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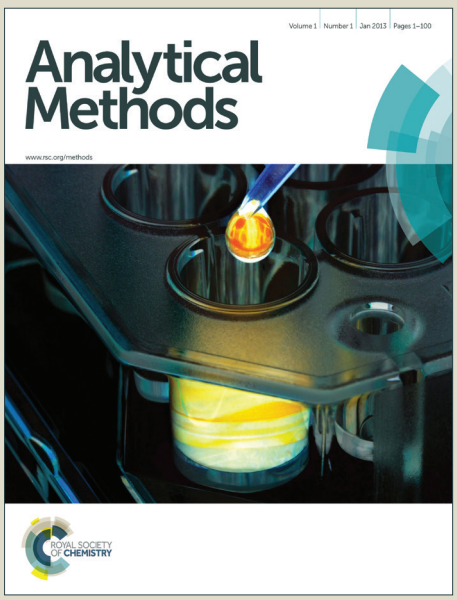

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High-performance liquid chromatography with resonance Rayleigh scattering detection for determining four tetracycline antibiotics

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Running title:

HPLC-RRS for determining four tetracycline antibiotics

List of nonstandard abbreviations:

resonance Rayleigh scattering (RRS), tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DOTC), Titan yellow (TY), Britton–Robinson buffer solution (BR).

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Abstract

A rapid high-performance liquid chromatography (HPLC) technique incorporating resonance Rayleigh scattering (RRS) detection was developed for determination of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DOTC). This method was based on the weak intensity of the RRS of TCs and its enhancement with addition of Titan yellow (TY) and $CuSO_4$ in pH 3.5 Britton–Robinson buffer solution. The RRS signal was detected at $\lambda_{ex} = \lambda_{em} = 450$ nm. The chromatographic separation used (25:75, v/v) methanol–phosphate buffer (pH 3) as the mobile phase. The analytical technique was validated for intra- and inter-day variations and the detection limits of the assay were 0.668 μg mL^{-1} for TC, 0.342 μg mL^{-1} for OTC, 0.480 μg mL^{-1} for CTC and 0.132 μg mL^{-1} for DOTC at a signal-to-noise ratio of 3.0. The conditions for separation and detection were optimized and the reasons for the RRS enhancement were evaluated. The developed method was validated for analysis of TCs in water samples. The recoveries were acceptable (range 97.4–102.4%).

Keywords:

High-performance liquid chromatography; Resonance Rayleigh scattering; Tetracycline antibiotics

1. Introduction

Tetracycline antibiotics (TCs) such as oxytetracycline (OTC), tetracycline (TC), doxycycline (DOTC) and chlortetracycline (CTC) have broad spectrum antibiotic activity and can be used for resistant Gram-positive and Gram-negative bacterial infections [1]. They are also used as therapeutic agents against cholera [2] and fluorescent probes for cancer diagnoses [3]. In addition, they are widely added to animal feed to prevent diseases and increase animal growth rates [4]. Because of their widespread use, TCs are present in the aquatic environment. Although they are present at only trace levels, consumption of TCs contaminated water for a long time has potential risks. For example, it will increase resistance of bacteria[5,6] and have impact on the health of consumers[7]. Therefore, development of a highly sensitive, rapid and simple method for determining trace TCs in water samples is important.

As reported in the literature, methods of analysis of TCs include microbiological [8,9], fluorescent [10], spectrophotometric [11,12] and chromatographic methods [13,14]. Microbiological methods are expensive, time-consuming and have poor sensitivity and specificity. Spectrophotometric and fluorescent methods have higher sensitivity than microbiological methods, but suffer from matrix interference. Among these methods, chromatographic methods are used most often for TCs analysis because they are simple and highly selective. In Chinese Pharmacopoeia [15], high-performance liquid chromatography (HPLC) is described as a method for the determination of OTC, TC, DOTC and CTC. However, the sensitivity of HPLC-UV and even HPLC-FL is not sufficient for detecting trace amounts of antibiotics.

