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Simultaneous determination of quinocetone and its major metabolites in chicken tissues by high-performance liquid chromatography tandem mass spectrometry

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ABSTRACT

A convenient, rapid and sensitive liquid chromatography-tandem mass spectrometry method was firstly established for the simultaneous determination of quinocetone and its 4 major metabolites: 1-desoxyquinocetone, di-deoxyquinocetone, carbonyl reduced metabolite from di-deoxyquinocetone and 3-methyl-quinoxaline-2-carboxylic acid in chicken muscle, liver, kidney and fat. Sample was extracted with acetonitrile and chloroform, and further purified by Oasis MAX SPE cartridge. Analysis was performed on a C_{18} column by detection with mass spectrometry in multiple reaction monitoring mode and using a gradient elution program with 0.1% formic acid solution and acetonitrile. The correlation coefficients (r) for each calibration curves are higher than 0.99 within the experimental concentration range. The recoveries of the five target analytes at three spiking levels were between 77.1% and 95.2%, with relative standard deviations less than 15%. The decision limits of the five analytes in chicken edible tissues ranged from 0.24 to 0.76 $\mu g\,kg^{-1}$, and the detection capabilities were below 2.34 $\mu g\,kg^{-1}$. The developed method demonstrated a satisfactory applicability in incurred chicken tissue samples.

1. Introduction

As a new quinoxaline 1,4-dioxide derivative, quinocetone (QCT) was synthesized by the Lanzhou Institute of Animal Husbandry and Veterinary Medicine(Lanzhou, China). It is broadly used as a medicinal feed additive in China due to its growth-promoting, low toxicity and rapid excretion in animals [1,2]. It is known to us all, the analogs of QCT such as carbadox (CBX) and olaquindox (OLA), are banned or strictly limited for the use in food-producing animals because of their genetic or potential toxicities [3,4]. Although some toxicity tests of QCT prototype show that the toxicity of QCT is significantly lower than that of CBX and OLA [1,2,5], it has been proved that quinoxalines metabolites (such as CBX and OLA) are closely associated with their toxicities [6,7]. Metabolic studies showed that QCT could be metabolized extensively into several metabolites like its analogs (CBX, OLA, cyadox and mequindox) because of their great similarity in structure [8–11], and these metabolites are potentially toxic to animals or further endanger human health. To our knowledge, no studies have been published to demonstrate which is the marker residue of QCT and its maximum residue limit (MRL) has not yet been established. Therefore, it is necessary to accurately determine and analyze QCT and its main metabolites for pharmacokinetic study and food safety concerns.

Up to now, many studies about determination of quinoxaline 1,4-dioxide, such as CBX, OLA, cyadox and mequindox and their related metabolites in animal tissues or plasma have been reported [12–15], and however, the simultaneous determination of QCT and its major metabolites in animal edible tissues was seldom reported. In order to clarify its metabolism and residue levels in swine and chicken, Huang et al. [16] developed a liquid chromatographic method for the determination of QCT and its major metabolites, such as desoxyquinocetone and 3-methyl-quinoxaline-2-carboxylic acid (MQCA) in edible swine and chicken tissues, respectively. The limits of detection (LOD) were above 50 $\mu g\,kg^{-1}$ for all three target analytes. Fang et al. [17] established a sensitive and quantitative liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for the simultaneous determination of QCT and its major two metabolites (1-desoxyquinocetone, Q_2 and di-deoxyquinocetone, Q_6) in chicken liver and muscle tissues. It was shown that the quinocetone and its metabolite Q_2 and Q_6 residues were found only in chicken liver, and the detection limits for all the compounds were below 3 $\mu g\,kg^{-1}$. However, on the basis of our preliminary experiments, MQCA and carbonyl reduced metabolite from di-deoxyquinocetone (Q_{43}) could be detected out in chicken muscle, liver, kidney and fat tissues.

The present paper described a convenient and rapid sample preparation procedure for the simultaneous determination of QCT and its four major metabolites including Q_2, Q_6 Q_{43} and MQCA

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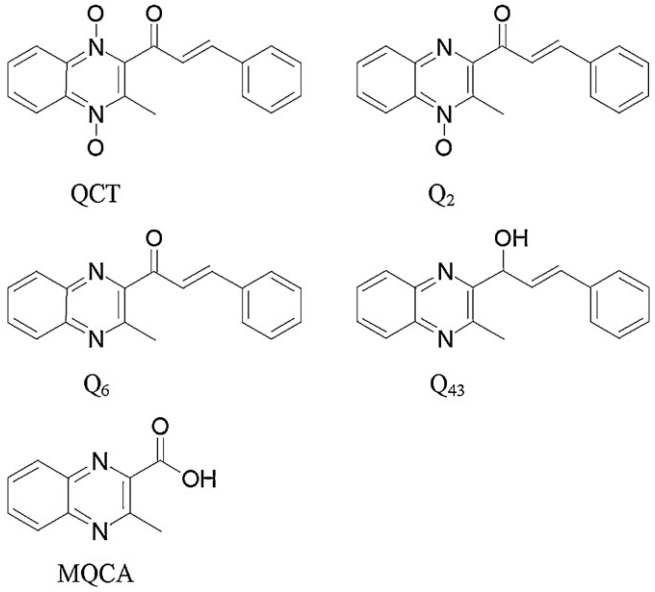

Fig. 1. Chemical structures of quinocetone and its major metabolites. QCT, Q_2, Q_6, Q_{43} and MQCA represent quinocetone, 1-desoxyquinocetone, di-deoxyquinocetone, di-deoxyquinocetone, and 3-methyl-quinoxaline-2-carboxylic acid, respectively.

