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Determination of quinolones residues in prawn using high-performance liquid chromatography with Ce(IV)–Ru(bpy)$_3^{2+}$–HNO_3 chemiluminescence detection

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Abstract

A novel method was developed for the determination of quinolone (QN) residues such as ofloxacin, norfloxacin, ciprofloxacin and lomefloxacin by high-performance liquid chromatography (HPLC) coupled with chemiluminescence (CL) detection. The procedure was based on the chemiluminescent enhancement by QNs of the $Ce(SO_4)_2$–Ru(bpy)$_3^{2+}$–HNO_3 system. The separation was carried out with an isocratic elution using the mobile phase of 3:15:82 (v/v/v) acetonitrile–methanol–ammonium acetate buffer (containing 7.5×10^{-4} M TBAB, 0.8% (v/v) TEA and 1.0×10^{-4} M ammonium acetate, pH 3.65) at a flow rate of 1.0 ml/min. For the four QNs, the detection limits at a signal-to-noise of 3 ranged from 0.36 to 2.4 ng/ml. The relative standard deviations for the determination of QNs ranged from 1.6 to 4.5% within a day ($n = 11$) and from 3.7 to 6.2% in three days ($n = 15$), respectively. The method was successfully applied to the determination of QNs in prawn samples. The possible mechanism of the CL reaction was also discussed briefly.

Keywords: Quinolones; HPLC; Chemiluminescence; Prawn; Ce(IV); Ru(bpy)$_3^{2+}$

1. Introduction

The quinolone antibiotics (Fig. 1) are synthetic antimicrobial agents with a broad spectrum of activity widely used in human and veterinary medicine. Extensive use of antibiotics in veterinarian medicine and medicated feed play a crucial role in intensive animal production in food-producing animals and farmed fish such as prawn, salmon and catfish [1,2]. It leads to a significant increase in antibiotic resistance and allergic reactions, having therefore important consequences on public health. Moreover, quinolone-induced acute arthropathy has been observed in several animal species. Although severe cases of arthropathy have been observed only rarely in humans, incidents of transient arthralgia have also been reported [3]. These observations have precluded the use of quinolones in children and pregnant women. To safeguard human health, the European Union set a Maximum Residue Limits (MRLs) of 30 ppb for the sum of enrofloxacin and its metabolite, ciprofloxacin, in muscle, kidney and liver [4]. Because of the great varieties of quinolones, and the possibility of trace residues in edible tissue, it is necessary to develop sensitive multi-residue screening methods for the determination of quinolones (QNs).

The methods for the determination of QNs in biological samples have been reviewed [5–7]. Numerous biological and chemical techniques such as microbiological assay [8], spectrophotometry [9], fluorimetry [10–12], electrochemical detection [13], capillary electrophoresis (CE) [14–16], high-performance liquid chromatography (HPLC) [17–25] and flow-injection–chemiluminescence methods [26–28] have been reported. The analysis of QNs has traditionally been performed using microbiological methods. However, these techniques are slow and suffer from poor precision and specificity. Now most of analytical methods developed for multi-residue quinolones detection is focused on liquid chromatography with different detection modes such as spectrophotometry [17,18], fluorescence [19–23] and mass spectrometry (MS) [24,25] because they

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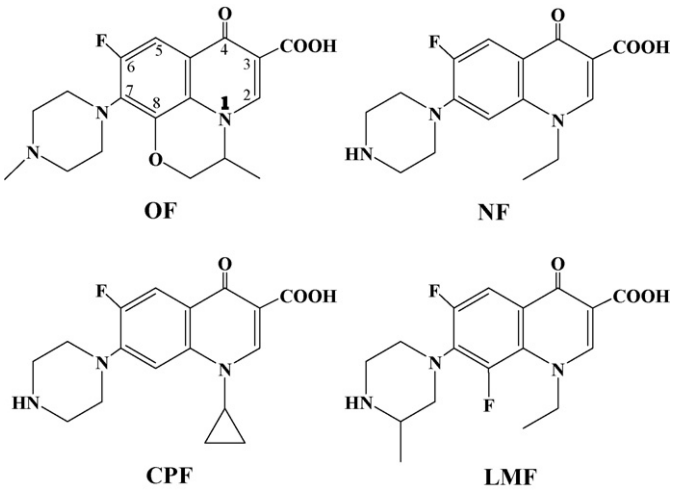

Fig. 1. Structural formulae of QNs.

can offer the advantages of better sensitivity and specificity. Of these detection modes, mass spectrometry can offer high sensitivity and selectivity for the determination of QN residues, 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. Francis and Adcock [29] reviewed the CL methods for the determination of ofloxacin involving in acidic $Ru(bpy)_3^{2+}$ system, alkaline $KMnO_4$ system and $NaNO_3$–H_2O_2 system, etc. However, these methods are restricted to flow-injection procedure and cannot be used for simultaneous determination of several QNs. Moreover, sensitivity is not good enough for the determination of QN residues. In the present work, we found that QNs could strongly enhance the CL of the $Ce(SO_4)_2$–$Ru(bpy)_3^{2+}$–HNO_3 system, the enhanced CL signal was much stronger than the $Ce(SO_4)_2$–$Ru(bpy)_3^{2+}$ system reported by Aly et al. [27] using flow-injection CL method, and the detection limits were lower than that of reported. To our knowledge, there is no report using HPLC–CL to detect QN residues. On this basis, a highly sensitive method was developed for the simultaneous determination of QN residues in prawn by coupling HPLC with this CL reaction.

2. Experimental

2.1. Chemicals and solutions

Methanol and acetonitrile were of HPLC grade and provided by Jiangsu Hanbang Technology Company (Jiangsu, China). $Ru(bpy)_3Cl_2{\cdot}6H_2O$ was purchased from Sigma (Sigma, USA). Ofloxacin (OF), norfloxacin (NF), ciprofloxacin (CPF) and lomefloxacin (LMF) were obtained from the Institute of Pharmaceutical and Biomaterial Authentication of China (Beijing, China). Tetrabutyl ammonium bromide (TBAB), triethylamine (TEA), $Ce(SO_4)_2$ and HNO_3 were analytical-reagent grade and obtained from Shanghai Chemicals Company (Shanghai, China).

