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ORIGINAL PAPER

Spectrofluorimetric Determination of Fluoroquinolones in Honey with 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone in the Presence of β-cyclodextrin

Qinghai Xia · Yaling Yang · Mousheng Liu

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Abstract A simple and sensitive spectrofluorimetric method was developed for the determination of four fluoroquinolone antibacterials namely norfloxacin (NOR), ofloxacin (OFL), ciprofloxacin (CIP) and gatifloxacin (GAT) in honey through charge transfer (CT) complex formation with 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), and then the inclusion complexes of FQs-DDQ with β-cyclodextrin (β-CD) were formed, which resulted in drastic fluorescence enhancement. The effect of several parameters including the concentration of reactants, reaction temperature, time and ultrasonic treatment on the efficiency of the proposed method involving CT reaction and inclusion interaction was systematically investigated. Under the optimum conditions, the limits of detection (LODs) for four FQs in honey varied from 11.6 to 15.4 μg/kg (signal-to-noise ratio (S/N)=3). The intra- and interday relative standard deviations (RSDs) were 1.6–4.0 % (n=5) for four FQs. The calibration graph was linear from 42.8 to 1346.8 μg/kg with correlation coefficients not less than 0.9905. The recoveries of four FQs at three different spiked concentrations in honey samples ranged from 80.9 % to 92.8 %. The results indicated that the method was successfully applied for analyzing FQs in honey.

Keywords Fluoroquinolones · Honey · 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone · β-cyclodextrin · Charge transfer · Inclusion complexes

Q. Xia
Faculty of Environmental Science and Engineering, Kunming University of Science and Technology, Kunming 650500, People's Republic of China

Q. Xia · Y. Yang (✉) · M. Liu
Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming 650500, People's Republic of China
e-mail: yilyil8@163.com

Introduction

Fluoroquinolones (FQs), piperazinyl derivates of the quinolone nadixilic acid, were a large class of synthetic antibiotics, and were initially employed in the treatment of a wide variety of diseases with abroad spectrum activity against Gram-positive and Gram-negative bacteria [1–3], However, as the use of fluoroquinolones has increased, a continual increase in bacterial resistance to these drugs has been widely recognized, it is well known that their residues can appear in foods of animal origin like meat, honey, milk or its derivatives [4–6].

Honey is a natural product produced by bees from the nectar of plants, which is a nutritious wholesome food with world-wide consumption especially among elderly and children. In recent years increasing public concern about possible health risks of food consumption is being observed. For example, chemical residues of veterinary drugs like streptomycin, chloramphenicol, sulfonamides, tetracycline antimicrobials and other contaminants such as pesticides, heavy metals, etc. have been found in honey [7–12] with its subsequent concern of the population. Consequently, it is necessary to monitor and control residual levels of these compounds in order to meet regulatory requirements and especially to protect the consumer and the environment.

More and more analysis methods for FQs were reported, which received close attention in analytical chemistry. For quality control purpose, methods such as the following were developed: spectrophotometry [13, 14], spectrofluorometry [15–17], chromatography [18–22], chemiluminescence [23–26], electrochemical analysis [27–29], immunoassay [30], etc. Fluorescence spectrometry, its main advantage is its simplicity and rapidity. Compared with spectrophotometric method, fluorescence spectroscopy processes good analytical selectivity, higher capacity against blank interference, and can improve the limit of detection. The method has already been

described for determination of FQs through sensitization of fluorescence by complex formation with small molecules such as chloranilic acid (CL) [31], tetracyanoethylene (TCNE) [32], 7,7,8,8-tetracyanoquinodimethane (TCNQ) [33], 2,3,5,6-tetrabromo-1,4-benzoquinone (TBBQ) [34], *p*-amino benzoic acid (PABA) [35], biological macromolecules [36–38], metal ions [39–41], etc.

In this work, a simple, rapid, highly sensitive and low-cost method was developed for the determination of the FQs in honey by DDQ sensitized fluorescence through CT complex formation coupled with inclusion complexes by β-cyclodextrin. To the best knowledge of the authors, the method has been firstly applied for determining FQs residues in honey samples.

Experimental

Chemicals and Reagents

All the chemicals and reagents involved in this study were of analytical grade and were used as supplied by the manufacturer. The standards of NOR, OFL, CIP and GAT were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and were used without further purification. Their chemical structures were shown in Fig. 1. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone, β-cyclodextrin, were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methanol was purchased from Merck (Darmstadt, Germany). The double distilled water was used as experimental water throughout the research.

The stock solutions of NOR, OFL, CIP, GAT (100 μg/mL), their mixed stock solution (100 μg/mL) and DDQ (1 mg/mL) were prepared in methanol. They were all kept in the freezer at 4 °C and can be stable for 2 months, β-cyclodextrin saturated solution (18.5 mg/mL, 25 °C, dissolved by heating and ultrasonic treatment) was prepared in double distilled water and kept in the dark at room temperature. The working solutions of four FQs were obtained by appropriate dilution of the stock solution with methanol.