(Fig. 1) residues in chicken edible tissues by LC–MS/MS. The method developed would facilitate the further pharmacokinetic study and to find the marker residue of QCT.

2. Experimental

2.1. Reagents and standards

QCT, Q_2, Q_6 and Q_{43} were provided by Institute of Veterinary Pharmaceuticals, Huazhong Agricultural University (Wuhan, China). MQCA was purchased from Sigma–Aldrich (Milwaukee, USA). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Fair lawn, NJ, USA). Oasis MAX (60 mg 3 mL^{-1}) solid phase extraction (SPE) cartridges were purchased from Waters Corp. (Milford, MA, USA). All the other reagents used in the experiment were of analytical grade and supplied by DAMAO Chemical Reagent Factory (Tianjin, China). Deionized water (Milli-Q; Millipore, Bedford, MA, USA) was used through this study.

Stock standard solutions (1.0 mg mL^{-1}) of the five drugs were prepared by dissolving each of the compound 10 mg in 1 mL dimethyl sulfoxide, and then add acetonitrile to 10 mL. Working mixed standard solution (10 μg mL^{-1}) was prepared weekly by diluting the stock standard solutions with acetonitrile. All solutions were kept in brown container and stored at 4 °C.

2.2. Apparatus

The chromatographic system composed of an Agilent 1200 series HPLC system, including quaternary pump and autosampler (Milford, MA, USA). The mass spectrometer included Applied Biosystems API 4000 triple quadrupole mass spectrometer with electrospray ionization (ESI) interface and Analyst 1.5 software (Foster City, CA, USA). MR23i Benchtop High-speed refrigerated centrifuge was purchased from Thermo Fisher Scientific (Oelde, Germany); KQ-100DE Ultrasonic generator was purchased from Kunshan Instruments Inc. (Kunshan, China).

2.3. Sample extraction and cleanup

An amount of 2.00 (±0.05) g chicken tissue homogenate sample was weighed into a 50 mL polypropylene centrifuge tube. Add 10 mL of chloroform/acetonitrile (1:2, v/v) into the tube, the sample was vortexed for 30 s, then sonicated for 10 min, and finally centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatant was collected into a 50 mL pear-shaped bottle. The residues were re-extracted with 5 mL of chloroform/acetonitrile (1:2, v/v). All supernatants (organic layer) were combined in the bottle.

Then add 1 mL of 1 mol L^{-1} hydrochloric acid into the sediments, the tube was vigorously vortexed for 1 min and placed in water bath at 90 °C for 1 h (hydrolysing the bound and conjugated metabolites). When cooled to room temperature, the mixture was sonicated for 5 min with 10 mL of chloroform/acetonitrile (1:2, v/v) and centrifuged at 10,000 × *g* for 10 min at 4 °C. Collect the organic layer into the same pear-shaped bottle and evaporate to dryness under a stream of nitrogen at 40 °C. The residue was dissolved in 6 mL of methanol/water solution (1:5, v/v). After being frozen for 1 h at 4 °C, the solution was centrifuged for 10 min at 10,000 × *g*, and then the supernatant was transferred to another 15 mL centrifuge tube, for further purification on Oasis MAX SPE cartridge.

The MAX cartridge was pre-conditioned with 3 mL methanol, followed by 3 mL water, then loaded the sample solution and washed with 3 mL water. After drying the cartridge under a weak vacuum, the analytes were eluted with 1 mL formic acid/acetonitrile (2:98, v/v) into a 1.5 mL sharp-bottomed centrifuge tube. The eluates were centrifuged at 15,000 × *g* for 10 min and the supernatant was filtered to brown microvials through 0.22 μm syringe filter for LC–MS/MS analysis.

2.4. LC–MS/MS analysis

The chromatographic separation was accomplished with gradient elution on a Luna C_{18} (150 × 2.0 mm i.d., 5 μm) column, which was purchased from Phenomenex (Torrance, CA). The mobile phase consisted of 0.1% formic acid solution (A) and acetonitrile (B). The detailed linear gradient elution program was as follows: 0–2.0 min: 98–20% A, 2–80% B; 2.0–8.0 min: 20% A, 80% B; 8.0–9.0 min: 20–98% A, 80–2% B; 9.0–15.0 min: 98% A, 2% B. An injection volume of 10 μL and a flow rate of 0.25 mL min^{-1} were used in LC–MS/MS analysis.

The mass analyses were performed using an electrospray source in positive ionization mode. The operation conditions were as follows: ion spray voltage, 4.2 kV; source temperature at 650 °C; curtain gas, 20 psi; ion source gas 1, gas 2 at 55 psi and 60 psi, respectively. Multiple reaction monitoring (MRM) experiments were carried out. The optimization of main mass spectrometric parameters was performed by flow injection analysis for each compound. Table 1 shows the optimized parameter values used for the confirmation and quantification of quinocetone and its major metabolites (Q_2, Q_6, Q_{43} and MQCA).