Stock solutions of OF, CPF and LMF (0.1 mg/ml) were prepared weekly using double distilled water, while stock solution of NF (0.1 mg/ml) was prepared weekly using methanol–double distilled water for its poor solubility. The stock solutions were stored at 4 °C in a refrigerator. A stock solution of $Ru(bpy)_3^{2+}$ (1.0×10^{-2} M) was prepared using double distilled water. The solution of $Ce(SO_4)_2$ (1.0×10^{-3} M) was prepared by dissolving $Ce(SO_4)_2$ in 3.6×10^{-2} M sulfuric acid. All working solutions were freshly prepared each day with double distilled 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 [30] 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 thermostatic column compartment, a diode array detector (DAD), a manual sample valve injector with a 100-μl loop, and an analytical column (Zorbax Eclipse XDB-C_8, 150 mm × 4.6 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 (i.d. 1 mm, 13 cm length 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.

Absorption spectra were acquired using a Shimadzu UV-2401 Spectrophotometer (Tokyo, Japan). CL spectra were measured on a Shimadzu RF-5301 Spectrofluorometer (Tokyo, Japan). pH measurement was carried out on a REX pHS-3B meter (Shanghai REX, China).

2.3. Procedure

The quinolones were separated by a XDB-C_8 column at 30 °C with an isocratic elution at a flow rate of 1.0 ml/min. The mobile phase consisted of 3:15:82 (v/v/v) acetonitrile (A)–methanol (B)–ammonium acetate buffer (C containing 7.5×10^{-4} M TBAB, 0.8% (v/v) TEA and 1.0×10^{-4} M ammonium acetate, pH 3.65). The separation was carried out in 8 min and typical retention time of OF, CPF, NF and LMF was 5.6, 6.2, 6.8 and 7.6 min, respectively. The UV–vis detector was set at 278 nm for NF and CPF and 295 nm for OF and LMF. The column effluent from DAD was first mixed with the solution of HNO_3 at a mixing tee via a PEEK tube (600 mm × 0.25 mm i.d., Agilent Technologies), then mixed with the combined stream of $Ru(bpy)_3^{2+}$ and $Ce(SO_4)_2$ solution. The CL solutions of HNO_3, $Ce(SO_4)_2$ and $Ru(bpy)_3^{2+}$ were pumped at a flow rate of 5.0 ml/min, respectively. Light emission was monitored by the photomultiplier tube. The quantitative determination was based on the net CL intensity $I = I_S - I_0$, where I_S is the CL intensity in the presence of QNs and I_0 is the CL intensity of blank signal.

2.4. Sample preparation

The sample-preparation method was based on the national standard of the People's Republic of China (GDFB 236—2004, LC or LC–MS–MS method for the determination of quinolone residues in animal food) [31]. For extraction of the prawn sample, 5 g (accurate to 0.1 g) of the sample tissue mince was dissolved in 30 ml of 1% acetic acid ethanol solution, homogenized and centrifuged at 4500 r/min for 5 min. The sample solutions were poured into the SPE cartridge (Bond-Elut SCX, Supelco, USA) at a rate of 3 ml/min, and the quinolone residues were adsorbed by the resin on the column. The quinolone residues adsorbed were washed with 10 ml of methanol, water, methanol in order by controlling the flow rate to 2.0 ml/min. Finally, the QN residues absorbed on SPE cartridge were eluted with 10 ml of 25% ammonia methanol, at a flow rate of 1.5 ml/min. The eluent was evaporated by a nitrogen stream at 35 °C and dissolved in mobile phase. The residue was filtered through 0.45-μm filter into a LC plastic vial. For the validation purpose, the recovery experiments were spiked with 10 ng/ml OF, NF, CPF and LMF standard solution in the prawn sample. All values measured were the average values of three replicates.

3. Results and discussion

The chemiluminescence of Ce(IV)–$Ru(bpy)_3^{2+}$ has been studied previously [27,31,32]. QNs were observed to enhance the net CL intensity in Ce(IV)–$Ru(bpy)_3^{2+}$ system [27]. We found that when HNO_3 was added to the system, the enhanced CL signals of QNs were increased sharply. Some acids (phosphoric acid, sulfuric acid, hydrochloric acid, nitric acid and acetic acid) with 1.0 M concentration were examined and the net CL intensity was found to be highest in nitric acid solution. This may be due to the strong oxidizability of nitric acid solution. The kinetic characteristics of the CL enhancement by OF (80 ng/ml) of Ce(IV)–$Ru(bpy)_3^{2+}$ and Ce(IV)–$Ru(bpy)_3^{2+}$–HNO_3 systems are shown in Fig. 2 by a flow-injection system under reaction conditions: 3.6×10^{-2} M H_2SO_4, 0.30 M HNO_3, 2.5×10^{-4} M Ce(IV) and 5.0 ml/min flow rate. The results indicated that the enhancing signal of OF was stronger in the Ce(IV)–$Ru(bpy)_3^{2+}$–HNO_3 system than that in the Ce(IV)–$Ru(bpy)_3^{2+}$ system. Thus, the Ce(IV)–$Ru(bpy)_3^{2+}$–HNO_3 system was chosen for the further experiments.

The enhanced net CL intensity was sensitive to a variety of factors such as solvent, pH and HPLC mobile phases. The strongest net CL intensity was obtained by optimization of such facts.

3.1. Optimization of HPLC conditions

As for HPLC–CL detection, the mobile phase of HPLC should be not only suitable for the separation of OF, NF, CPF and LMF, but also compatible with the CL reaction. Therefore, much attention was paid to the compatibility of chromatographic system and CL detection. Methanol or acetonitrile often used in mobile phase were not compatible with many CL systems, but compatible with this CL system.

