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Determination of tetracyclines residues in honey using high-performance liquid chromatography with potassium permanganate–sodium sulfite–β-cyclodextrin chemiluminescence detection

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Abstract

A novel method was developed for the simultaneous determination of tetracycline antibiotic (TCA) residues such as oxytetracycline (OTC), tetracycline (TC), and metacycline (MTC) by high-performance liquid chromatography (HPLC) coupled with chemiluminescence (CL) detection. The procedure was based on the chemiluminescent enhancement by TCAs of the potassium permanganate–sodium sulfite–β-cyclodextrin system in a phosphoric acid medium. The separation was carried out with an isocratic elution using a mixture of acetonitrile and 0.001 M phosphoric acid. For the three TCAs, the detection limits at a signal-to-noise of 3 ranged from 0.9 to 5.0 ng/ml. The relative standard deviations for the determination of TCAs ranged from 3.1 to 7.4% within a day ($n = 11$) and ranged from 2.2 to 8.6% in 3 days ($n = 9$), respectively. The method was successfully applied to the determination of TCA residues in honey samples. The possible mechanism of the CL reaction was also discussed.

Keywords: Tetracycline antibiotics; HPLC; Chemiluminescence; Residues; Honey

1. Introduction

Tetracycline antibiotics (TCAs) are commonly used both for the treatment of infectious diseases and as an additive to animal feeds for their broad-spectrum antibacterial activity and cost effectiveness. If recommendations for drug withdrawal are not respected or if veterinary drugs are used unlicensed, there is a significant risk of detecting TCA residues in honey, milk, and some edible animal tissues [1]. Moreover, TCAs can be added directly to plants in the orchard environment during blossom. The contamination of the blossom with high concentrations of antibiotic implies the risk of a carry-over of residues into honey [2]. Relatively high levels of TCA residues in food products present a potential hazard to the consumers in terms of allergic reaction and the development of bacterial resistance [3]. Therefore, regulatory authorities have established maximum residue limits (MRLs) for TCAs in food. Some countries do not have fixed MRLs for honey because TCAs are illegal for use with bees at any level, while some countries apply an action level of 50 ng/g.

Many methods have been described for the determination of TCAs such as microbiological assay [4,5], enzyme immunoassay [6], spectrophotometry [7], fluorimetry [8], electrochemical detection [9], flow-injection-chemiluminescence methods [10,11], high-performance liquid chromatography (HPLC) [12–23], and capillary electrophoresis (CE) [24]. However, few methods are applied to determine TCA residues in honey, mainly due to the lack of sufficient sensitivity for practical application and the interferences suffered from honey complex matrix. Currently, TCA residues in honey are determined mainly by microbiological assay [5], enzyme immunoassay [6], and chromatographic methods [17,21]. The official methods of assay in China are

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the bioassay approaches. These methods are not only expensive and time consuming but they also do not distinguish among TCAs, thus their sensitivity and specificity are limited. This is why methods now developed for TCA detection in honey are focused on chromatographic methods because they offer the advantages of better sensitivity and specificity.

TCA residues in various biological matrices can be determined using reversed-phase liquid chromatography (LC) with different detection modes such as spectrophotometry [12–17], fluorescence [18,19], mass spectrometry (MS) [20–22], and electrochemical detection [23]. Mass spectrometry can detect residual TCAs with high sensitivity and selectivity, but the instrumentation is expensive. In recent years, chemiluminescence (CL) has become an attractive detection method for liquid chromatography due to its high sensitivity and wide linear working ranges, which can be obtained with relatively simple instrumentation. However, to our knowledge, there has been no published report using HPLC-CL to detect TCA residues.

It was reported that the reaction between acidic potassium permanganate and sodium sulfite could give rise to chemiluminescence from 450 to 600 nm [25]. The mechanism of the CL reaction of sodium sulfite with acidic potassium permanganate [26] was suggested to be due to electronically excited state of sulphur dioxide molecules. In the present study, we found that the reaction of potassium permanganate with sodium sulfite in the presence of β-cyclodextrin could yield intensive CL and TCAs could strongly enhance the CL of potassium permanganate–sodium sulfite–β-cyclodextrin system. On this basis, a highly sensitive method was developed for the determination of TCA residues in honey by coupling HPLC with this CL reaction.

2. Experimental

2.1. Chemicals and solutions

Acetonitrile of HPLC grade and sodium sulfite anhydrous (Na_2SO_3) were from Beijing Chemicals Company (Beijing, China). Potassium permanganate ($KMnO_4$), β-cyclodextrin (β-CD), and phosphoric acid (H_3PO_4) were obtained from Shanghai Chemicals Company (Shanghai, China). Oxytetracycline (OTC), tetracycline (TC) and metacycline (MTC) were obtained from the Institute of Pharmaceutical and Biomaterial Authentication of China (Beijing, China). All other chemicals were of analytical-reagent grade.