Apparatus

Fluorescence measurements were carried out on Shimadzu RF-5301 PC spectrofluorimeter (Kyoto, Japan) equipped with a hydrogen discharge lamp and 1.0 cm quartz cell. The slit width of both excitation and emission monochromators was set at 5 nm and scan rate was 250 nm/min. Sample centrifugation was performed on a model H-1650 centrifuge (Xiangyi Centrifuge Instrument Co., Ltd., Changsha, China). Ultrasonic treatment was performed on a model SK5210LHC ultrasonic cleaner, working at 35–53 kHz with maximum output power of 100 W (Shanghai Kudos Ultrasonic Instrument Co., Ltd., China). A digital display temperature control water-bath Model XMTB (Central Experimental Furnace Co., Ltd., Tianjin, China) was used to control the experimental temperature. A Model MixPlus vortex mixer (ABSON Scientific Instruments Company, Hefei, China) was used to mix honey samples and solutions.

Preparation of Honey Samples

Honey samples were obtained from a local supermarket in Kunming. Preliminary analyses showed that there were no residues of target analytes in the samples and they were used as blank matrices. The samples were stored at 4 °C and analysed within 24 h.

3.0 g honey sample was accurately weighed into a 10 mL PTFE centrifuge tube, 6 mL of methanol was added and mixed with a vortex mixer for complete dissolution, then spiked with known variable amounts of FQs, incubated for 10 min at room temperature, the samples were diluted with methanol to 10.0 mL and mixed for a further 2 min, and then centrifuged for 5 min at 8,000 rpm. The supernatant solution was collected and filtered through a 0.45 μm micropore filter prior to CT reaction and inclusion interaction with analytes. Blank samples were prepared in the same way as above but without the compound-spiking step.

CT Reaction Procedures

8 mL treated sample containing spiked FQs was pipetted into a 10 ml graduated glass tube, and 0.1 ml 1 mg/ml of DDQ solution was added, then the solution was diluted to volume with methanol and mixed thoroughly. The solution in the tube was sealed with stopper and thermostated at 55.0±0.5 °C for 90 min, and then cooled to room temperature. The fluorescence intensities for complexes of NOR, OFL, CIP, and GAT with DDQ were measured at λ_{ex}=280, 294, 278, and 290 nm against a reagent blank, respectively.

Inclusion Complexes Procedures

In a 10 mL graduated glass tube, 8.0 mL FQs-DDQ solution and 1.0 mL β-CD solution were added, and the mixed solution was diluted to final volume with methanol and shaken thoroughly. Then the solution in the tube sealed with stopper was thermostated at 45.0±0.5 °C for 60 min and the fluorescence intensities were measured at optimal excitation wavelength.

Results and Discussion

Fluorescence Spectra

Figure 2 and Table 1 showed the excitation and emission spectra for studied FQs and their complexes with DDQ and