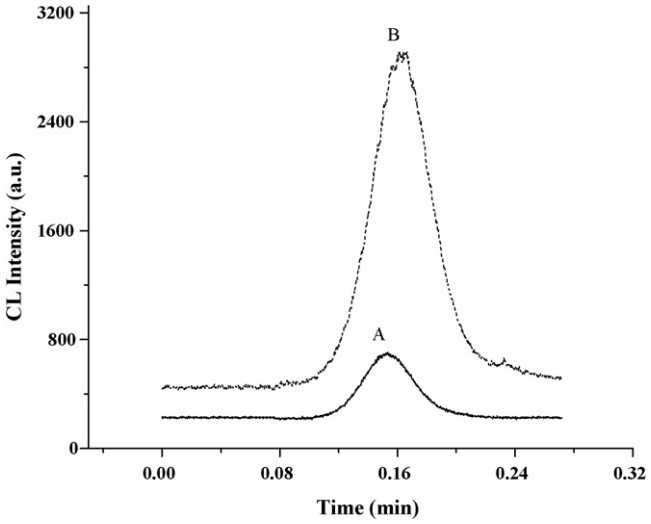

Fig. 2. Kinetic characteristics of CL systems in the presence and absence of HNO_3. Reaction conditions: H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; Ce(IV): 2.5×10^{-4} M; flow rate: 5.0 ml/min; OF: 80 ng/ml. (A) Ce(IV)–$Ru(bpy)_3^{2+}$ and (B) Ce(IV)–$Ru(bpy)_3^{2+}$–HNO_3.

Several mobile phases have been reported for the separation of QNs on a reversed phase column, such as acetonitrile–water [18,19,21,24,25], methanol–water [22], acetonitrile–methanol–water [17,23] and acetonitrile–tetrahydrofuran–water [20]. The ternary mixtures of acetonitrile–methanol–water (v/v/v) 3:15:82 were found to be suitable for good separation of these compounds and compatible with the CL system. In order to improve the chromatography separation, tetrabutyl ammonium bromide (TBAB) was added as an ion-paired reagent and triethylamine (TEA) was added as a tail-reducing agent, which competed with the analytes for the active residual silanol groups. Good chromatographic separation of quinolones was achieved by the use of a ternary mixture of acetonitrile (A)–methanol (B)–ammonium acetate buffer solution (C) containing TBAB and TEA, adjusting pH to 3.65 with acetic acid. Separation was carried out at 30 °C for reducing mobile phase viscosity and decreasing back pressure.

Under the condition of complete separation, the concentration of TBAB, TEA and the pH of buffer solution were optimized to obtain maximal CL intensity.

The concentration of TBAB ranged from 0 to 10^{-3} M was studied. Baseline separation could not be obtained with the concentration lower than 7.5×10^{-4} M, good separation was achieved with the concentration of 7.5×10^{-4} M and peaks overlapped with each other with the concentration higher than 7.5×10^{-4} M. Thus, the concentration of 7.5×10^{-4} M was chosen.

In the range of 0–0.8% (v/v), the effect of TEA concentration on separation and CL intensity was tested. With an increase in TEA concentration, the chromatography separation improved but the separation time increased and the net CL intensity decreased. Therefore, the concentration of 0.8% (v/v) was used.

The effect of ammonium acetate buffer solution on CL behaviors was studied. When concentration of ammonium acetate was greater than 10^{-4} M, the baseline separation could be obtained. Net CL intensity reached to its maximum at 10^{-4} M ammonium acetate and did not change with further increase of ammonium acetate concentration. Thus, the concentration of ammonium acetate was chosen as 10^{-4} M.

pH ranged from 3.35 to 3.95 was tested. The chromatographic peaks of OF, NF and CPF overlapped when the pH was lower than 3.65. When pH increased, good separation could be achieved and the net CL intensity reached its maximum at pH 3.65.

Therefore, good chromatographic separation of quinolones was obtained by the use of a mobile phase of acetonitrile–methanol–ammonium acetate buffer (3:15:82, v/v/v) (containing 7.5×10^{-4} M TBAB, 0.8% (v/v) TEA and 1.0×10^{-4} M ammonium acetate, pH 3.65). The chromatograms obtained from DAD and CL detector are shown in Fig. 3.

3.2. Optimization of CL conditions

The effect of HNO_3 concentration on the net CL intensity was studied in the range of 0.0–6.0 M. The optimal concentration of HNO_3 was 0.3 M for OF, and 1.8 M for NF and CPF (Fig. 4). The CL intensity of LMF was increased slowly with the concentration of the HNO_3. In order to reduce the consumption of the reagents and find an optimum concentration of HNO_3 suitable for QNs, 1.8 M HNO_3 was chosen for LMF as a compromise.

The effect of Ce(IV) concentration on the net CL intensity was investigated in the range of 1.0×10^{-4} to 2.5×10^{-3} M in 3.6×10^{-2} M H_2SO_4 (Fig. 5). The curve of the net CL intensity versus the concentration of Ce(IV) changed with QNs in different ways. The enhanced CL intensity reached its maximal at the concentration of 2.5×10^{-4} M for OF and 10^{-3} M for NF, CPF and LMF. Therefore, the optimal concentration of Ce(IV) was 2.5×10^{-4} M for OF and 10^{-3} M for the other QNs.

The effect of H_2SO_4 concentration on the net CL intensity was studied in the range of 9.0×10^{-3}–1.1×10^{-1} M. The net CL intensity of all the QNs increased in the range of 9.0×10^{-3}–3.6×10^{-2} M, and decreased in the range of 3.6×10^{-2}–1.1×10^{-1} M. The optimal concentration of H_2SO_4 was 3.6×10^{-2} M.