2.5. Method validation

The method was validated in accordance with the criteria described in Commission Decision 2002/657/EC [18]. On the basis of the criteria, various parameters such as specificity, linearity, decision limit (CCα), detection capability (CCβ), accuracy (measured as mean recovery), precision (expressed as relative standard deviation, RSD) and stability test were evaluated.

2.5.1. Specificity

The specificity was checked by analyzing 20 blank chicken muscle, liver, kidney and fat samples from different sources to evaluate possible endogenous interferences. The results were evaluated by

Table 1
The optimized parameter values used for the confirmation and quantification of quinocetone and its major metabolites.

Compound	Precursor ion m/z	Production ions m/z	DP (V)	CE (eV)
QCT	307.1	273.1[a]	90	28
		131.1	90	35
Q_2	291.1	159.0[a]	85	30
		245.2	85	30
Q_6	275.2	143.1[a]	80	36
		247.2	86	27
Q_{43}	277.1	259.3[a]	47	23
		145.0	65	40
MQCA	189.1	145.3[a]	60	21
		171.1	55	15

Note: CE represents collision energy; QCT, Q_2, Q_6, Q_{43} and MQCA represents quinocetone, 1-desoxyquinocetone, di-deoxyquinocetone, di-deoxyquinocetone, and 3-methyl-quinoxaline-2-carboxylic acid, respectively.

[a] Represents quantitative ion; precursor ions are $[M+H]^+$; DP represents declustering potential.

the presence of interfering substances around the analyte retention time.

2.5.2. Matrix-matched calibration curve

The matrix-matched calibration curves were prepared and used for quantification and testing the linearity of the method developed. Blank tissues were prepared as described above. The eluates from blank chicken muscle and fat tissues were used to prepare the concentrations of 1.0, 2.5, 5.0, 10, 25, 50 and 100 $\mu g\,kg^{-1}$; and the eluates from blank chicken liver and kidney were prepared the concentrations of 1.5, 5.0, 10, 25, 50, 100 and 250 $\mu g\,kg^{-1}$, respectively. Four replicates of each concentration were performed and repeated on each of three days. The calibration curves were generated by using the peak area of analyte versus the corresponding concentration in the matrix solution. The acceptance criterion was that the coefficient of correlation (r^2) must be more than 0.99.

2.5.3. CCα and CCβ

The CCα is the lowest concentration at which a method can discriminate with a statistical certainty of $1-\alpha$ that the analyte is present. In this case, CCα was established by the following: 20 blank matrix samples of chicken muscle, liver, kidney and fat were analyzed and the *S/N* is calculated at the time window in which the analyte is expected. CCα values were defined as three times of *S/N*. The CCβ is the concentration at which the method is able to detect truly contaminated samples with a statistical certainty of $1-\beta$. CCβ was calculated by analyzing 20 blank chicken muscle, liver, kidney and fat samples spiked with the analytes at CCα and then the CCα value plus 1.64 times the corresponding standard deviation (SD) is equal to CCβ ($\beta = 5\%$).

2.5.4. Accuracy and precision

The accuracy and the precision of the method were evaluated from spiked tissue samples at three concentration levels. An aliquot of 0.1 mL of the mixed standard solution (0.050, 0.50 or 2.00 $\mu g\,mL^{-1}$) was added into blank chicken muscle and fat samples in order to obtain the spiked levels at 2.50, 25.0 and 100 $\mu g\,kg^{-1}$. 0.1 mL of the mixed standard solution (0.100, 1.00 or 5.00 $\mu g\,mL^{-1}$) was added into blank liver and kidney samples in order to obtain the spiked levels at 5.00, 50.0 and 250 $\mu g\,kg^{-1}$. Five replicates for each concentration level were performed and repeated in three consecutive days. The extraction recoveries of QCT, Q_2, Q_6, Q_{43} and MQCA at the spiked samples were determined by measuring the peak area response from samples spiked with particular working solution of QCT and metabolites before extraction with those from blank tissues samples extracted and spiked with same concentration of analytes after extraction.

2.5.5. Stability test

The stability of five analytes in chicken tissues extracts and neat solvent at room temperature and 4 °C was assessed within a week. The measured values were compared with those freshly prepared pure solvent or matrix standard solutions at 10 $\mu g\,kg^{-1}$ concentration level in triplicate.

2.6. Incurred samples validation

Incurred samples were obtained from 72 chickens fed with QCT for 7 consecutive days at a dose of 50 $mg\,kg^{-1}$ b.w. (oral), twice a day. Six chickens were killed at the time of 4, 6, 12, 24, 72, 120, 168, 216, 264, 336, 408 and 504 h after last administration. Tissues of muscle, liver, kidney and fat were obtained from each chicken. Controlled samples were obtained from 6 chickens fed normally for 7 days. All samples were homogenized (chicken kidney was collected in bulk) in a high-speed food blender and then frozen at −20 °C until analysis. The samples were then prepared and analyzed using the described LC–MS/MS method.