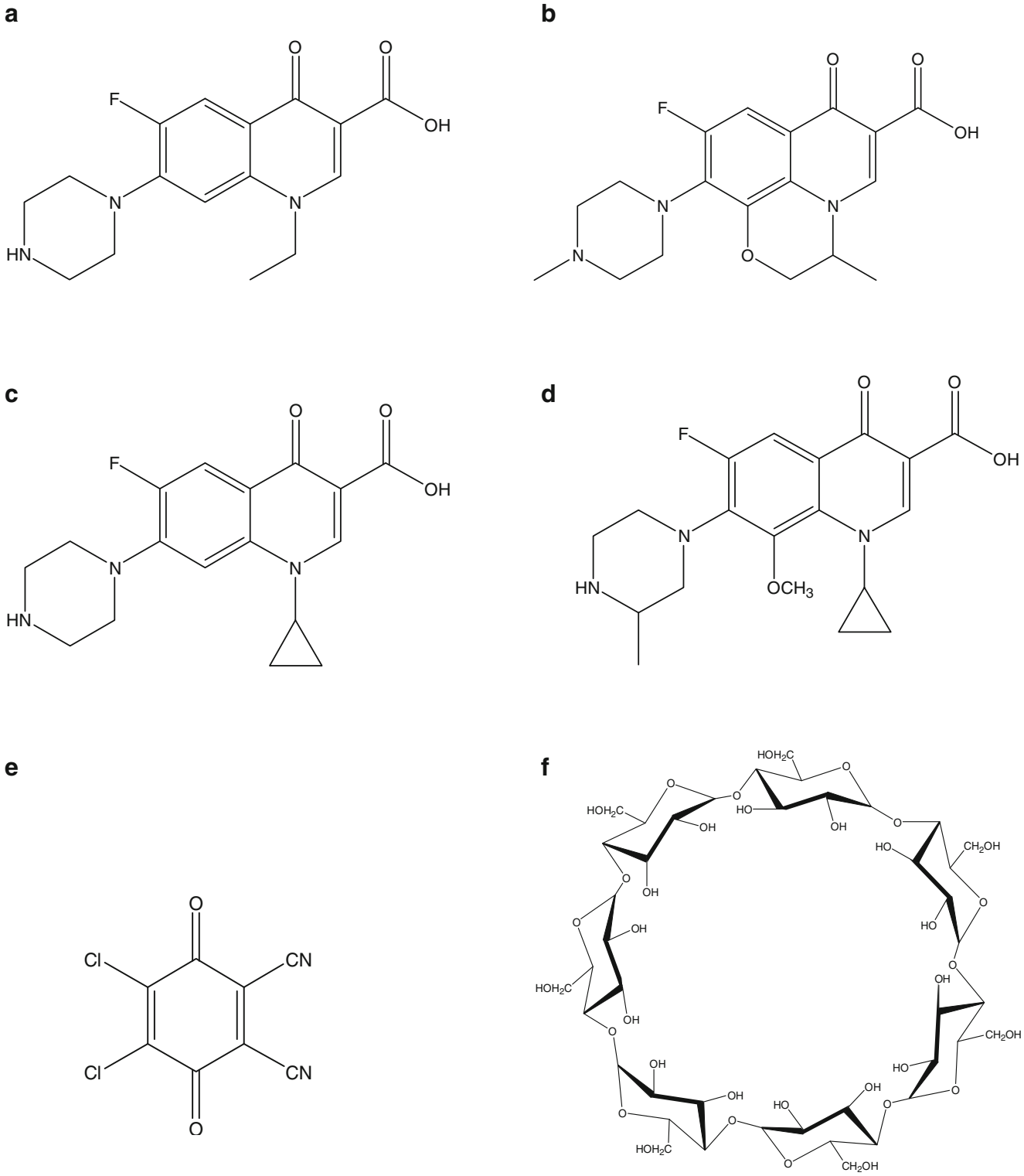

Fig. 1 Structure of NOR(**a**), OFL(**b**), CIP(**c**), GAT(**d**), DDQ(**e**) and β-CD(**f**)

β-CD. It can be seen that the solution of the four FQs has weak native fluorescence. However, through the CT reaction with DDQ, the fluorescence intensity increases substantially, the sensitivity is enhanced by at least a factor of 1.5, and the complexes move to longer wavelengths showed in Table 1. The modification of the features of the fluorescence spectra is considered to be a result of complex formation between FQs and DDQ.

