The EpiOTC was easily converted to OTC, but the conversion of OTC to EpiOTC was more problematic. The epimerization results in the sample extraction process were in accordance with the epimerization rule of TCs [18], and this may explain the stability of the sample in the extraction solution. Sample stability was also studied in 2 weeks, and the determination was that results changed less than 10% for ten times during the freeze–thaw procedure. Because the royal jelly samples must be kept at −20 °C to maintain the activity, any epimerization should have occurred during the sample preparation process. The chromatography curves of TCs and their epimers are shown in Fig. 3 for both the standard solution and spiked samples.

Because of the transformations, it was difficult to quantify the residue by TC and EpiTC calibration curves, respectively. Fortunately, the EU regulations require that the total residue composed of the three TCs and their corresponding epimers is reported. Due to the transformation between TCs and EpiTCs in the sample preparation process, and taking into account that the RFs ranged from 0.80 to 0.90, the calculated results could be calibrated using the TC calibration curves. In this way, it is not necessary to produce a second calibration curve for the standard EpiTCs. The recovery results were calibrated using the TC curves only and are listed in Table 4.

In the TC determination process, the results were influenced by the matrix effect and recovery in addition to the relative response factor. In this experiment, the accuracy of the method was evaluated using a negative sample spiked with different levels of standard. The results are listed in Table 4 and show that, due to many factors, including the extraction and instrument analysis process, the recoveries can only be in an acceptable range. The average recoveries ranged from 62% to 115%.

There are three main effects, which influence the quantification of TC residues in royal jelly. The first is the matrix effect. It has been reported that the matrix effect enhances the signals [16]. The enhancement ratio can be estimated by comparing the matrix calibration and solvent calibration as shown in Fig. 4. The enhancement was about 30–50% for the different TCs. The second factor is the recovery. Due to losses incurred during the extraction process the recovery of TCs will be less than 100%. In this way, the recovery can be estimated by comparing the matrix calibration and extrac-

Table 4
Extraction recoveries and the relative standard deviation of the TCs from royal jelly samples spiked with different concentrations

Spike concentration	TC		OTC		CTC		DC	
5 μg/kg								
Average (μg/kg)	4.923	5.349	5.396	5.577	4.858	6.018	4.977	5.148
Recoveries (%)	98.5	107	107.9	111.5	97.2	120.4	99.5	103.0
RSD% (intra-day)	6.8	14.1	3.4	11.5	7.2	9.7	9.2	14.2
RSD% (inter-day)	15.7		12.0		12.1		16.9	
10 μg/kg								
Average (μg/kg)	9.199	9.384	10.551	10.388	11.454	9.222	7.755	6.492
Recoveries (%)	92.0	93.8	105.5	103.9	114.5	92.2	77.5	64.9
RSD% (intra-day)	16.3	12.1	15.5	10.4	9.9	6.1	13.4	12.0
RSD% (inter-day)	20.3		18.7		11.6		18.0	
40 μg/kg								
Average (μg/kg)	24.887	33.826	25.98	39.996	30.907	30.855	26.274	27.244
Recoveries (%)	62.2	84.6	64.9	100.0	77.3	77.1	65.7	68.1
RSD% (intra-day)	9.9	8.2	8.9	7.3	8.2	9.9	6.1	8.8
RSD% (inter-day)	12.9		11.5		12.9		10.7	