OTC, TC, and MTC stock standard solutions (0.1 mg/ml) were prepared weekly using redistilled water and were stored at 4 °C in a refrigerator. A stock solution of β-CD (1×10^{-3} M) was prepared by dissolving 0.28375 g β-CD in 250 ml redistilled water. The solution of Na_2SO_3 (9.6×10^{-4} M) was prepared by dissolving Na_2SO_3 in 5×10^{-8} M β-CD. The solution of $KMnO_4$ (1.5×10^{-4} M) was prepared by dissolving $KMnO_4$ in 0.075 M phosphoric acid. All working solutions were freshly prepared each day with redistilled water. The HPLC mobile phases were freshly prepared each day, filtered through a 0.22-μm membrane filter (Xinya Company, Shanghai), and then degassed before use.

2.2. Instrumentation

The schematic diagram as described previously [27] illustrated the HPLC-CL detection system used in our experiments. The HPLC system was Agilent 1100 series (Agilent Technologies, USA), including a binary pump, a thermostat column compartment, a diode array and multiple wavelength detector (DAD), a manual sample valve injector with a 100-μl loop, and an analytical column (Zorbax Eclipse XDB-C_{18}, 150 mm × 2.1 mm I.D., 5 μm; Agilent Technologies, USA). CL detection was conducted on a flow-injection-chemiluminescence system (Remax, China) consisting of a model IFFM-D peristaltic pump, a mixing tee and a model IFFS-A CL detector equipped with a glass coil (used as reaction coil and detection cell), and a photomultiplier. The data from the CL detector were acquired by Agilent Interface 35900E and processed by Chemstation A.08.03 running on a DELL smartpc 100 personal computer. Fluorescence spectrum was recorded offline by LS55 fluorimeter (Hitachi, Japan) after correction. CL spectrum was obtained by inserting cut-off filters at wavelengths of 360, 380, 400, 420, 430, 470, 490, 510, 535, 550, 565, 580, 600, 630, and 650 nm (light cannot pass at wavelengths lower than these values) between the detection cell and a photomultiplier in flow injection CL system [28].

2.3. Procedure

TCAs were separated by XDB-C_{18} column at 25 °C with an isocratic elution program at a flow rate of 0.5 ml/min. The mobile phase consisted of acetonitrile (A) and 0.001 M phosphoric acid (B). The isocratic elution program was 16% A for 11 min. The UV–vis detector was set at 274 nm for OTC and TC and 350 nm for MTC. Typical retention time of OTC, TC, and MTC was 2.6, 3.2, and 10.3 min, respectively. Solutions of $KMnO_4$ and Na_2SO_3 were combined with the peristaltic pump at a flow rate of 1.8 ml/min, respectively, and then mixed with the column effluent from DAD at a mixing tee via a PEEK tube (600 mm × 0.25 mm I.D., Agilent Technologies). Light emission was monitored by the photomultiplier tube. The quantitative determination was based on the relative CL intensity $\Delta I = I_S - I_0$, where I_S was the CL intensity of TCA compounds and I_0 was the intensity of blank signal.

2.4. Sample preparation

For extraction of the honey sample, 50 g (accurate to 0.1 g) of the sample was weighted and dissolved in 200 ml of 2% citric acid buffer solution (pH 4.0). The pH was adjusted to 4.5 with 2% citric acid or 40% NaOH solu-

tion, and 60 ml of pH 4.5 phosphate buffer solution (PBS, 0.1 M) was added. The sample solutions were poured into the chromatographic column packed with XAD-2 resin at a rate of 100 ml/h, and the tetracycline residues were adsorbed by the resin on the column. The tetracycline residues adsorbed were washed with 200 ml of water by controlling the flow rate of the water to 150 ml/h. Finally, the TCA residues eluted with 90 ml of 60% methanol, with a flow rate of 90 ml/h. The eluent was ready for use after it was transferred into a round-bottomed flask, concentrated at 40 °C under reduced pressure to dryness, then dissolved and diluted to 100 ml with water. The recovery experiments were spiked 50 ng/ml OTC and TC and 250 ng/ml MTC in the honey sample. All values measured were the average values of three replicates.

3. Results and discussion

In acidic medium, $KMnO_4$ reacted with Na_2SO_3 to generate CL. TCAs, including OTC, TC, and MTC, enhanced the CL intensity of the $KMnO_4$–Na_2SO_3–β-CD system. The enhanced CL intensity was sensitive to a variety of factors such as solvent, pH, and HPLC mobile phases. The strongest CL intensity was obtained by optimization of the following conditions.