3. Results and discussion

3.1. Optimization of instrumental conditions

On the basis of structures of QCT and its major metabolites such as Q_2, Q_6 and Q_{43}, which are neutral or weak basic compounds, therefore, the positive electrospray ionization mode should be selected in LC–MS/MS analysis. As containing a carboxyl group within MQCA molecule, the negative electrospray ionization mode is generally more sensitive than the positive ion mode. But it is experimental that MQCA is also enough sensitive in the positive mode. For the simultaneous determination of QCT, Q_2, Q_6, Q_{43} and MQCA, and avoiding the loss of sensitivity due to switch between the positive and negative mode, the five compounds are expected to be monitored in the same ionization mode. So the positive ion mode was finally selected for the simultaneous determination of the five compounds in this study. Firstly, MS parameters for the target analytes were optimized in positive electrospray ionization full scan mode. In a second phase, the MS/MS conditions were adjusted in collision-induced dissociation (CID) mode under various collision energies. The five analytes are preferably ionized in the positive mode, and form a stable $[M+H]^+$ ion. Therefore, $[M+H]^+$ molecular ions, which were predominant for QCT, Q_2, Q_6, Q_{43} and MQCA were selected as the precursor ions. Two MRM transitions were used for confirmation and the most intense MRM transition was selected for quantification (Table 1).

QCT, Q_2, Q_6 and Q_{43} are weak basic compounds, and their retention times are not very relevant with the pH value of the mobile phase. MQCA is weak acidic molecule containing carboxyl group in its structure, and its retention time is affected by the pH value of the mobile phase. The simple water-acetonitrile (or methanol) mobile phase system has a pH of around 7, in which, MQCA will partially dissociate carboxyl group to weakly anion so that it is not capable of being well retained in C_{18} column. Based on our trials, when the water mobile phase containing 0.1% formic acid and pure acetonitrile solvent was employed, the five target analytes were separated well and good peak shapes for all of the analytes were achieved under the optimized gradient elution program.

3.2. Samples preparation

On basis of the structures of the analytes and their solubility properties, several organic reagents, such as ethyl acetate, chloroform and acetonitrile were chosen as solvents of extraction. When

Table 2
Calibration curves for five compounds in four chicken tissues.

Matrix	Analytes	Concentration ranges ($\mu g\,kg^{-1}$)	Regression equations $Y=(\text{slope}\pm\text{SD})\cdot X+(\text{intercept}\pm\text{SD})$	Correlation coefficients (r^2)
Muscle	QCT	1.0–100	$Y=(4.29\pm0.21)\times10^3\cdot X+(1.60\pm0.09)\times10^4$	0.9954
	Q2	1.0–100	$Y=(9.70\pm0.62)\times10^3\cdot X-(5.98\pm0.34)\times10^3$	0.9933
	Q6	1.0–100	$Y=(2.20\pm0.07)\times10^4\cdot X+(4.94\pm0.42)\times10^3$	0.9999
	Q43	1.0–100	$Y=(1.10\pm0.13)\times10^5\cdot X+(2.70\pm0.31)\times10^5$	0.9957
	MQCA	1.0–100	$Y=(2.05\pm0.24)\times10^4\cdot X-(4.71\pm0.53)\times10^3$	0.9997
Fat	QCT	1.0–100	$Y=(4.15\pm0.28)\times10^3\cdot X+(1.07\pm0.13)\times10^3$	0.9991
	Q2	1.0–100	$Y=(1.08\pm0.07)\times10^4\cdot X+(7.03\pm1.01)\times10^3$	0.9981
	Q6	1.0–100	$Y=(2.36\pm0.18)\times10^4\cdot X-(3.47\pm0.64)\times10^3$	0.9999
	Q43	1.0–100	$Y=(1.22\pm0.03)\times10^5\cdot X+(1.03\pm0.12)\times10^5$	0.9976
	MQCA	1.0–100	$Y=(1.30\pm0.33)\times10^4\cdot X+(1.37\pm0.16)\times10^4$	0.9991
Liver	QCT	1.5–250	$Y=(6.13\pm0.36)\times10^3\cdot X+(2.36\pm0.14)\times10^4$	0.9984
	Q2	1.5–250	$Y=(1.17\pm0.06)\times10^4\cdot X+(3.72\pm0.38)\times10^4$	0.9987
	Q6	1.5–250	$Y=(3.89\pm0.17)\times10^4\cdot X+(4.11\pm0.31)\times10^4$	0.9972
	Q43	1.5–250	$Y=(1.88\pm0.09)\times10^5\cdot X+(3.69\pm0.24)\times10^5$	0.9981
	MQCA	1.5–250	$Y=(2.37\pm0.14)\times10^4\cdot X-(2.19\pm0.23)\times10^4$	0.9994
Kidney	QCT	1.5–250	$Y=(3.59\pm0.23)\times10^3\cdot X+(8.66\pm0.83)\times10^3$	0.9989
	Q2	1.5–250		0.9951
	Q6	1.5–250	$Y=(1.71\pm0.12)\times10^4\cdot X+(1.03\pm0.02)\times10^4$	0.9996
	Q43	1.5–250	$Y=(5.89\pm0.41)\times10^4\cdot X+(2.93\pm0.11)\times10^5$	0.9968
	MQCA	1.5–250	$Y=(9.10\pm0.35)\times10^3\cdot X+(4.59\pm0.28)\times10^4$	0.9954

Note: SD represents standard deviation ($n=12$).

the three kinds of organic solvents were used separately for extraction of the analytes from chicken tissues, a similar extraction effect was obtained for chloroform and ethyl acetate. They could extract more than 90% of Q_2 and Q_6, but QCT and Q_{43} were less than 70%. Acetonitrile could extract 90% of QCT and Q_{43}, but the recovery rates were low for Q_2 and Q_6, only about 50%. So a mixture of acetonitrile and chloroform or ethyl acetate was tested. When acetonitrile and ethyl acetate were used for extraction together, the recovery rates of most analytes reached 85%, but Q_{43} was about 60%. While the mixture of chloroform and acetonitrile was used, the recovery rate of Q_{43} improved obviously. Thus, the different ratio of chloroform to acetonitrile as extractive solvent, further experiments were carried out. The ideal extraction solvent was the mixture of chloroform/acetonitrile (1:2, v/v). Under this condition, the mean recoveries of the four analytes including QCT, Q_2, Q_6 and Q_{43} were more than 85% (Fig. 2).