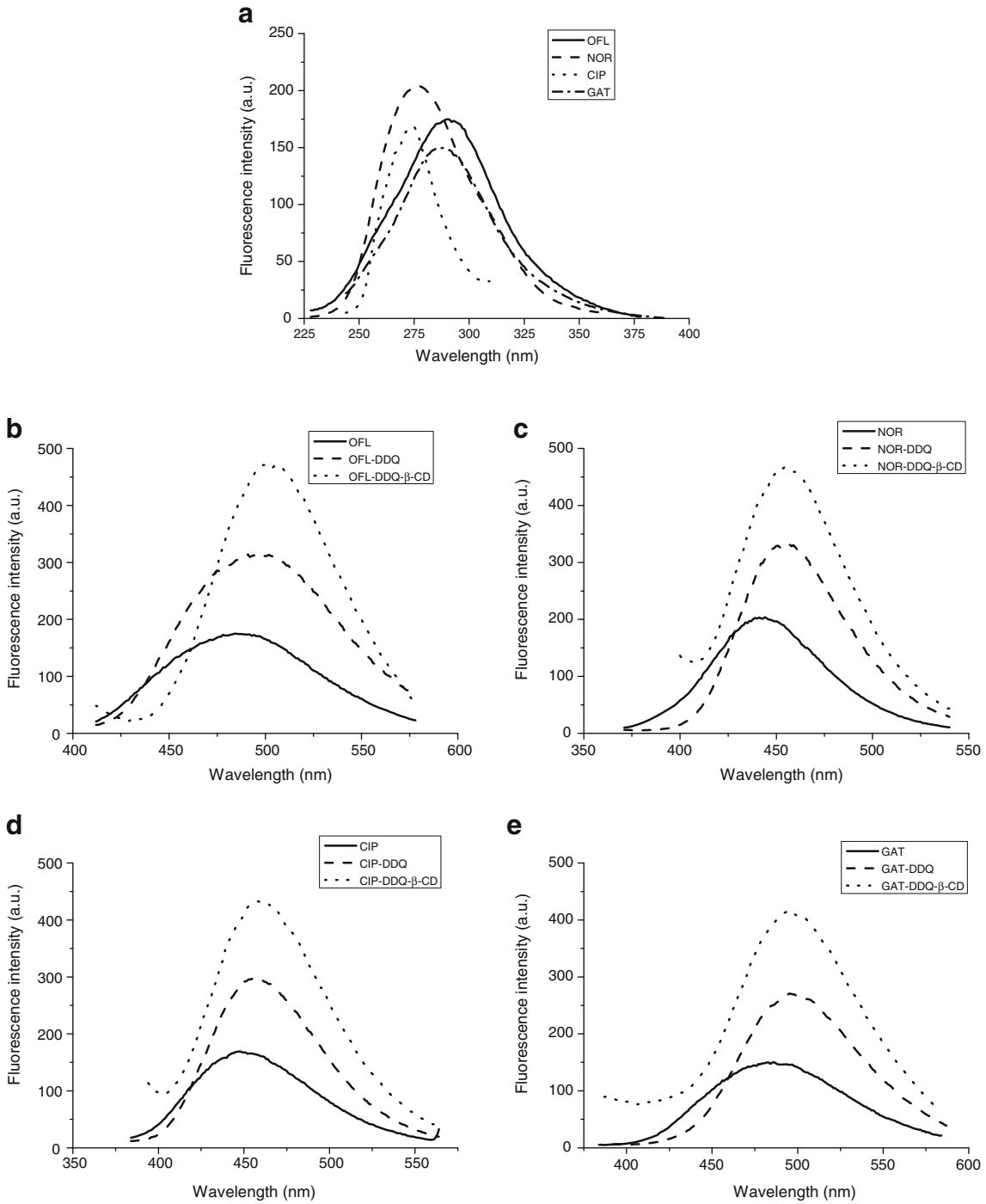

Fig. 2 Fluorescence excitation spectra of the four FQs (**a**) (0.1 μg/mL), emission spectra of OFL (**b**), NOR (**c**), CIP (**d**), GAT (**e**) and their complexes with DDQ and β-CD

The fluorescence excitation and emission spectra of FQs-DDQ-(β-CD) system was showed in Fig. 2. There is no shift of their complex emission peaks in the presence of β-CD, but the fluorescence intensity of FQs-DDQ is enhanced by more than a factor of 1.4 in the presence of β-CD.

Optimization of CT Reaction

Four FQs studied in this paper have similar molecular structure, there is almost no difference on the effect of conditions for CT reaction and inclusion interaction.

Table 1 Excitation and emission wavelengths of four FQs (0.1 μg/mL), FQs-DDQ and FQs-DDQ-(β-CD) complexes

Species	Excitation wavelengths(nm)	Emission wavelengths(nm)	Fluorescence intensity (a.u.)
NOR	277	444	204
OFL	290	490	175
CIP	274	448	169
GAT	286	486	150
NOR-DDQ	280	452	326
OFL-DDQ	294	502	314
CIP-DDQ	278	458	297
GAT-DDQ	290	495	271
NOR-DDQ-(β-CD)	280	452	468
OFL-DDQ-(β-CD)	294	502	451
CIP-DDQ-(β-CD)	278	458	433
GAT-DDQ-(β-CD)	290	495	415

Here OFL was chosen for description of the optimization of reaction conditions.

Effect of DDQ Concentration

The influence of DDQ concentration was studied in the range of 1×10^{-5}–1×10^{-2} mg/mL for 0.1 μg/mL FQs spilked in honey simple. The relative fluorescence intensity increased with increasing DDQ concentration up to 5×10^{-3} mg/mL and there was no enhancement for fluorescence intensity of FQs-DDQ system at higher concentrations. The final DDQ concentration of 5×10^{-3} mg/mL was accordingly used for all of the studied FQs.

Effect of Reaction Temperature and Time

The effect of temperature on the formed FQs-DDQ complexes was studied in the range of 20–80 °C. The fluorescence intensity was found to depend on formation of FQs-DDQ complexes, whose fluorescence intensity increased in the range 20–55 °C, and the relative fluorescence intensity decreased due to dissociation of the complexes at temperature higher than 55 °C. Therefore, the temperature for CT reaction of FQs and DDQ was carried out at 55±0.5 °C. It was further found that it took about 80 min to fully form the complexes which were stable for at least 24 h.

Optimization of Inclusion Complexes

Effect of β-CD Concentration

The influence of β-CD concentration in the range of 1×10^{-4}–1×10^{-3} mol/L on the fluorescence intensity of FQs-DDQ was studied (Fig. 3). The fluorescence intensity was enhanced with increasing of β-CD concentration in the range of 1×10^{-4}–5.0×10^{-4} mol/L and there was almost no enhancement for further increasing concentration. The results showed that 5.0×10^{-4} mol/L β-CD was appropriate for maximum fluorescence intensity of FQs-DDQ complexes.

Effect of Ultrasonic Treatment

In β-CD inclusion procedure, the effect of ultrasonic treatment on inclusion of FQs-DDQ complexes was studied, it was found that the addition of ultrasonic could shorten the time of inclusion and the inclusion efficiency was also improved, which can reflect through determination of fluorescence intensity of FQs-DDQ-(β-CD) system. When ultrasonic power is 50 W at 53 kHz, and ultrasonic time is 60 min at 40 °C, there is a maximum of fluorescence intensity of 454 compared to 421 without ultrasound-assisted inclusion of FQs-DDQ complexes. The results were showed in Fig. 4.