The effect of $Ru(bpy)_3^{2+}$ concentration on net CL intensity was examined in the range of 5.0×10^{-6} to 1.0×10^{-4} M. With higher concentrations of $Ru(bpy)_3^{2+}$, net CL intensity for all the QNs increased, which could be due to more efficiency of $Ru(bpy)_3^{2+}$ at high concentration. In order to reduce the consumption of reagents, the optimal concentration of $Ru(bpy)_3^{2+}$ was chosen as 1.0×10^{-4} M.

The effect of the flow rate from 1.2 to 5.5 ml/min on the net CL intensity was studied. The maximal net CL intensity was obtained for different QNs at different flow rate. Lower flow rates resulted in peak tailing of the chromatography, whereas higher rates led to excessive consumption of reagents. The flow rate of 5.0 ml/min was acceptable for all QNs.

The optimal CL reaction conditions for QNs are shown in Table 1. It was noted that the optimal CL reaction conditions for NF, CPF and LMF were consistent, but the optimal CL reaction conditions for OF were different from the former three compounds, which might be due to difference in molecular structure such as substituent and its position. If the reaction conditions of 1.8 M HNO_3 and 1.0×10^{-3} M Ce(IV) are chosen for all the QNs as a compromise, the sensitivity for the detection of OF will be poor. Alternatively, if the reaction conditions of 0.3 M HNO_3 and 2.5×10^{-4} M Ce(IV) for OF other than NF, CPF and LMF were chosen, two injections will be needed and good sensitivity for the detection of OF will be obtained. However,

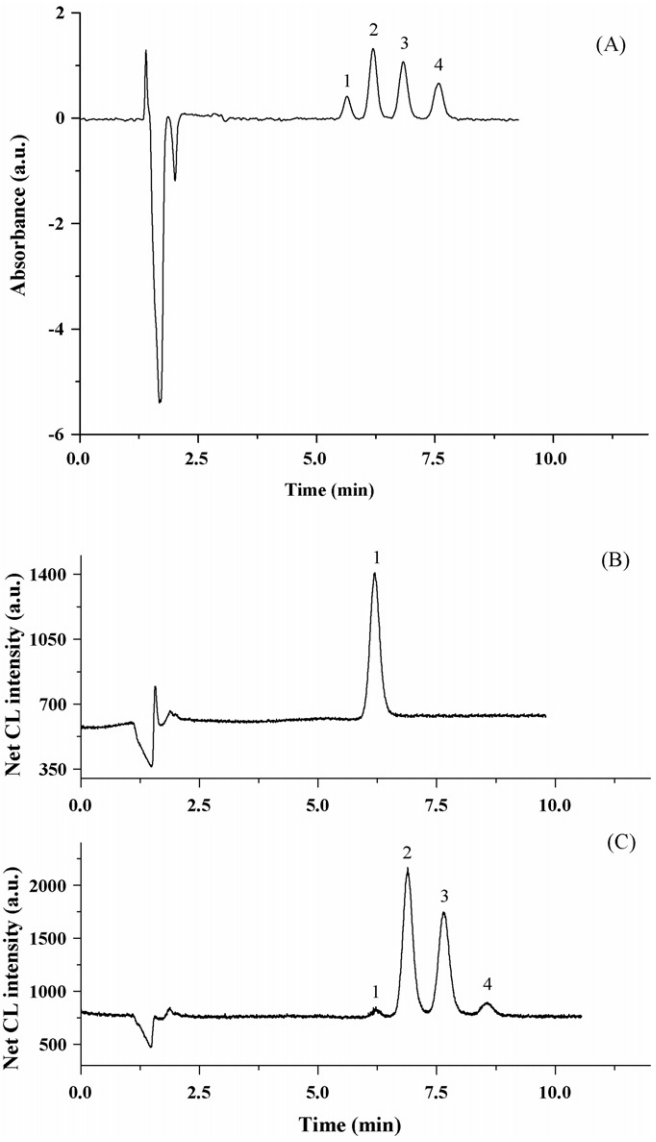

Fig. 3. Chromatograms of QNs with DAD at 278 nm and CL detection. QNs: 50 ng/ml; peaks: 1: OF, 2: NF, 3: CPF, 4: LMF. (A) Chromatogram of a mixture of standard QNs with DAD detection. Separation condition: isocratic elution with the mobile phase of 3:15:82 (v/v/v) acetonitrile–methanol–ammonium acetate buffer (containing 7.5×10^{-4} M TBAB, 0.8% (v/v) TEA and 1.0×10^{-4} M ammonium acetate, pH 3.65), 1.0 ml/min. (B) Chromatograms of a mixture of standard QNs with CL detection. Reaction conditions: HNO_3: 3.0×10^{-1} M; Ce(IV): 2.5×10^{-4} M; H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; flow rate: 5.0 ml/min. (C) Chromatogram of a mixture of standard QNs with CL detection. Reaction conditions: HNO_3: 1.8 M; Ce(IV): 1.0×10^{-3} M; H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; flow rate: 5.0 ml/min.

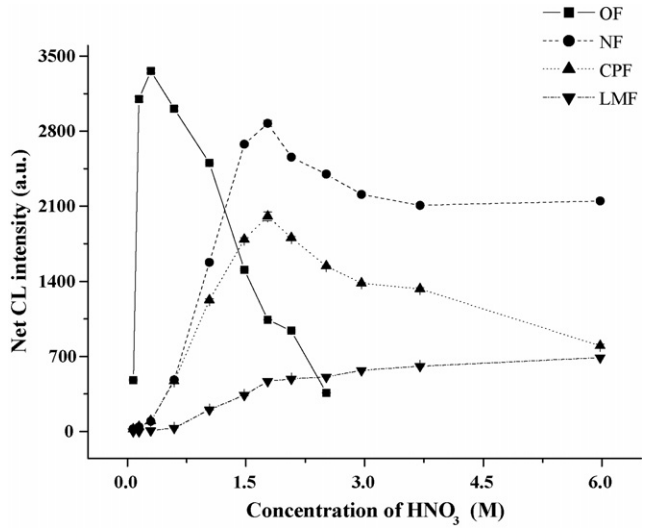

Fig. 4. Effect of the HNO_3 concentration on the net CL intensity. All values are average values of three replicates. Standard deviations are given as error bars. Separation condition was same as Fig. 2. Reaction conditions: H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; Ce(IV): 2.5×10^{-4} M for OF; Ce(IV): 1.0×10^{-3} M for NF, CPF and LMF; flow rate: 5.0 ml/min; QNs: 100 ng/ml.