Considering some MQCA might exist in the tissues as its conjugated form, enzymatic digestion procedure was firstly tested. Because more complex sample matrix solution was produced after digestion, the extraction recovery of MQCA was lower than that obtained by hydrolyzing with inorganic strong acid. Therefore, in this study we chose to hydrolyze chicken tissues under acidic conditions and then extract MQCA (including bound and unbound) from the solution. Since MQCA is weak acid, its recovery was very low if chloroform/acetonitrile (1:2, v/v) is as extractive solvent. It was experimentally found that high recovery of MQCA could be obtained when hydrochloric acid-chloroform/acetonitrile (1:2, v/v) was used. However, the recoveries of the metabolites, including Q_2, Q_6 and Q_{43} were very low. So a reasonable pretreatment procedure was as follows: firstly, extract QCT, Q_2, Q_6 and Q_{43} with chloroform/acetonitrile (1:2, v/v), and then extract MQCA with hydrochloric acid-chloroform/acetonitrile (1:2, v/v). Thus, the recoveries of five target analytes were more than 85%.

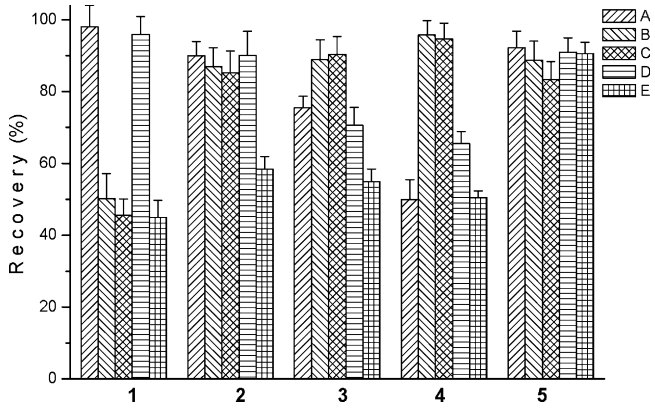

Fig. 2. Recoveries of QCT, Q_2, Q_6, Q_{43} and MQCA extracted by different solvent systems from muscle samples spiked at 25 $\mu g\,kg^{-1}$. A, B, C, D and E represent QCT, Q_2, Q_6, Q_{43} and MQCA, respectively. ACN, CF and HCl represent acetonitrile, chloroform and hydrochloric acid, respectively; M represents mol L^{-1}; 1, 2, 3, 4, and 5 represent ACN, CF:ACN (1:2), CF:ACN (1:1), CF:ACN (2:1), CF:ACN (1:2) → 1 M HCl + CF:ACN (1:2), respectively. The error bars represent standard deviations ($n=3$).

In order to remove lipids and other lipophilic impurities co-extracted with target analytes from chicken tissues, a further defatted step is necessary before SPE cleanup. Due to its strong lipophilicity, the recovery of target compound Q_6 is seriously affected by the traditional defatted method using hexane or chloroform. It was found that good recoveries were obtained for all five analytes when the defatted procedure by freezing was used.

Oasis HLB and MAX cartridges were investigated for SPE cleanup. It was shown that low recovery (below 50%) of Q_6 was obtained with the HLB cartridge, and high recoveries (above 95%) and good cleanup efficiency were obtained for all analytes when the MAX cartridge was used. Therefore, the MAX cartridge was selected for the following experiment. Different volumes and percentages of formic acid in acetonitrile were used to elute the analytes. The results showed that the target analytes could be completely eluted from the cartridge using 1 mL of 2% formic acid in acetonitrile and resulting in the least matrix interference.

3.3. Method performance characteristics

3.3.1. Specificity

The specificity of the method was evaluated by analyzing different blank tissues samples. Compared with the background noise in chicken edible tissues matrices, the results demonstrated that, there were no interfering peaks that could be detected within the 2.5% margin of the relative retention time of these target analytes. The typical MRM chromatograms of blank and spiked chicken muscle sample are showed in Fig. 3.