Effects of Temperature and Time

Response surface methodology (RSM) was applied to identify optimum levels of two variables of the temperature (°C) and time (min) which affected inclusion of FQs-DDQ complexes, The temperature was tested in the range of 20–70 °C and time was tested in the range of 20–80 min. The experiments were designed according to the Box-Behnken design (BBD) used to develop correlation among the variables of the temperature, time and fluorescence intensity. Data were analyzed by multiple regressions through the least-square method. The results showed that the temperature of 45± 0.5 °C and the time of 50 min was optimal to form the

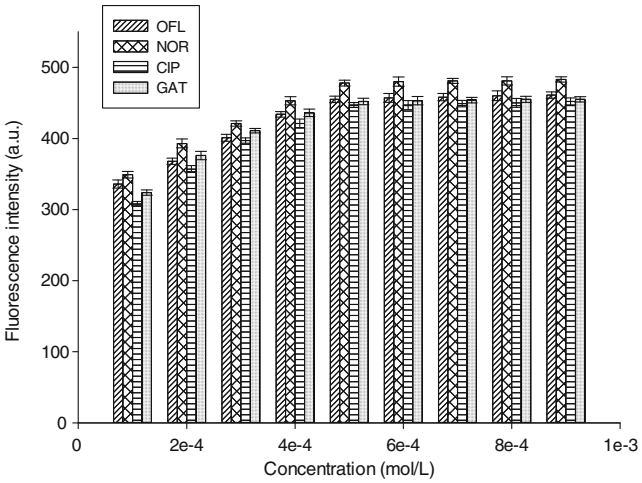

Fig. 3 Effect of β-CD concentration on inclusion of four FQs-DDQ complexes

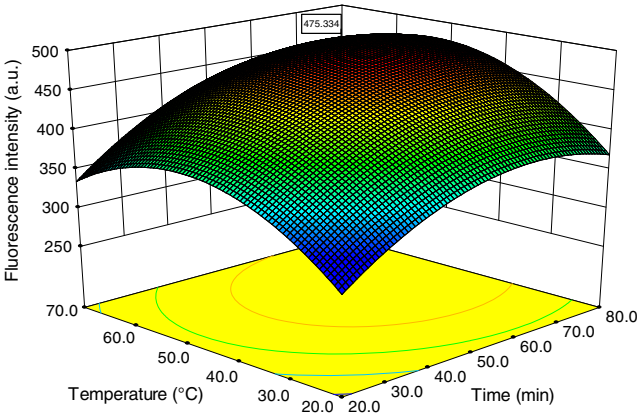

Fig. 5 Response surfaces of effects of temperature and time in the Box-Behnken design for the focused OFL-DDQ complex inclusion optimization

inclusion complexes completely since there was a maximum fluorescence intensity of 475 for FQs-DDQ-(β-CD) system (Fig. 5).

Discussion of Interaction Mechanism

CT Reaction Mechanism

CT reaction within a molecular complex forming an electron donor *D* and electron acceptor *A* involves a resonance with a transfer of charge from *D* to *A*, the resulting electrostatic attraction provides a stabilizing force for the molecular complex. CT reaction has been extensively utilized for the determination of electron donating basic nitrogenous compounds. NOR, OFL, CIP and GAT are nitrogenous compounds that act as *n* donors to the π acceptors such as DDQ, which could