3.1. Optimization of HPLC system

As for HPLC-CL detection, the mobile phase of HPLC is not only suitable for the separation of MTC, OTC, TC, but also compatible with the CL reaction. Several mobile phases have been reported for the separation of TCAs on a RP-C_{18} column, such as acetonitrile–phosphoric acid–water [13,14], acetonitrile–acetate buffer–tetrahydrofuran [12], acetonitrile–oxalic acid–water [15,17], acetonitrile–citric acid–water [20], and methanol–formic acid–water [21]. The CL intensity was intensely inhibited by solvents such as methanol and oxalic acid, but the mobile phase of acetonitrile–phosphoric acid–water was found suitable for the separation of these compounds and compatible with the $KMnO_4$–Na_2SO_3–β-CD system in acidic medium. Under the condition of complete separation, the effects of phosphoric acid concentration and mobile phase composition on the CL intensity were studied.

The effects of phosphoric acid concentration ranged from 1×10^{-5} M to 1×10^{-2} M. When the concentration of phosphoric acid was 1×10^{-3} M, the separation was good, and the CL intensities of TCA compounds were almost maximal. The concentration of acetonitrile ranged from 8 to 25%. The chromatographic peaks of OTC and TC overlapped when the concentration of acetonitrile was greater than 19%, whereas, peak tailing of TCAs occurred with 10% acetonitrile and separation time was about 40 min. Good separation and maximal relative CL intensity were obtained when 16% was chosen for the acetonitrile concentration.

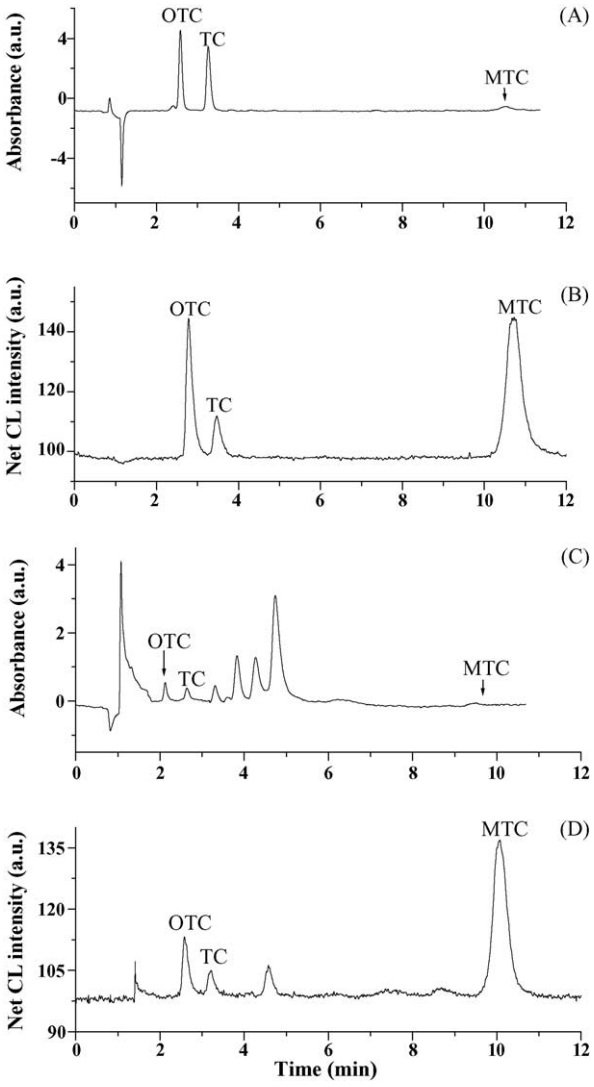

Fig. 1. Chromatograms of TCAs with DAD at 350 nm and CL detection. Separation condition: isocratic elution with acetonitrile and 0.001 M phosphoric acid (16:84, v:v). CL reaction condition: H_3PO_4: 0.075 M; $KMnO_4$: 1.5×10^{-4} M; Na_2SO_3: 9.6×10^{-4} M; β-CD:5×10^{-8} M; flow rate: 1.8 ml/min. (A) Chromatogram of a mixture of standard TCAs with DAD detection. (B) Chromatogram of a mixture of standard TCAs with CL detection. (A, B: TCAs: OTC, TC 250 ng/ml, MTC 50 ng/ml). (C) Chromatogram of honey sample with DAD detection. (D) Chromatogram of honey sample with CL detection. (C, D: honey sample was spiked with the TCAs: OTC, TC 50 ng/ml, MTC 250 ng/ml).

The chromatograms obtained from DAD and CL detector are shown in Fig. 1. Compared with DAD, CL detector was predominantly superior for the detection of OTC and MTC. The results demonstrated that the potassium permanganate–sodium sulfite–β-cyclodextrin system was highly compatible with the mobile phase of HPLC.