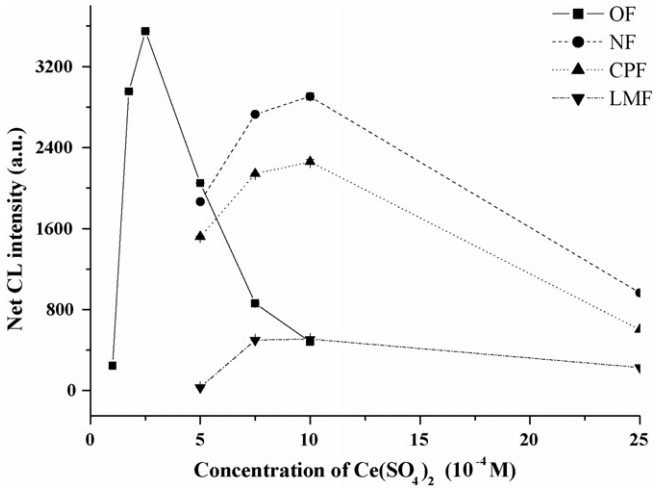

Fig. 5. Effect of the Ce(IV) concentration on the net CL intensity. All values are average values of three replicates. Standard deviations are given as error bars. Separation condition was same as Fig. 2. Reaction conditions: H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; HNO_3: 3.0 × 10^{-1} M for OF; HNO_3: 1.8 M for NF, CPF and LMF; flow rate: 5.0 ml/min; QNs: 100 ng/ml.

Table 2
Parameters of regression for QNs

QNs	Linear range (ng/ml)	Regression equation[a], $\Delta I = aC + b$	Correlation coefficient (r)
OF[b]	0.5–250	$\Delta I = 12.780 + 1.682 \times 10^{10}C$	0.9992
OF[c]	25–250	$\Delta I = 11.436 + 1.926 \times 10^{9}C$	0.9997
NF	0.5–250	$\Delta I = 26.983 + 3.234 \times 10^{10}C$	0.9989
CPF	0.5–250	$\Delta I = 36.237 + 2.208 \times 10^{10}C$	0.9987
LMF	2.5–250	$\Delta I = 30.388 + 5.019 \times 10^{10}C$	0.9998

[a] ΔI: net CL intensity; C: concentration of TCAs.
[b] Reaction conditions: HNO_3: 3.0×10^{-1} M; Ce(IV): 2.5×10^{-4} M; H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; flow rate: 5.0 ml/min.
[c] Reaction conditions: HNO_3: 1.8 M; Ce(IV): 1.0×10^{-3} M; H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; flow rate: 5.0 ml/min.

when the concentration of OF is very low, it deserve to use two injections. As an alternative, two kinds of reaction conditions were provided in this case.

3.3. Linearity, sensitivity and precision

Under the optimum conditions with two injections (first injection for the detection of OF using 3.0×10^{-1} M HNO_3 and 2.5×10^{-4} M Ce(IV) and second injection for the detection of NF, CPF and LMF using 1.8 M HNO_3 and 1.0×10^{-3} M Ce(IV)), working curves of the OF, NF, CPF and LMF were obtained in the concentration range of 0.5–250 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 QNs as shown in Tables 1 and 2. For the four tested QNs, 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 0.36 to 2.4 ng/ml. The precision and stability of the proposed method were studied by assaying the standard solutions of QNs (50 ng/ml) within a day and between days. The relative standard deviations (Table 1) ranged from 1.6 to 4.5% within a day ($n = 11$) and from 3.7 to 6.2% in three days ($n = 15$), respectively. Therefore, the precision and stability of the proposed method were acceptable. Table 3 summarizes the detection limit and linear range with different methods for the determination of four QN residues. The CL detection limits for

Table 1
The optimal CL conditions and the performance of this CL method for the determination of QNs

QNs	Concentration (M)				Detection limit (ng/ml)		Precision		
	HNO_3	Ce(IV)	H_2SO_4	$Ru(bpy)_3^{2+}$	CL	DAD	ng/ml		R.S.D. (%)
OF	0.30	2.5×10^{-4}	3.6×10^{-2}	1.0×10^{-4}	0.43	9.1[a]	4.5[b]	5.1[c]	50
	1.78	1.0×10^{-3}	3.6×10^{-2}	1.0×10^{-4}	10.4	9.1[a]	3.7[b]	6.3[c]	50
NF	1.78	1.0×10^{-3}	3.6×10^{-2}	1.0×10^{-4}	0.36	7.8[d]	2.6[b]	3.7[c]	50
CPF	1.78	1.0×10^{-3}	3.6×10^{-2}	1.0×10^{-4}	0.40	15.0[d]	2.0[b]	6.2[c]	50
LMF	1.78	1.0×10^{-3}	3.6×10^{-2}	1.0×10^{-4}	2.4	16.5[a]	1.6[d]	4.8[c]	50

[a] Wavelength: 295 nm.
[b] Precision in one day ($n = 15$).
[c] Precision in three days ($n = 9$).
[d] Wavelength: 278 nm.