Resonance Rayleigh scattering (RRS) can be used for simple quantitative analysis of trace levels of compounds. But its selectivity is not as good as that of HPLC [16-21]. If there are several compounds present in one sample, RRS cannot determine them at the same time.

Effective combination of these two methods could provide a highly sensitive and selective tool for quantitative analysis of antibiotics. LC-MS method[22,23] is the current state of art in analysis of tetracyclines in water samples. But it cannot be popularize because of high cost. HPLC-RRS technique has been applied for the

determination of proteins and pharmaceutical [24-25]. It has a good sensitivity and linear correlation in the detection of them. As a result, it proved the feasibility of this method. In the present study, HPLC-RRS method was developed for detecting antibiotics in water samples. To the best of our knowledge, this is the first report on HPLC-RRS of a ternary ion-association complex. This strategy is simple and shows excellent analytical performance. It could be applied in substances that lack useful spectroscopic and electrochemical properties for detection.

2. Experimental

2.1 Instrumentation

A HPLC (Shimadzu, Japan) consisting of a DGU-20A5R degassing unit, two LC-20AD pumps and RF-20A fluorescence detector was used. A PCX-BT post-column derivatization instrument was purchased from Tian Mei Da Scientific Instruments Co. Ltd. (Shenyang, China). RRS spectra were obtained with a Hitachi F-2500 spectrofluorophotometer (Tokyo, Japan), while the absorption spectra were measured by a UV-Vis 8500 spectrophotometer (Shanghai, China). The surface of ion-association complexes were observed by scanning electron microscopy (SEM, S-4800, Hitachi, Tokyo, Japan) at an acceleration voltage of 20 kV. The pH measurements were made with a model PHS-FE20 pH meter (Mettler-Toledo Instruments Co. Ltd., Shanghai, China). Double distilled water was prepared by a Millipore SZ-93 system (Shanghai Yarong Biochemical Apparatus Co., Shanghai, China).

2.2 Chemicals and reagents

The following chemicals were obtained: TC (Dr. Ehrenstorfer, Augsburg, Germany), OTC, CTC, DOTC and Titan yellow (TY) (Aladdin Industrial Corporation, Shanghai, China). HPLP-grade acetonitrile, methanol and isopropanol were purchased from Kermel (Tianjin, China). Britton–Robinson buffer solutions (BR, pH 3.5) were prepared by mixing together 0.2 mol L^{-1} NaOH and 0.4 mol L^{-1} solutions of H_3PO_4, HAc and H_3BO_3. Phosphate buffer (pH 3) was prepared by mixing together 0.025 mol L^{-1} solutions of H_3PO_4 and NaH_2PO_4. These solutions were purchased from the Chemistry Reagent Factory (Chongqing, China). $CuSO_4$ was purchased from

Rgent (Tianjin, China). All reagents were filtered through a 0.2-μm pore–size filter membrane (Millipore, Billerica, MA, USA) before use.

2.3 Preparation of sample solutions

Samples of TC, OTC, CTC and DOTC were weighed accurately and dissolved in water to prepare 1 mmol L^{-1} stock solutions. The standard solutions were stored at 4 °C in darkness. BR and phosphate buffer solutions with different pH values were prepared by mixing the appropriate solutions in different proportions. $CuSO_4$ solution was prepared by accurately weighing a sample and dissolving it in water. All stock solutions were kept at 0–4 °C during the experiment. Plasma was obtained after vortex–mixing for 1 min and centrifuging at 8000 r min^{-1} for 10 min. Acetonitrile of twice volume was added to 200 μL human serum to precipitate proteins. The supernatant was transferred into a 1.5 mL centrifuge tube and the organic phase was evaporated to dryness in a vacuum drying oven at 40 °C. The residue was dissolved in 100 μL mobile phase and was vortex–mixed for 30s and centrifuged at 8000 r min^{-1} for 10 min.