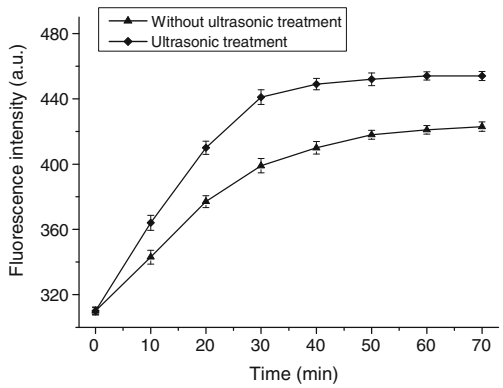

Fig. 4 Effect of ultrasonic treatment on inclusion of OFL-DDQ complex by β-CD

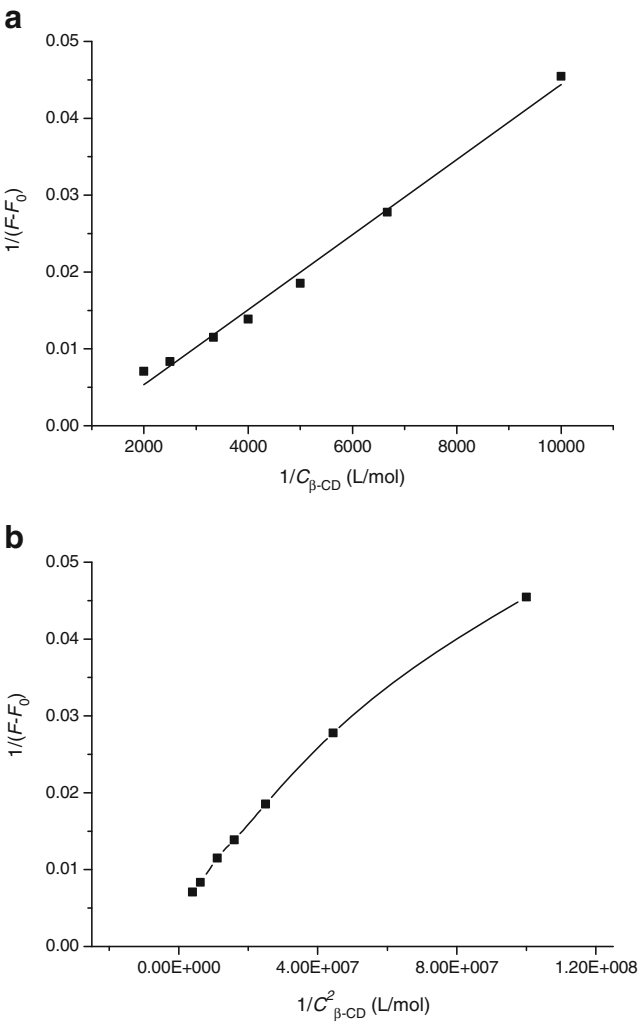

Fig. 6 **a** Double reciprocal plot obtained from $1/(F\text{-}F_0)$ plotted against $1/C_{\beta\text{-CD}}$ at pH7.0, **b** Double reciprocal plot obtained from $1/(F\text{-}F_0)$ plotted against $1/C^2_{\beta\text{-CD}}$ at pH7.0

form n-π complexes with these drugs by CT reaction as shown in the following equation:

$$\underset{\text{Donor}}{\ddot{D}} + \underset{\text{Acceptor}}{A} \rightarrow \underset{\text{D-A complex}}{\left[\ddot{D} \rightarrow A\right]} \rightarrow \underset{\text{Radical ions}}{\dot{D}^{+} + \dot{A}^{-}}$$

The stoichiometry of the reactions was studied by Job's method, and it was found that the ratios were 1:1 (donor/acceptor) for four FQs. This indicates that only one nitrogen atom is responsible for the complex formation although four studied FQs have three nitrogen atoms. This ratio may be due to the presence of the fluorine atom acting as an electron drawing group in the molecule of four FQs. The benzene ring has lower electron density, but nitrogen atom in four of piperazingl has more electron density and less sterically hindered. So n-π CT complexes were formed.

Determination of Stoichiometry and Inclusion Constant

CDs are untoxic macrocyclic oligosaccharides, consisting of (α-1,4)-linked α-L-glucopyranose units, the most common CDs used as formulation vehicles are α-, β- and γ-CDs. β-CDs are composed of seven glucopyranose units with the shape of a torus which has a hydrophilic exterior and a hydrophobic interior. They are in host guest chemistry due to their unique ability to form inclusion complexes with numerous compounds.

It was desirable for noticing the formation of inclusion complex with a ratio of 1:1 or 1:2. Employing the typical double reciprocal plots we obtained the stoichiometry and inclusion constant for each host-guest combination from the analysis of the sequential changes of fluorescence intensity at various host concentrations:

$$\frac{1}{F - F_0} = \frac{1}{(F_\infty - F_0)KC_{\beta-CD}} + \frac{1}{(F_\infty - F_0)} \quad (1)$$

$$\frac{1}{F - F_0} = \frac{1}{(F_\infty - F_0)K'C^2_{\beta-CD}} + \frac{1}{(F_\infty - F_0)} \quad (2)$$

Where F was the determined fluorescence intensity of the FQs-DDQ solutions at each β-CD concentration. F_0 and F_∞ were the fluorescence intensity in the absence of β-CD and when all the FQs molecules were included, respectively. K and K' were the inclusion constants.

$1/(F\text{-}F_0)$ against $1/C_{\beta\text{-CD}}$ and $1/(F\text{-}F_0)$ against $1\Big/C^2_{\beta-\text{CD}}$ were plotted. Good linear relationship (r=0.9910) was observed in Fig. 6(a). Figure 6(b) showed that when $1/(F\text{-}F_0)$ was ploted against $1\Big/C^2_{\beta-\text{CD}}$, no linear relationship was gained. These confirmed that β-CD and FQs molecules formed host-guest complexes in 1:1 stoichiometry. The inclusion constant (K) was calculated to be 4.84×10^2L/mol and the RSD was 4.2 % (n=5).

The interaction of guest molecules OFL-DDQ with β-CD led to apparent changes in their chemical properties and the interaction process was showed in Fig. 7.