3.2. Optimization of CL system

In acid media, $KMnO_4$ can react with Na_2SO_3 to produce weak CL. TCAs were found to enhance the CL

intensity in $KMnO_4$–Na_2SO_3 solution. When β-CD was added to the system, both the CL intensity (S) and the noise (N) increased and the ratio of the peak height of CL signal-to-noise (S/N), i.e. relative CL intensity, also increased sharply. The kinetic characteristics of the CL enhancement by TCAs, for example MTC (200 ng/ml), of $KMnO_4$–Na_2SO_3 and $KMnO_4$–Na_2SO_3–β-CD systems were studied by a flow injection system under reaction conditions: 0.075 M H_3PO_4, 1.5×10^{-4} M $KMnO_4$, 9.6×10^{-4} M Na_2SO_3, 5×10^{-8} M β-CD, 1.8 ml/min flow rate. The results indicated that the enhancing signal of MTC was stronger in the $KMnO_4$–Na_2SO_3–β-CD system than that in the $KMnO_4$–Na_2SO_3. The S/N in the $KMnO_4$–Na_2SO_3 and the $KMnO_4$–Na_2SO_3–β-CD systems was 11.8 and 14.1, respectively. Thus, the $KMnO_4$–Na_2SO_3–β-CD system was chosen for the subsequent experiments. To obtain maximal net CL intensity, the effects of the concentrations of $KMnO_4$, Na_2SO_3, H_3PO_4, β-CD, and flow rate on net CL intensity were investigated.

There are some reports on the chemiluminescence of $KMnO_4$ in acid media [25]. Some acids (phosphoric acid, polyphosphate acid, sulfuric acid, hydrochloric acid, nitric acid, acetic acid) with 0.01 M concentration were examined and the CL intensity was found to be highest in phosphoric acid solution. The optimal concentration of H_3PO_4 was 0.075 M (Fig. 2). The effect of $KMnO_4$ concentration on the net CL intensity ranged from 7.5×10^{-5} M to 2.25×10^{-4} M in 0.075 M H_3PO_4. The maximum enhanced CL intensity was reached at a concentration of 1.5×10^{-4} M for TCAs tested (Fig. 3). With lower concentrations of $KMnO_4$, net CL intensity was weak. With higher concentrations of $KMnO_4$, net CL intensity decreased, which could be due to permanganate absorption of the CL emission at high concentration. Therefore, the optimal concentration of $KMnO_4$ was 1.5×10^{-4} M.

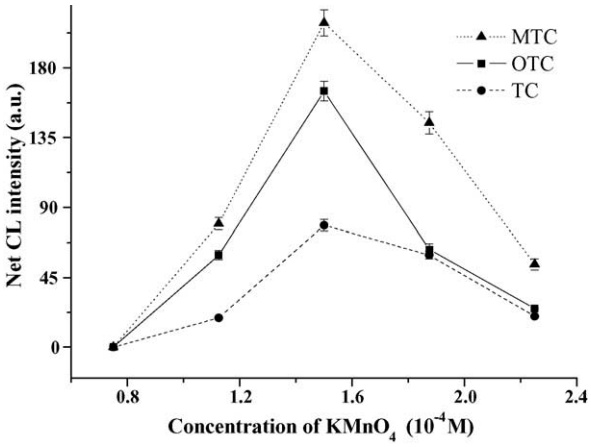

Fig. 3. Effect of the $KMnO_4$ concentration in H_3PO_4 solution on the relative CL intensity. All values are average values of three replicates. Standard deviations are given as error bars. Mobile phase: acetonitrile and 0.001 M phosphoric acid (16:84, v:v). Reaction condition: H_3PO_4: 0.075 M; Na_2SO_3: 9.6×10^{-4} M; β-CD: 5×10^{-8} M; flow rate: 1.8 ml/min. TCAs: OTC, TC 300 ng/ml, MTC 60 ng/ml.

The effect of Na_2SO_3 concentration on net CL intensity ranged from 1.6×10^{-4} M to 1.2×10^{-3} M (Fig. 4). The net CL intensity reached maximum for TCAs when the concentration of Na_2SO_3 was 9.6×10^{-4} M. Then it decreased greatly beyond 9.6×10^{-4} M in which some tiny air bubbles were produced. Thus, the concentration of Na_2SO_3 chosen was 9.6×10^{-4} M.

Fig. 5 shows the effect of β-CD concentration on CL intensity. The maximal relative CL intensity was achieved at 5.0×10^{-8} M, which could probably be ascribed to the formation of inclusion complex micelle with TCAs in acid at this concentration [29]. As a result, 5.0×10^{-8} M β-CD concentration was chosen.

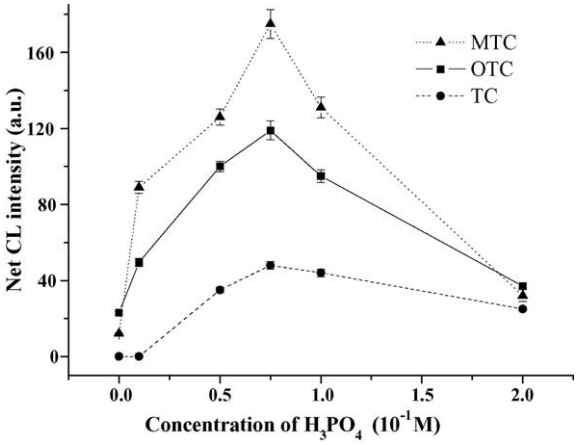

Fig. 2. Effect of the H_3PO_4concentration in $KMnO_4$ solution on the relative CL intensity. All values are average values of three replicates. Standard deviations are given as error bars. Mobile phase: acetonitrile and 0.001 M phosphoric acid (16:84, v:v). Reaction condition: $KMnO_4$: 1.5×10^{-4} M; Na_2SO_3: 9.6×10^{-4} M; β-CD: 5×10^{-8} M; flow rate: 1.8 ml/min. TCAs: OTC, TC 300 ng/ml, MTC 60 ng/ml.