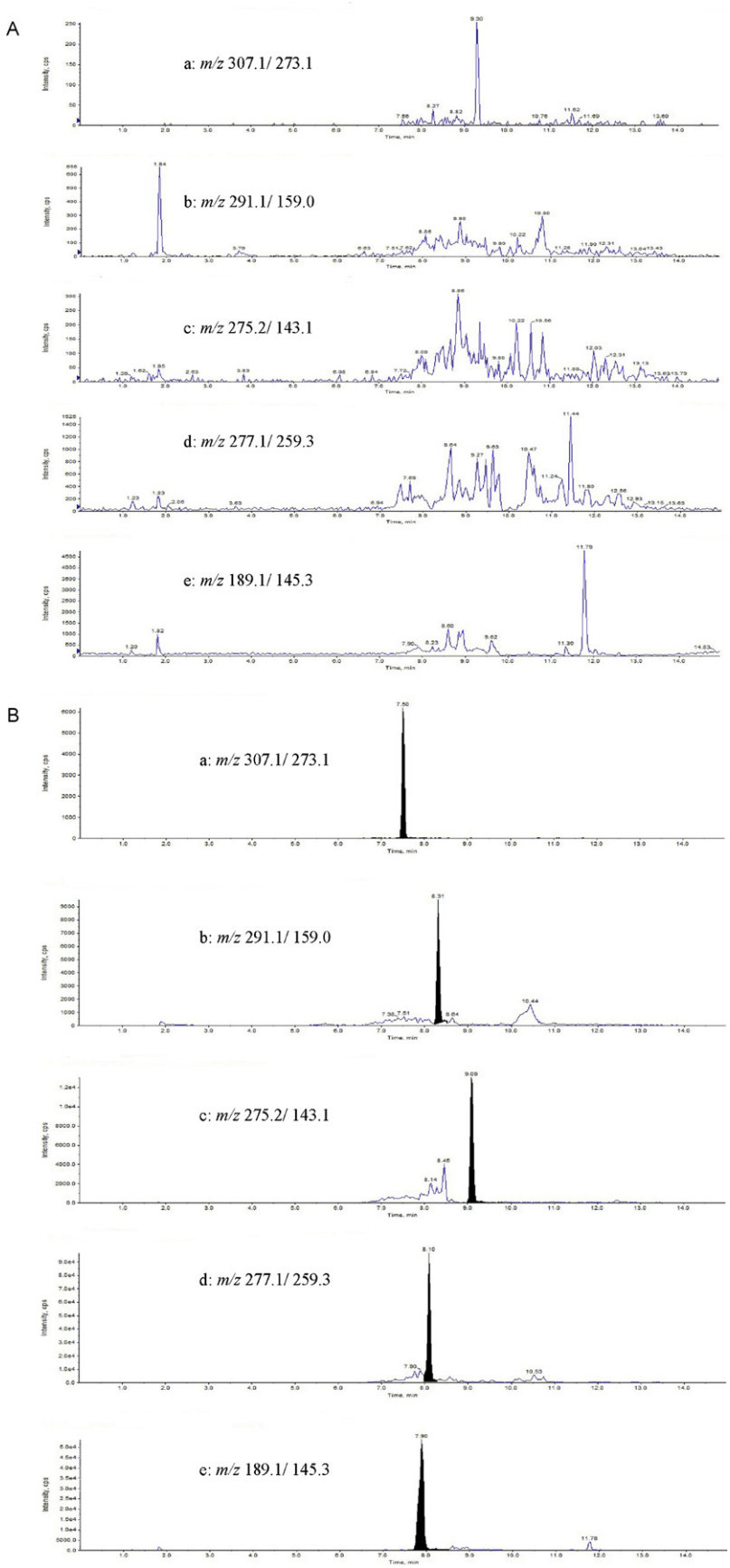

Fig. 3. Typical MRM chromatograms of blank chicken muscle (A) and spiked chicken muscle sample at the level of 2.5 μg kg^{-1} (B). a, b, c, d and e represent QCT, Q_2, Q_6, Q_{43} and MQCA, respectively.

Table 3
Decision limit (CCα) and detection capability (CCβ) of the method in chicken edible tissues.

Compound	Muscle ($\mu g\,kg^{-1}$)	Fat ($\mu g\,kg^{-1}$)	Liver ($\mu g\,kg^{-1}$)	Kidney ($\mu g\,kg^{-1}$)
QCT				
CCα	0.37	0.28	0.41	0.27
CCβ	1.15	0.95	1.25	0.85
Q_2				
CCα	0.47	0.36	0.24	0.60
CCβ	1.54	1.20	0.80	1.39
Q_6				
CCα	0.76	0.29	0.59	0.43
CCβ	2.34	0.96	1.69	1.43
Q_{43}				
CCα	0.52	0.48	0.44	0.61
CCβ	1.94	1.59	1.46	2.03
MQCA				
CCα	0.34	0.42	0.56	0.49
CCβ	1.06	1.31	1.62	1.44

Table 4
Accuracy and precision of the method for quinocetone and its major metabolites in spiked chicken muscle and fat tissues.

Compound	Spiked level ($\mu g\,kg^{-1}$)	Muscle			Fat		
		Recovery (%)	Intra-day RSD, %	Inter-day RSD, %	Recovery (%)	Intra-day RSD, %	Inter-day RSD, %
QCT	2.50	95.2	8.3	8.9	88.4	4.2	4.1
	25.0	85.9	10.2	9.8	79.7	8.7	8.4
	100	81.4	6.5	6.7	79.8	5.2	5.6
Q_2	2.50	90.4	10.2	10.7	81.0	6.9	6.7
	25.0	77.1	10.3	10.6	83.5	8.9	10.2
	100	82.0	7.8	7.6	81.1	5.0	5.2
Q_6	2.50	90.2	4.2	4.3	80.4	5.9	5.7
	25.0	79.0	7.1	8.1	80.8	4.6	5.3
	100	81.3	8.2	9.0	81.4	9.0	9.1
Q_{43}	2.50	88.4	11.4	10.6	79.4	5.4	5.6
	25.0	81.5	9.7	9.4	82.5	7.2	7.0
	100	80.9	4.3	4.2	80.9	9.1	8.7
MQCA	2.50	93.2	4.8	4.4	90.2	5.7	5.4
	25.0	90.4	3.9	4.1	89.7	4.8	4.2
	100	88.6	5.8	5.6	92.6	7.3	6.9

Note: RSD represents relative standard deviation ($n = 5$).