Table 3
Detection of QN residues in food with different methods

QNs	Methods	Linear range (ng/ml)	Detection limit (ng/ml)	Reference
OF	This CL method	0.5–250	0.43	
	HPLC–UV	1.2–4000	1	[17]
	HPLC–FL	45–10000		[20]
	HPLC–MS		21	[25]
	Polarography	3600–18000	110	[13]
	Fluorimetry	20–1000	6	[9]
	FIA–CL	3–700	1.98	[27]
	Microbiological assay	12000–27000		[7]
NF	This CL method	0.5–250	0.36	
	HPLC–UV	6–2000	5	[17]
	HPLC–FL	30–6500		[20]
	HPLC–MS		2	[24]
	Fluorimetry		0.54	[10]
	FIA–CL	50–7000	9.9	[27]
CPF	This CL method	0.5–250	0.40	
	HPLC–UV	6–2800	5	[17]
	HPLC–FL	30–6500		[20]
	HPLC–MS		1	[24]
	CE		1000	[16]
	Fluorimetry		0.40	[10]
	FIA–CL	50–6000	9.6	[27]
LMF	This CL method	2.5–250	2.4	
	HPLC–FL	30–7500		[20]
	HPLC–MS		0.5	[24]
	Fluorimetry	50–2200	16	[9]

the determination of OF, NF and CPF were comparable with MS detection [22,23] and fluorimetry detection [10,11,21]. For LMF, the CL detection limit was lower than that MS detection [22]. The results demonstrate that the HPLC–CL method offers an alternative and sensitive approach for the detection of four tested QNs and can be used in MRL analysis.

Under the optimum conditions with one injection for the simultaneous detection of four QNs using 1.8 M HNO_3 and 1.0×10^{-3} M Ce(IV)), the detection limits and the linear ranges for NF, CPF and LMF were the same as with two injections. For OF, the detection limits were 0.43 ng/ml with two injections and 10.4 ng/ml with one injection, respectively; the linear ranges were 0.5–250 ng/ml with two injections and 25–250 ng/ml with one injection, respectively. The results show that the method with two injections for the detection of OF can offer more than one order of magnitude higher sensitivity than the method with one injection. As an option, one injection is enough when the OF concentration is not low. However, two injections are necessary when the OF concentration is very low.

3.4. Application

To evaluate the applicability of the present method in real samples, the prawn samples provided by the Anhui Province Entry-Exit Inspection and Quarantine Bureau (EIQB, Anhui, PR China) were analyzed by this HPLC–CL method. The chromatograms are shown in Fig. 6 and the results are presented in Table 4. The chromatograms obtained with CL detector were very simple (only a few peaks) and baseline was stable with a very low background. The recoveries of the prawn sample spiked with 10 ng/ml OF, NF, CPF and LMF reached to 88.3–109.4% and were acceptable for the determination of QNs at such trace level. The repeatability of the method by measuring the spiked 10 ng/ml QNs prawn samples ranged from 4.7 to 6.8%. Therefore, the proposed method was applicable for the detection of QN residues in food.

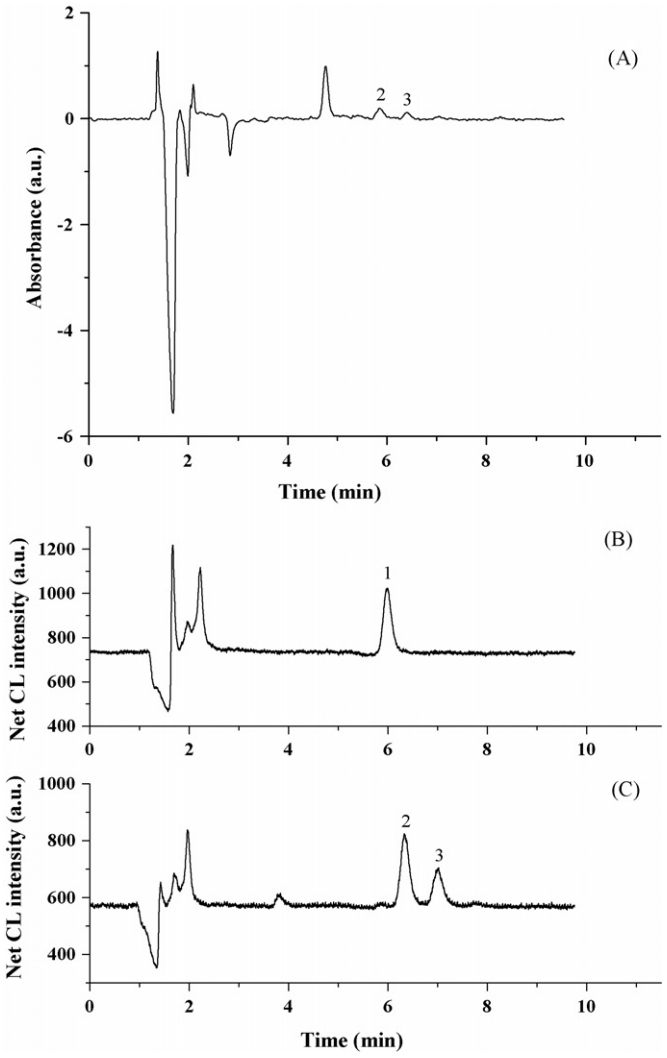

Fig. 6. Chromatograms of prawn sample with DAD detection at 278 nm and CL detection. Sample 1 without spiked QNs in Table 4; peaks: 1: OF, 2: NF, 3: CPF. (A) Chromatogram of prawn sample with DAD detection. Separation condition was same as Fig. 2. (B) Chromatogram of prawn sample with CL detection. Reaction conditions: HNO_3: 3.0×10^{-1} M; Ce(IV): 2.5×10^{-4} M; H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; flow rate: 5.0 ml/min. (C) Chromatogram of prawn sample with CL detection. Reaction conditions: HNO_3: 1.8 M; Ce(IV): 1.0×10^{-3} M; H_2SO_4: 3.6×10^{-2} M; $Ru(bpy)_3^{2+}$: 1.0×10^{-4} M; flow rate: 5.0 ml/min.