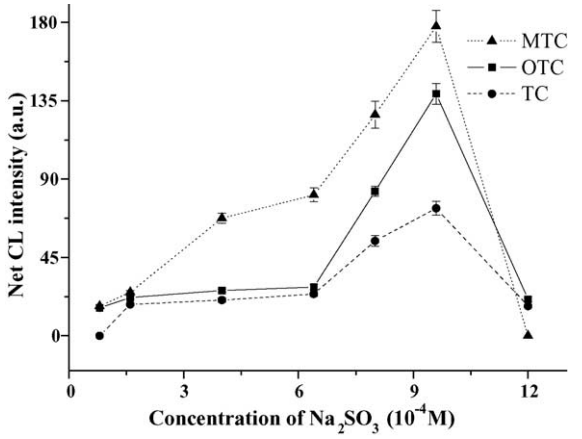

Fig. 4. Effect of the Na_2SO_3 concentration on the relative CL intensity. All values are average values of three replicates. Standard deviations are given as error bars. Mobile phase: acetonitrile and 0.001 M phosphoric acid (16:84, v:v). Reaction condition: H_3PO_4: 0.075 M; $KMnO_4$: 1.5×10^{-4} M; β-CD: 5×10^{-8} M; flow rate: 1.8 ml/min. TCAs: OTC, TC 300 ng/ml, MTC 60 ng/ml.

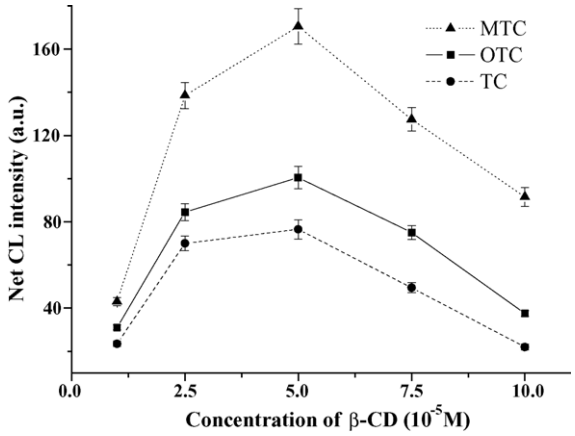

Fig. 5. Effect of the β-CD concentration on the relative CL intensity. All values are average values of three replicates. Standard deviations are given as error bars. Mobile phase: acetonitrile and 0.001 M phosphoric acid (16:84, v:v). Reaction condition: H_3PO_4: 0.075 M; $KMnO_4$: 1.5×10^{-4} M; Na_2SO_3: 9.6×10^{-4} M; flow rate: 1.8 ml/min. TCAs: OTC, TC 300 ng/ml, MTC 60 ng/ml.

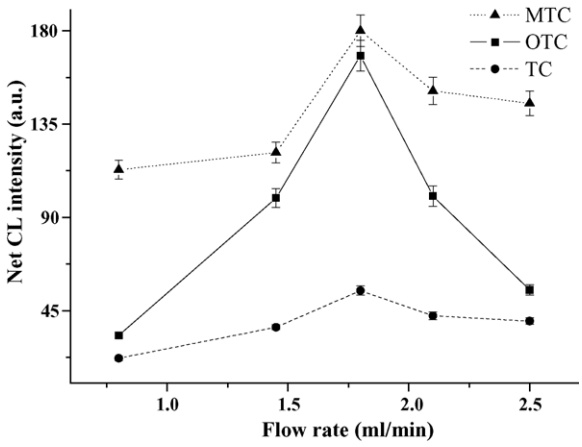

Fig. 6. Effect of flow rate on the relative CL intensity. All values are average values of three replicates. Standard deviations are given as error bars. Mobile phase: acetonitrile and 0.001 M phosphoric acid (16:84, v:v). Reaction condition: H_3PO_4: 0.075 M; $KMnO_4$: 1.5×10^{-4} M; Na_2SO_3: 9.6×10^{-4} M; β-CD: 5×10^{-8} M. TCAs: OTC, TC 300 ng/ml, MTC 60 ng/ml.

The effect of flow rate on the CL intensity ranged from 0.8 to 2.5 ml/min (Fig. 6). The relative CL intensity increased sharply in the range of 0.8–1.8 ml/min, and then decreased beyond 1.8 ml/min. Under optimal total flow rate condition, the flow rate of Na_2SO_3 and $KMnO_4$ was 1.8 ml/min.