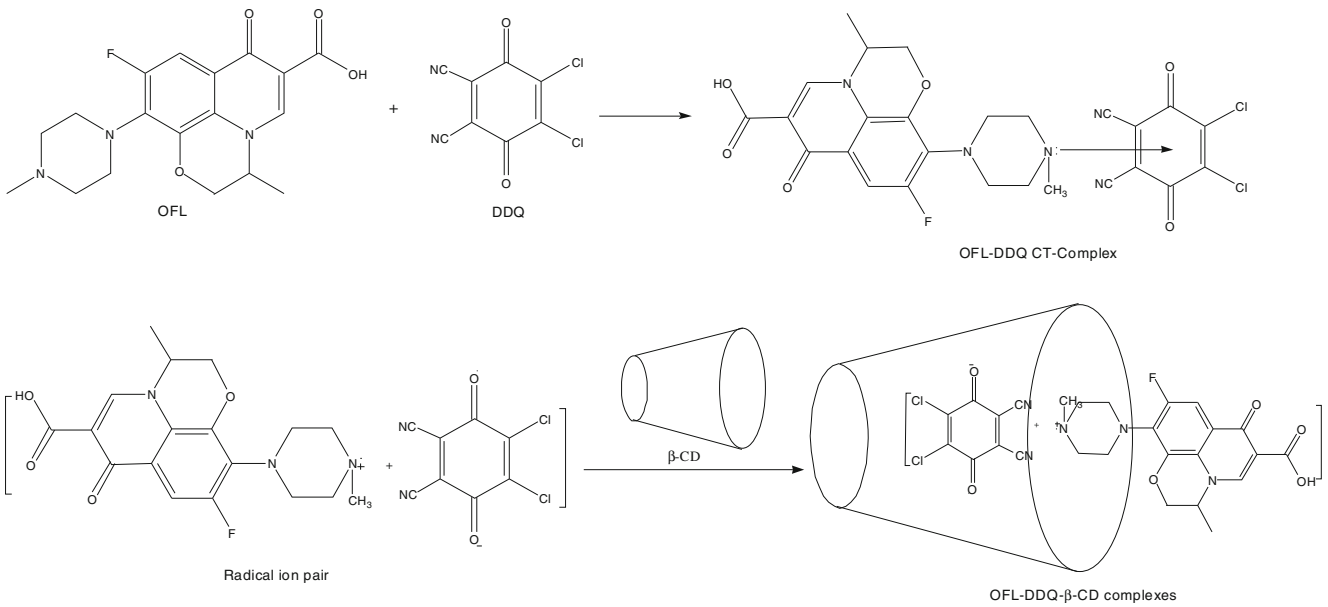

Fig. 7 Interaction process of the OFL-DDQ complex with β-CD for 1:1 inclusion complex

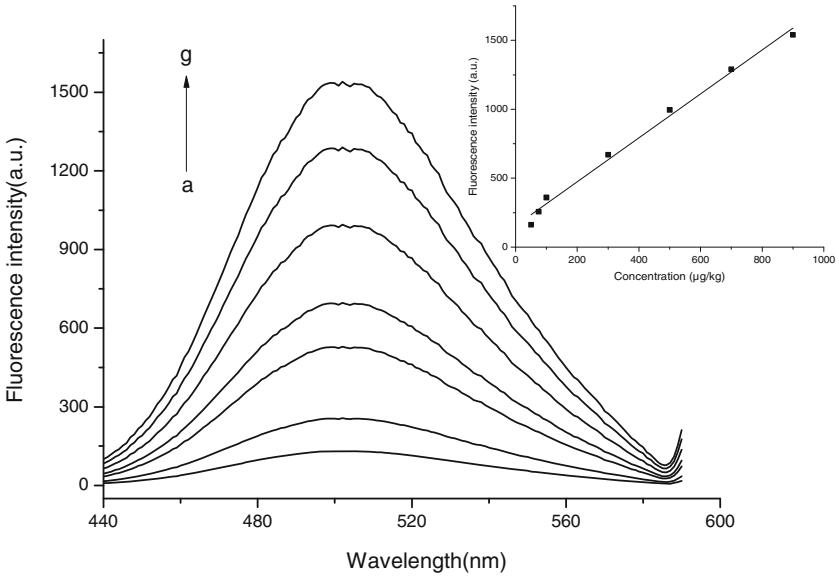

Fig. 8 Fluorescence emission spectra of OFL at different concentrations with DDQ in the presence of β-CD and its linear graph

Method Evaluation

Linearity, Limits of Detection and Limits of Quantification

Matrix-calibration curves for honey free of analytes and spiked with different concentration levels were established. The linear regression analyses were performed using spectral peak height ratios against the respective analyte concentration. The concentrations of NOR and OFL were in the range of 50–900 μg/kg, at seven levels of 50, 75, 100, 300, 500, 700, 900 μg/kg and the concentrations of CIP and GAT were in the range of 75–1,100 μg/kg at levels of 75, 100, 300, 500, 700, 900, 1,100 μg/kg. The four analytes exhibited good linearity with good squared regression coefficients, ranging from 0.9905 to 0.9946. These results were presented in Fig. 8 and Table 2. The LODs (S/N=3) and LOQs (S/N=10) for four FQs were found to be 11.6–15.4 μg/kg and 38.6–51.2 μg/kg, respectively. As it can be observed that the proposed method has low LODs and can be used for trace analysis of FQs in honey samples.