3.5. Mechanism of CL reaction

It was reported that the reaction between Ce(IV) and $Ru(bpy)_3^{3+}$ could give rise to chemiluminescence, which was suggested to be due to electronically excited state of $Ru(bpy)_3^{2+}$ molecules [27,32]. The emission spectra of the CL reaction

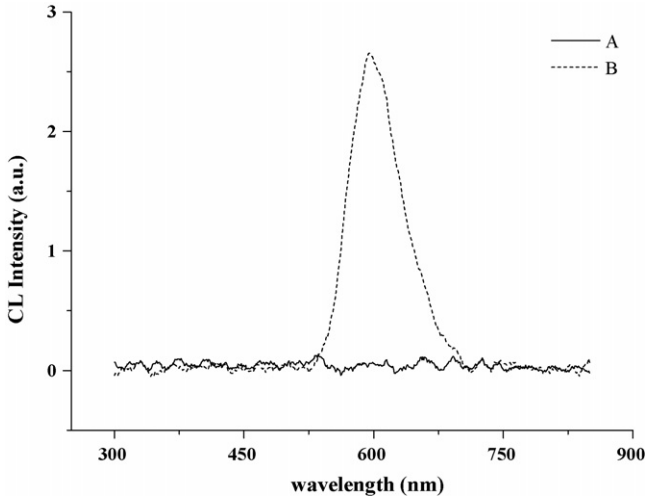

Fig. 7. Chemiluminescent spectra of Ce(IV)–Ru(bpy)$_3^{2+}$–HNO_3–CPF system. Reaction conditions: HNO_3: 1.8 M; Ce(IV): 1.0×10^{-3} M; H_2SO_4: 3.6×10^{-2} M; Ru(bpy)$_3^{2+}$: 1.0×10^{-4} M; CPF: 0.1 mg/ml. (A) Ce(IV)–Ru(bpy)$_3^{2+}$–HNO_3 and (B) Ce(IV)–Ru(bpy)$_3^{2+}$–HNO_3–CPF.

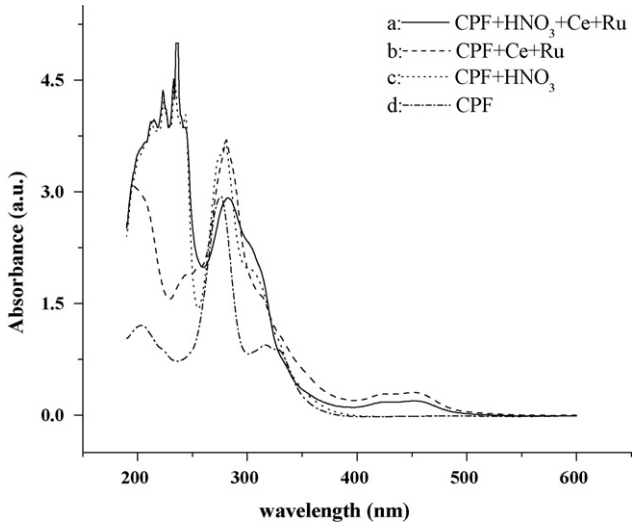

Fig. 9. UV–vis absorption spectra. The spectra were recorded as soon as possible after solutions were mixed. (a) Ce(IV)–Ru(bpy)$_3^{2+}$–HNO_3–CPF, (b) Ce(IV)–Ru(bpy)$_3^{2+}$–CPF, (c) HNO_3–CPF and (d) CPF. Reaction conditions: CPF: 0.1 mg/ml; HNO_3: 1.8 M; Ce(IV): 1.0×10^{-3} M; H_2SO_4: 3.6×10^{-2} M; Ru(bpy)$_3^{2+}$: 1.0×10^{-4} M.

showed that maximal emission wavelength is 605 nm in the absence and presence of CPF (Fig. 7), being identical with light emission of [Ru(bpy)$_3^{2+}$]*. Thus, it indicates that the emitter of the Ce(IV)–Ru(bpy)$_3^{2+}$–HNO_3–CPF system is [Ru(bpy)$_3^{2+}$]*.

The Ce(IV)–Ru(bpy)$_3^{2+}$ system has proven to be a very sensitive detection system for compounds which contain a secondary or tertiary aliphatic amine and the mechanism has been discussed [27]. The amine compounds and Ru(bpy)$_3^{2+}$ were oxidized by Ce(IV) to radicals and Ru(bpy)$_3^{3+}$, respectively, then radicals reacted with Ru(bpy)$_3^{3+}$ to form [Ru(bpy)$_3^{2+}$]*, leading to light emission. The studied QNs contain secondary amine (NF, CPF and LMF) or tertiary amine (OF) since they contain piperazine moiety. Thus, the CL reaction mechanism was presumably similar to that of Ce(IV)–Ru(bpy)$_3^{2+}$–amine compounds.

Du et al. proposed that QNs could form a quinoline (enol) structure (Fig. 8) in the strong acid medium [34]. UV–vis spectra in Fig. 9 indicate that the reaction between CPF and HNO_3 solution took place according to the fact that the absorption peak at 203 nm appeared after mixing. He et al. elucidated that this quinoline structure can form activated complexes rapidly with Ce(IV) and enhance greatly the CL from the Ce(IV)–Ru(bpy)$_3^{2+}$ system [32]. Accordingly [32–34], we deduced that QNs was first oxidized by HNO_3 to quinoline (enol) structure, followed by the reaction with Ce(IV) to give rise to the activated complexes.