3.3. Linearity, sensitivity, and precision

Under the optimum conditions described above, working curves of the OTC, TC, and MTC were obtained in the concentration range of 0.5–1000 ng/ml (at least 10 concentration points covering the whole range were used). Each point of the calibration graph corresponded to the mean value from three independent injections. The parameters of the regression equations, detection limits, and precisions were obtained with standard solutions of TCAs (Table 1). For the three tested TCAs, linear ranges of the CL detection were about 2 orders of magnitude and the detection limits at a signal-to-noise of 3 ranged from 0.9 to 5.0 ng/ml. The precision and stability of the proposed method were studied by assaying the standard solutions of TCAs (OTC, TC 250 ng/ml; MTC 50 ng/ml) within a day and between days. The relative standard deviations (Table 1) ranged from 3.1 to 7.4% within a day ($n = 11$) and ranged from 2.2 to 8.6% in 3 days ($n = 9$), respectively. Therefore, the precision and stability of the proposed method were acceptable. Table 2 summarizes the detection limit and linear range with different methods for the determination of three TCA residues in food products. The CL detection limits for the determination of OTC and TC were comparable with UV–vis and electrochemical detections [23], but were higher than that with MS detection [20]. For MTC, the CL detection limit was lower than that with UV–vis and electrochemical detection [23]. The results demonstrated that the HPLC-CL method offers an alternative and sensitive approach for the detection of three tested TCAs and can be used in MRL analysis.

3.4. Application

To evaluate the applicability of the present method in real samples, the honey samples provided by the Anhui Province Entry-Exit Inspection and Quarantine Bureau (EIQB, Anhui, PR China) were analyzed by this CL method. The chromatograms are shown in Fig. 1 and the results are presented in Table 3. The chromatograms obtained with CL detector were very simple (only a few peaks) and baseline was stable

Table 1
Performance of the proposed CL method for the determination of TCAs in honey matrix

TCA	Linear range (ng/ml) ($n = 10$)	Detection limit (ng/ml)		Precision			Regression equation[a] $\Delta I = aC + b$	Correlation coefficient (r)
		CL	DAD	(ng/ml)	R.S.D. (%)			
OTC	8–800	3.0	2.0[b]	250	2.9[d]	4.5[e]	$\Delta I = 4.064 \times 10^8 C + 21.987$	0.9963
TC	10–800	5.0	2.0[b]	250	7.4[d]	8.6[e]	$\Delta I = 1.925 \times 10^8 C + 12.616$	0.9925
MTC	1–800	0.9	9.0[c]	50	3.1[d]	2.2[e]	$\Delta I = 1.934 \times 10^9 C + 69.150$	0.9929

[a] ΔI: net CL intensity, C: concentration of TCAs.
[b] Wavelength: 274 nm.
[c] Wavelength: 350 nm.
[d] Precision in one day ($n = 11$).
[e] Precision in three days ($n = 9$).

Table 2
Detection of TCAs residues in food with different methods

TCAs	Method	Sample	Linear range (ng/ml)	Detection limit (ng/ml)	MRL (EU) (ng/ml)[a]	Reference
OTC	This HPLC-CL method	Honey	8–800	3.0	–[b]	
	This HPLC-DAD method	Honey	10–800	2.0	–	
	HPLC-UV	Fish		40	100	[16]
	HPLC-UV	Honey	50–650	9	–	[17]
	HPLC-EC	Milk	50–1000	12.5	100	[23]
	HPLC-MS	Muscle		0.8	100	[19]
		Liver		25.2	300	
		Kidney		4.8	600	
	HPLC-MS-MS	Honey		5	–	[20]
TC	This HPLC-CL method	Honey	10–800	5.0	–	
	This HPLC-DAD method	Honey	10–800	2.0	–	
	HPLC-UV	Honey	50–650	4	–	[17]
	HPLC-EC	Milk	50–1000	20	100	[23]
	HPLC-MS-MS	Honey		5	–	[20]
	EIA	Honey		20	–	[6]
MTC	This HPLC-CL method	Honey	1–800	0.9	–	
	This HPLC-DAD method	Honey	40–800	9.0	–	
	HPLC-UV	Honey	100–850	4	–	[17]
	HPLC-EC	Milk	50–1000	10	100	[23]

[a] MRL values for TCAs (parent residues and its 4-epimer) in food accordance with European Union regulation (EEC) No. 2377/90 and (EEC) 281/96.
[b] The dash (–) means that no MRL or tolerance limit for the TCAs have been established.

with a very low background. The recoveries from 92 to 122% were obtained for the three compounds and the results were comparable with DAD detection. The recovery of some samples was up to 120%, which was probably due to either the interference of sample matrices or experimental error. However, the recoveries were acceptable for the determination of TCAs at such trace level. The repeatability of the method by measuring the spiked 50 ng/ml OTC and TC and 250 ng/ml MTC honey samples ranged from 5.2 to 8.9%. Therefore, the proposed method was applicable for the detection of TCA residues in honey.