Precision

In this study, the precision of the proposed method was evaluated by determining intra- and inter-day RSDs of the analytes. The intra-day precision was performed by analyzing spiked honey samples six times in 1 day at three different concentrations of 50, 200 and 800 μg/kg. The inter-day precision was performed over 3 days. The results obtained were shown in Table 3. The RSDs of intra- and inter-day tests ranging from 1.8 % to 4.0 % and from 1.6 % to 3.6 % were obtained, respectively.

Extraction Recovery

In order to check the trueness of the proposed methodology, recovery experiments were carried out in honey samples.

Table 2 Characteristic parameters for inclusion complexes of FQs-DDQ with β-CD

Parameters	FQs-DDQ-(β-CD) derivatives			
	NOR	OFL	CIP	GAT
Linear equation	y = 1.36x + 13.5	y = 1.59x + 11.9	y = 1.21x + 12.7	y = 1.18x + 14.2
Correlation coefficients (r)	0.9946	0.9905	0.9915	0.9921
Linear range (μg/kg)	42.8–946.5	47.5–1235.6	51.2–1254.5	53.7–1346.8
Limit of detection (μg/kg)	11.6	13.7	14.6	15.4
Limit of quantitation (μg/kg)	38.6	45.5	48.5	51.2

Table 3 Intra-day and inter-day precision of FQs with DDQ in the presence of β-CD (n=5) (concentration: μg/kg)

Added	NOR-DDQ-(β-CD)		OFL-DDQ-(β-CD)		CIP-DDQ-(β-CD)		GAT-DDQ-(β-CD)	
	Found mean.	RSD (%)	Found mean.	RSD (%)	Found mean.	RSD (%)	Found mean.	RSD (%)
Intra-day								
50	45.2	2.9	44.3	3.1	42.3	3.6	42.7	4.0
200	179.4	2.6	186.7	2.9	174.8	3.3	176.3	3.7
800	686.6	1.8	729.0	2.6	664.4	2.7	659.8	3.2
Inter-day								
50	42.1	2.7	42.4	2.8	40.1	3.4	40.8	3.6
200	172.7	2.2	175.5	2.3	165.7	2.8	167.3	2.7
800	665.3	1.6	689.1	1.9	653.2	2.2	647.4	2.1

Samples were respectively spiked with standards of four FQs at three concentration levels. The results were listed in Table 4. The recoveries of the samples ranged from 80.9 % to 92.8 % and RSDs were in the range of 1.8–3.7 %, respectively. The preceding results demonstrated that the proposed method was feasible for the quantitative determination of FQs in honey samples.

Application to Real Samples

To demonstrate the applicability of the proposed method in genuine honey samples, matrix-matched calibration was used for the analysis of real honey samples. The honey samples were spiked with four FQs standards for the validation of the proposed method. As illustrated in Table 4, NOR, OFL, CIP and GAT were detected.

Conclusion

In summary, 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone sensitized fluorescence followed by inclusion using β-cyclodextrin has been developed for determination of FQs in honey samples. FQs can form complexes with DDQ and the fluorescence of drug is sensitized based on formation of charge-transfer complexes, the presence of β-CD in FQs-DDQ complexes system can greatly enhance fluorescence of FQs-DDQ through inclusion complexes. The purposed method can reduce the limit of detection of FQs residues in honey samples. It provides a simple, sensitive and economical method and is successfully applied to honey for analysis of FQs residues. This method is helpful to expand the analysis applicability of FQs in various solid and semisolid matrices.

Table 4 Analysis of four FQs and recovery of the proposed method in honey (n=5)

Sample analytes	Spiked honey				
	Originals (μg/kg)	Amount added (μg/kg)	Amount found (μg/kg)	Recovery (%) ± SD	RSD (%)
NOR	136.3	50	158.0	84.8±3.2	3.7
		100	213.1	90.2±2.0	2.2
		200	293.4	87.2±2.1	2.4
OFL	245.8	100	299.8	86.7±2.5	2.9
		200	413.9	92.8±1.7	1.8
		400	586.1	90.8±2.6	2.9
CIP	352.5	100	373.2	82.5±2.8	3.5
		300	564.6	86.5±1.7	2.0
		500	714.9	83.9±2.4	2.9
GAT	176.2	100	236.3	85.5±2.2	2.7
		200	333.8	88.7±1.9	2.2
		400	466.2	80.9±2.6	3.2

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