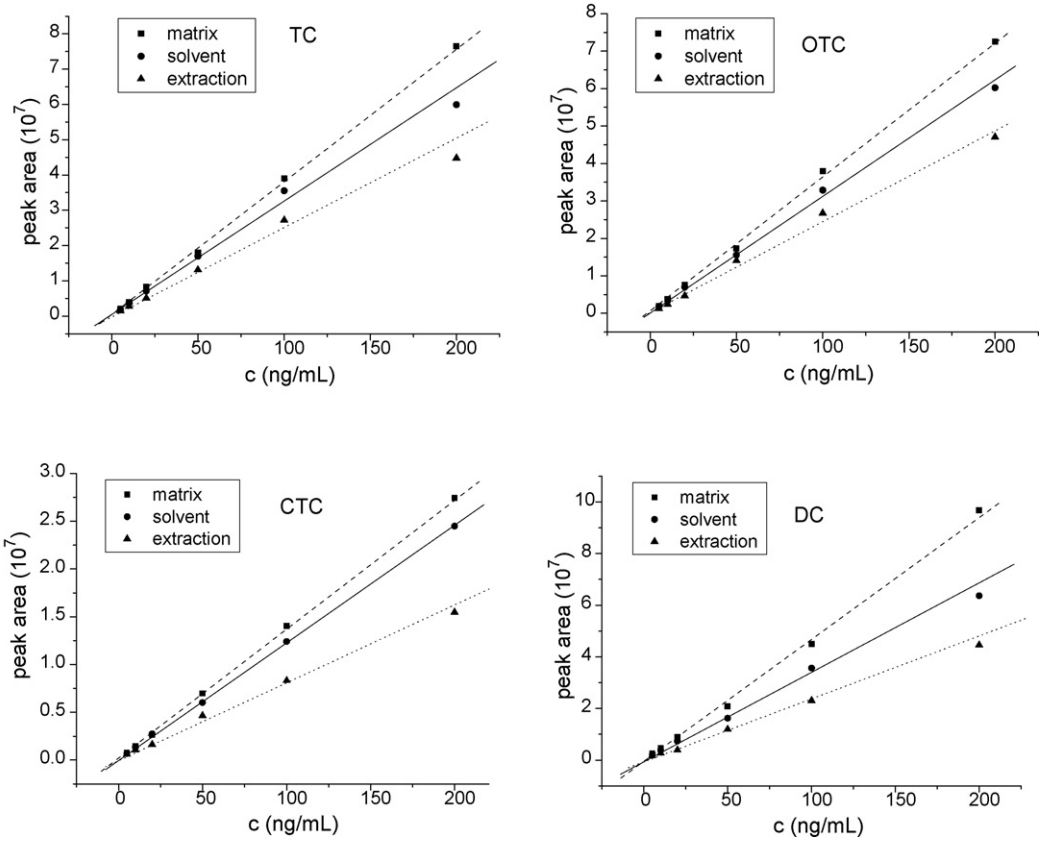

Fig. 4. Calibration curves for tetracyclines (TCs) obtained from the LC/ESI-MS/MS analysis of the standard solution (solvent), matrix-match (matrix) and extraction-match (extraction).

tion calibration as shown in Fig. 4. The actual recoveries were about 45–70% for the different TCs. The last factor is the relative response factors of the epimers. The RFs ranged from 0.80 to 0.90. In comparison to the matrix effect and the recovery factors, the RF had the least influence.

3.5. Accuracy and precision

The repeatability within a day and the precision of the method between days was measured by fortified matrix samples ($n = 6$) at three different concentration levels: 5.0, 10.0 and 40.0 μg/kg. Statistical evaluation revealed that relative standard deviations (RSDs) were in the range of 3.4–16.3% (intra-day) and 10.7–20.3% (inter-day), and that recoveries were in the range of 62.2–114.5% (Table 4).

3.6. Limit of quantification (LOQ) and limit of detection (LOD)

The LOQ was established by analyzing six negative samples, which were spiked with all the TCs under study at half of the MRL level, and 5 μg/kg of royal jelly. As discussed previously, these levels could be quantified within the regulatory accuracy and precision. Therefore, the LOQs were set for the method. It is possible to decrease these values further by increasing the injection volume or by increasing the initial weight of the sample to 5 g.

The LOD was determined by setting the signal-to-noise (S/N) = 3 as the criterion. The average S/N value of the MS/MS mass chromatograms of the six LOQ samples was determined. The LOD values were 0.5 μg/kg for TC and OTC, and 1.0 μg/kg for CTC and DC.

4. Conclusion

In this paper, an LC–ESI-MS/MS method is presented which is capable of identifying and quantifying four types of TC residue in royal jelly. The method was found to be capable of separating the epimers of TCs in a short period of time and facilitating the calculation of their concentrations as a total amount in the sample. The transformations between the TCs and their epimers were studied in the standard solution and during sample preparation. The method is currently used in our laboratory for the routine detection of TC residues in royal jelly.

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