3.5. Mechanism of CL reaction

Earlier work suggested that electronically excited sulphur dioxide molecules were the emitters of the CL reaction of sodium sulfite with acidic potassium permanganate [26]. The spectra of the CL reaction in the presence of MTC (Fig. 7) showed that there were two emission peaks and the maximum emissions were at 436 and 535 nm, respectively. The peak at 535 nm was identical with the CL spectrum of excited state sulphur dioxide molecules [26,30] and thus the emitters at 535 nm were the excited state sulphur dioxide

Table 3
Determination of TCAs in honey sample

Sample	TCAs	Original[a] (ng/ml)		Added (ng/ml)	Found[a] (ng/ml)		Recovery[a] (%)	
		HPLC-CL	HPLC-DAD		HPLC-CL	HPLC-DAD	HPLC-CL	HPLC-DAD
1	OTC	ND	ND	50	59 ± 0.84	54 ± 0.52	118.0	108.0
	TC	ND	ND	50	61 ± 0.24	58 ± 0.69	122.0	116.0
	MEC	ND	ND	250	252 ± 0.48	253 ± 0.75	100.8	101.2
2	OTC	ND	ND	50	59 ± 0.65	59 ± 0.37	118.0	118.0
	TC	ND	ND	50	49 ± 0.45	51 ± 0.39	98.0	102.0
	MEC	ND	ND	250	256 ± 0.48	258 ± 0.85	102.4	103.2
3	OTC	ND	ND	50	52 ± 0.38	48 ± 0.46	104.0	96.0
	TC	ND	ND	50	46 ± 0.72	48 ± 0.62	92.0	96.0
	MEC	ND	ND	250	264 ± 0.41	243 ± 0.47	105.6	97.2
4	OTC	14 ± 0.81	ND	50	67 ± 0.54	62 ± 0.23	106.0	124.0
	TC	ND	ND	50	47 ± 0.75	48 ± 0.29	94.0	96.0
	MEC	ND	ND	250	249 ± 0.87	246 ± 0.51	99.6	98.4
5	OTC	21 ± 0.93	18 ± 0.12	50	73 ± 0.46	67 ± 0.18	104.0	98.0
	TC	25 ± 0.72	32 ± 0.41	50	80 ± 0.57	79 ± 0.31	110.0	94.0
	MEC	ND	ND	250	256 ± 0.28	258 ± 0.67	102.4	103.2

[a] Mean value ± R.S.D. (n = 3). ND: not detected (<LD).

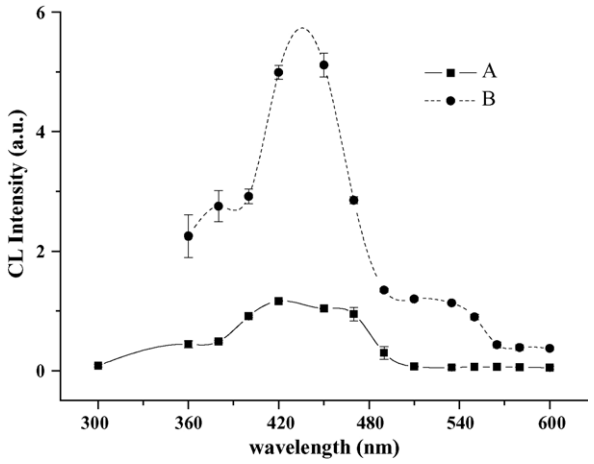

Fig. 7. Chemiluminescence spectra of $KMnO_4$–Na_2SO_3–β-CD–MTC system. All values are average values of three replicates. Standard deviations are given as error bars. Reaction condition: H_3PO_4: 0.075 M; $KMnO_4$: 1.5×10^{-4} M; Na_2SO_3: 9.6×10^{-4} M; β-CD: 5×10^{-8} M; MTC: 0.1 mg/ml. (A) $KMnO_4$–Na_2SO_3–MTC; (B) $KMnO_4$–Na_2SO_3–β-CD–MTC.

molecules. Another CL emission occurred at 436 nm, and this was in agreement with the fluorescence spectra of TCA oxidation products (Fig. 8). It was reported that TCAs could be easily converted into anhydro-derivatives in acid medium with strong oxidizing agents such as H_2O_2 and $KMnO_4$ [31,32]. These anhydro-derivatives of TCAs were fluorescent compounds for conjugated skeleton. Moreover, Yao et al. proposed that β-CD could form an inclusion complex with anhydro-derivatives of TCAs in acid medium [29]. This inclusion complex of β-CD and anhydro-derivatives of TCAs had strong fluorescence at 435–480 nm. Therefore, TCA oxidation products were the inclusion complex of β-CD and anhydro-derivatives of TCAs, which were responsible for the