3.3.2. Matrix-matched calibration curve

Matrix-matched calibration curves were obtained by least-squares regression, and used to test the linearity of the method by applying the extraction procedure to the analysis of spiked tissues at seven different concentrations ranged from 1.00 to 100 $\mu g\,kg^{-1}$ in chicken muscle and fat samples, from 1.5 to 250 $\mu g\,kg^{-1}$ in liver and kidney samples. It was shown that the method was good linearity over the experimental concentration ranges and the correlation coefficients (r^2) were higher than 0.99 for all the analytes. The detailed data of calibration curves for five compounds in four chicken tissues matrixes are shown Table 2.

Table 5
Accuracy and precision of the method for quinocetone and its major metabolites in spiked chicken liver and kidney tissues.

Compound	Spiked level ($\mu g\,kg^{-1}$)	Liver			Kidney		
		Recovery (%)	Intra-day RSD, %	Inter-day RSD, %	Recovery (%)	Intra-day RSD, %	Inter-day RSD, %
QCT	5.00	92.7	8.0	8.0	94.3	5.2	6.6
	50.0	83.1	6.4	6.2	93.7	6.6	7.1
	250	84.4	5.6	5.9	84.6	5.2	4.9
Q_2	5.00	85.9	6.0	6.0	80.1	9.1	9.5
	50.0	81.1	8.1	7.8	85.8	7.5	7.7
	250	81.4	8.2	8.9	80.6	6.4	6.2
Q_6	5.00	89.8	7.1	6.9	83.6	3.9	3.8
	50.0	81.0	10.2	10.3	85.6	5.4	5.4
	250	80.4	6.2	6.3	80.3	9.6	9.5
Q_{43}	5.00	91.4	7.6	7.5	90.0	7.3	7.3
	50.0	82.1	4.0	4.3	83.3	4.6	4.5
	250	84.3	6.5	6.6	81.5	7.5	7.6
MQCA	5.00	94.2	4.9	5.2	94.2	3.2	3.0
	50.0	92.7	4.1	3.7	90.1	5.3	5.6
	250	90.8	6.8	6.4	92.5	4.8	4.6

Note: RSD represents relative standard deviation ($n = 5$).