The activated complex may generate the radicals according to the literature [32], which reacted with Ru(bpy)$_3^{3+}$ generated by the reaction of Ru(bpy)$_3^{2+}$ with Ce(IV) to form [Ru(bpy)$_3^{2+}$]*, leading to light emission. The overall reaction pathways may be as follows [32]:

$$\mathrm{Ru(bpy)_3^{2+} + Ce(IV) \rightarrow Ce(III) + Ru(bpy)_3^{3+}} \quad (1)$$

$$\mathrm{CPF + HNO_3 \rightarrow CPF\ quinoline\ structure\ product} \quad (2)$$

$$\mathrm{CPF\ quinoline\ structure\ product + Ce(IV) \rightarrow [CPF\ quinoline\ structure\ product - Ce(IV)]} \quad (3)$$

$$\mathrm{[CPF\ quinoline\ structure\ product - Ce(IV)] \rightarrow [^{\bullet}CPF\ quinoline\ structure\ product] + Ce(III)} \quad (4)$$

$$\mathrm{Ru(bpy)_3^{3+} + [^{\bullet}CPF\ quinoline\ structure\ product] \rightarrow [Ru(bpy)_3^{2+}]^* + CPF - product} \quad (5)$$

$$\mathrm{[Ru(bpy)_3^{2+}]^* \rightarrow Ru(bpy)_3^{2+} + h\nu(605\ nm)} \quad (6)$$

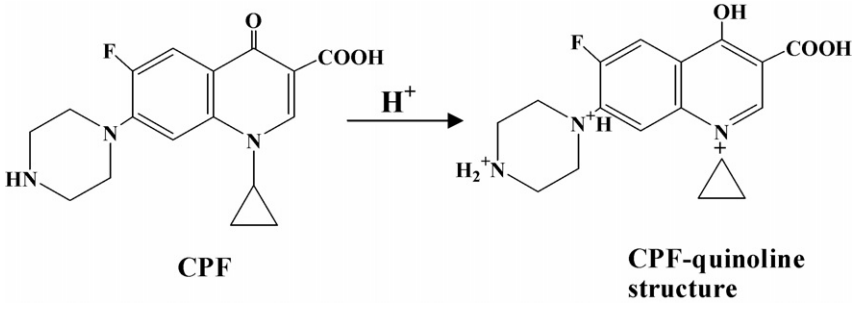

Fig. 8. Reaction scheme of CPF with HNO_3.

Table 4
Determination of QNs in prawn sample

Sample	Quinolone	Found[a] (ng/ml) (HPLC–CL)	Added (ng/ml)	Recovered[a] (ng/ml) (HPLC–CL)	Recovery[a] (%) (HPLC–CL)
A[b]					
1	OF	17.86 ± 0.47	10	10.14 ± 0.52	101.4
	NF	6.99 ± 0.71	10	8.95 ± 0.35	89.5
	CPF	4.11 ± 0.15	10	8.80 ± 0.28	88.0
	LOME	ND	10	9.44 ± 0.80	94.4
2	OF	5.09 ± 0.74	10	10.08 ± 0.39	100.8
	NF	7.32 ± 0.16	10	10.44 ± 0.45	104.4
	CPF	6.13 ± 0.09	10	10.45 ± 0.27	104.5
	LOME	ND	10	9.73 ± 0.31	97.3
3	OF	5.93 ± 0.67	10	9.03 ± 0.43	90.3
	NF	3.53 ± 0.75	10	8.89 ± 0.58	88.9
	CPF	2.86 ± 0.89	10	8.86 ± 0.55	88.6
	LOME	ND	10	9.73 ± 0.14	97.3
4	OF	3.74 ± 0.71	10	9.60 ± 0.62	96.0
	NF	3.73 ± 0.91	10	10.78 ± 0.81	107.8
	CPF	2.98 ± 0.21	10	10.72 ± 0.37	107.2
	LOME	ND	10	10.60 ± 0.22	106.0
5	OF	4.19 ± 0.82	10	9.62 ± 0.72	96.2
	NF	6.35 ± 0.73	10	9.15 ± 0.59	91.5
	CPF	5.23 ± 0.74	10	9.29 ± 0.80	92.9
	LOME	ND	10	10.02 ± 0.73	100.2
B[c]					
1	OF	2.47 ± 0.23	10	8.83 ± 0.50	88.3
	NF	6.25 ± 0.57	10	10.51 ± 0.41	105.1
	CPF	4.35 ± 0.91	10	10.30 ± 0.93	103.0
	LOME	ND	10	9.62 ± 0.28	96.2
2	OF	5.59 ± 0.28	10	9.58 ± 0.35	95.8
	NF	4.63 ± 0.30	10	9.59 ± 0.17	95.9
	CPF	2.81 ± 0.68	10	9.12 ± 0.24	91.2
	LOME	ND	10	8.89 ± 0.20	88.90
3	OF	1.48 ± 0.47	10	8.87 ± 0.59	88.7
	NF	4.38 ± 0.43	10	9.92 ± 0.52	99.2
	CPF	2.34 ± 0.32	10	10.01 ± 0.49	100.1
	LOME	ND	10	9.26 ± 0.53	92.6
4	OF	0.94 ± 0.80	10	9.96 ± 0.67	99.6
	NF	2.87 ± 0.72	10	10.94 ± 0.16	109.4
	CPF	1.25 ± 0.34	10	10.70 ± 0.29	107.0
	LOME	ND	10	10.34 ± 0.38	103.4
5	OF	5.50 ± 0.73	10	9.98 ± 0.67	99.8
	NF	2.33 ± 0.69	10	102.5 ± 0.57	102.5
	CPF	1.91 ± 0.89	10	9.93 ± 0.90	99.3
	LOME	ND	10	9.62 ± 0.31	96.2

ND: not detected.
[a] Mean value ± R.S.D. ($n = 3$).
[b] Prawn sample with shell.
[c] Peeled prawn sample without shell.

4. Conclusion

A novel HPLC–CL detection method was established for the determination of QN residues in prawn. This method was based on enhancement by QNs of the CL from the Ce(IV)–$Ru(bpy)_3^{2+}$–HNO_3 system. The method allows for sensitive detection of ofloxacin (OF), norfloxacin (NF), ciprofloxacin (CPF) and lomefloxacin (LMF) residues in prawn. 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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