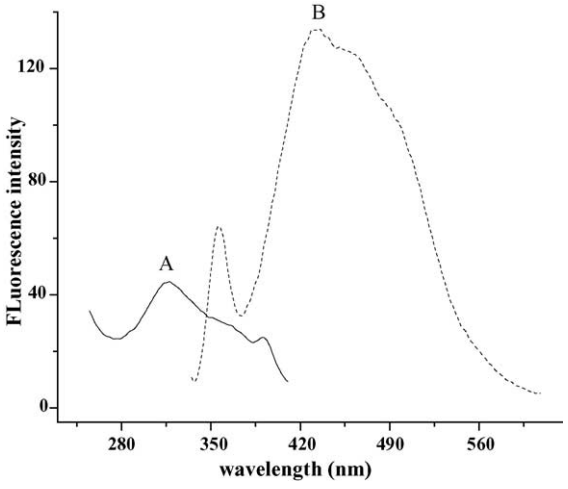

Fig. 8. Fluorescence spectra of $KMnO_4$–Na_2SO_3–β-CD–MTC system. Reaction condition: H_3PO_4: 0.075 M; $KMnO_4$: 7.5×10^{-4} M; Na_2SO_3: 5×10^{-3} M; β-CD: 5×10^{-8} M; MTC: 0.1 mg/ml. (A) Fluorescence excitation spectrum (emission wavelength = 436 nm); (B) Fluorescence emission spectrum (excitation wavelength = 320 nm).

emission at 436 nm. Thus, the excited state sulphur dioxide and the excited state inclusion complex of β-CD and anhydro-derivatives of TCAs might be the emitters in this system. We suggest that the CL reactions might undergo two routes:

(1) The reaction of HSO_3^- with MnO_4^- formed the excited state sulphur dioxide, which followed the pathways proposed by Kato et al. [33].
(2) MTC was oxidized by MnO_4^- in acidic medium to anhydro-derivatives of TCAs [31], followed by the reaction with β-CD to give rise to the inclusion complex of β-CD and anhydro-derivatives of TCAs.

The inclusion complex may react further with HSO_3^- and MnO_4^- to generate the excited state inclusion complex of β-CD and anhydro-derivatives of TCAs [29,34]. The overall reaction pathways may be as follows:

$$HSO_3^- + MnO_4^- \rightarrow HSO_3^\bullet + \text{manganese complex [30, 33]} \quad (1)$$

$$2HSO_3^\bullet \rightarrow S_2O_6^{2-} + 2H^+ \text{ [30, 33]} \quad (4)$$

$$S_2O_6^{2-} \rightarrow SO_4^{2-} + SO_2^* \quad (5)$$

$$SO_2^* \rightarrow SO_2 + h\nu\,(535\text{ nm}) \quad (6)$$

$$MnO_4^- + \text{MTC} + H^+ \rightarrow [\text{anhydro-MTC}]^+ + \text{manganesecomplex [31]} \quad (2)$$

$$\beta\text{-CD} + [\text{anhydro-MTC}]^+ \rightarrow [\text{anhydro-MTC-}\beta\text{-CD}]^+ \text{ [29]} \quad (3)$$

$$HSO_3^- + [\text{anhydro-MTC-}\beta\text{-CD}]^+ \rightarrow HSO_3^\bullet + [\text{anhydro-MTC-}\beta\text{-CD}]_{ox}^\bullet \quad (7)$$

$$[\text{anhydro-MTC-}\beta\text{-CD}]_{ox}^\bullet + MnO_4^- \rightarrow [\text{anhydro-MTC-}\beta\text{-CD}]_{ox}^{+*} + \text{manganesecomplex [34]} \quad (8)$$

$$[\text{anhydro-MTC-}\beta\text{-CD}]_{ox}^{+*} \rightarrow [\text{anhydro-MTC-}\beta\text{-CD}]_{ox}^{+} + h\nu(436\text{ nm}) \quad (9)$$

Yao et al. gave a model of inclusion complex and demonstrated that fluorescence intensity was dependent on the stability constants of inclusion complex affected by two main factors: electronic effect and stereoscopic effect. According to their studies, the stability constants of MTC and OTC were greater than that of TC, thus the fluorescence intensity of MTC and OTC were stronger than that of TC. This could be the reason why the CL signals of MTC and OTC were greater than TC in our work.

4. Conclusion

A novel HPLC-CL detection method was established for the determination of TCA residues in honey. This method was based on enhancement by TCAs of the CL from the potassium permanganate–sodium sulfite–β-cyclodextrin system in a phosphoric acid medium. The method allowed for simultaneous and sensitive detection of oxytetracycline, tetracycline, and metacycline residues in honey. Moreover, the CL reaction was highly compatible with the mobile phase used in the HPLC separation. The application potential of the HPLC-CL method to other analytes, especially residues in more complex matrices, is under further investigation.

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