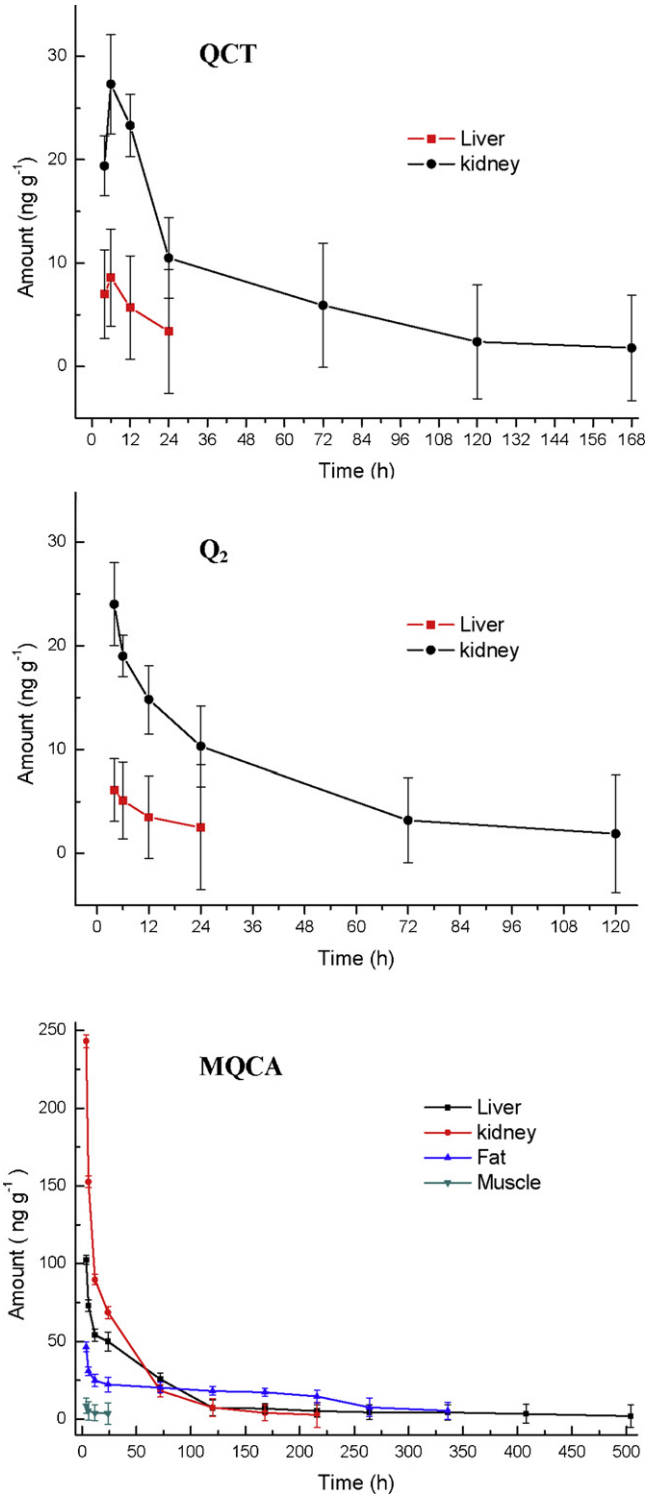

Fig. 4. The elimination curves of QCT, Q_2 and MQCA in chicken liver and kidney after QCT was administrated to chickens for 7 days at a dose of 50 mg kg^{-1} b.w. (oral), twice a day. The error bars represent standard deviations ($n = 3$).

3.3.3. CCα and CCβ

As shown in Table 3, under the conditions specified in the method, the CCαs of five compounds in chicken muscle, liver, kidney and fat tissues ranged from 0.24 to 0.76 μg kg^{-1}, and the values of CCβ ranged from 0.80 to 2.34 μg kg^{-1}. Compared with the published methods, the higher sensitivity was obtained for QCT, Q_2, Q_6, Q_{43} and MQCA.

3.3.4. Accuracy and precision

Mean recoveries for the five compounds were estimated by comparing the spiked sample with the matrix-matched standard solutions. Data are presented in Table 4 and Table 5. At the spiked three levels of 2.50, 25.0 and 100 μg kg^{-1}, the mean recoveries of QCT, Q_2, Q_6, Q_{43} and MQCA in chicken muscle and fat tissues were from 77.1% to 95.2%. The intra-day RSD was from 3.9% to 11.4% and the inter-day RSD was below 15% for the five analytes. At the spiked three levels of 5.00, 50.0 and 250 μg kg^{-1}, the mean recoveries of QCT, Q_2, Q_6, Q_{43} and MQCA in chicken liver and kidney tissues were from 80.1% to 94.3%. The intra-day RSD was between 3.2% and 10.2%, and the inter-day RSD was below 15% for all analytes of interest. It was shown that the accuracy and the precision of the method developed are acceptable to the residues analysis.

3.3.5. Stability

The results of stability test showed that under the conditions at 4 °C, QCT, Q_{43} and MQCA in chicken tissue extracts were stable within 5 days, in pure solvent no degradation was observed during a week. While Q_2 and Q_6 in tissue extracts remain stable in 3 days and in pure solvent for 4 days. In room temperature, QCT, Q_{43} and MQCA remained stable in tissue extracts for 4 days and in pure solvent for 6 days; Q_2 and Q_6 could remain stable only 2 days in tissue extracts and in pure solvent for 3 days. Therefore, the prepared samples must be injected within 2 days for good quantification.

3.4. Application to real samples

In order to check the applicability of the proposed method, the analytical procedures described above were used to extract and determine QCT and its major metabolites such as Q_2, Q_6, Q_{43} and MQCA in the incurred chicken tissues (muscle, liver, kidney and fat). The results show that the residue concentrations of chicken liver were the highest and those of chicken muscle were the lowest. QCT and its metabolites, including Q_2 and MQCA could be detected in all four chicken tissues. However, Q_6 could be detected only in chicken liver and kidney, and Q_{43} was detected only in chicken liver. Moreover, Low residues and rapid depletion of Q_6 and Q_{43} were observed in chicken liver and (or) kidney (undetectable after 6 h of the last administration). QCT and Q_2 could be detected in kidney at 168 h and 120 h after the last administration, respectively. It is doubtful that the quinocetone and its two metabolite (Q_2 and Q_6) residues were found only in chicken liver [17]. Although MQCA existed in four tissues of chicken muscle, liver, kidney and fat, it demonstrated tissue selectivity, with lowest residue level and shortest depletion time in muscle, highest residue level in kidney and longest depletion time in liver. The depletion of MQCA in chicken kidney was more rapid than that in liver, where it could be even detected after a withdrawal time of 21 days. After QCT was administrated to chickens for 7 days curves of concentration-time of QCT, Q_2 and MQCA in liver and kidney are shown in Fig. 4.

4. Conclusions

In conclusion, a convenient, rapid and sensitive LC–MS/MS method was developed for the simultaneous determination of quinocetone and its four metabolites (1-desoxyquinocetone, di-deoxyquinocetone, di-deoxyquinocetone, and 3-methyl-quinoxaline-2-carboxylic acid) in the incurred chicken muscle, liver, kidney and fat tissues. The method was rigorously validated over a wide concentration range (1.00–250 μg kg^{-1}). The CCαs of the five compounds in chicken edible tissues ranged from 0.24 to 0.76 μg kg^{-1}, and the CCβs were below 2.34 μg kg^{-1}. It is suggested that 3-methyl-quinoxaline-2-carboxylic acid might be as the marker residue for quinocetone in chicken edible tissues. The proposed method will be applied for the residue surveillance

and food safety evaluation of quinocetone and its metabolites in chicken edible tissues.

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