2.4 The HPLC-RRS system

The HPLC-RRS system is shown in Fig. 1. The chromatographic separation was achieved at 30 °C on a reversed-phase column (Phenomenex Luna 5 μm C18 100 Å, 250 mm × 4.6 mm). The mobile phase was 25:75 (v/v) methanol–phosphate buffer at the flow rate of 0.8 mL min^{-1}. After injection of a 50-μL aliquot of the sample solution, the separated components from the chromatogram column interacted with TY-$CuSO_4$ and BR. The RRS was monitored at $\lambda_{ex}=\lambda_{em}=450$ nm. The total analysis time was less than 20 min.

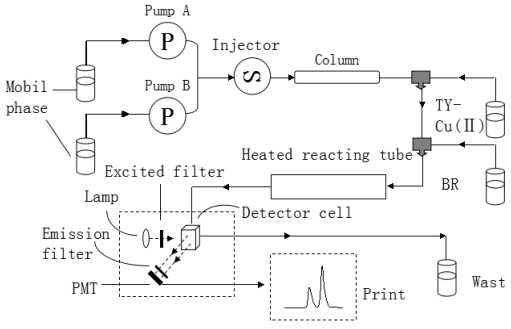

Fig. 1 The HPLC-RRS system.

3. Results and discussion

3.1 Investigation of the chromatographic conditions

3.1.1 Optimization of the detection wavelength

In this work, the RRS spectra of TY, Cu, TCs, TY-Cu(II) and their complexes were measured by the Hitachi F–2500 spectrofluorophotometer with synchronous scanning. RRS wavelengths from 400 nm to 500 nm were investigated to obtain the optimal detection wavelength for HPLC. The peak heights were maximized at 450 nm (Supplementary material, Fig.1) and this was selected as the detection wavelength.

3.1.2 Optimization mobile phase flow rate

The mobile phase flow rate affects the separation and RRS signal. Van Deemter studied from the kinetic theory only to find that enlarges chromatographic peak would influence plate height. And then, Van Deernter equations were put forward. It will lower the column efficiency if chromatographic separation which having an optimal flow rate was higher or lower than the velocity. In this work, when the flow rate was less than 0.6 mL min^{-1}, the analysis time was more than 25 minutes and the shape of the peak was even worse. The peak shape is short and wide. When the flow rate was higher than 1.1 mL min^{-1}, the resolution is low. As a result, OTC, TC, CTC and DOTC were not separated completely and the column pressure increased. From what we can see (Supplementary material, Fig. 2), when the flow rate was carried out at 0.8 mL min^{-1}, four TCs could be separated completely and the peaks were highest. Therefore, the flow rate was kept at 0.8 mL min^{-1} in our experiments.

3.1.3 Concentration and pH of phosphate buffer

When the concentration of the sodium dihydrogen phosphate buffer solution increased especially more than 0.050 mol L^{-1} , the retention times decreased but TC and OTC were not separated. When the concentration of the sodium dihydrogen phosphate buffer solution was reduced from 0.025 mol L^{-1}, the separation improved but the peak deformed and some are split peaks and trailed seriously. The optimum concentration of the sodium dihydrogen phosphate buffer solution was 0.025 mol L^{-1} (pH 3.0) and this concentration provided good separation and peak shapes.

The effect of concentration of phosphate buffer on separation may change the ionization mechanism about sample on the stationary phase. In the weak acid environment, the ionization degree of composition change with pH. Thereby

adjusting pH could change the separation selectivity on a wide range. TCs has three pKa values roughly about 3.0, 7.0 and 9.0, therefore in the aqueous solution of different pH would have three kinds of dissociative form[26]. In this experiment when the pH was 3.0, the reason for good separation of the TCs may be that the ionization of components is completely.

3.2 Selection of the RRS parameters

3.2.1 BR buffer solution and TY-Cu(II) selection

A series of BR buffer solutions (pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0) were tested (Supplementary material, Fig. 3). When the pH was between 2.0 and 5.0, the change of RRS intensity for four TCs was great and the highest peak occurred at 3.5. The result showed that when the pH was lower than 2.0, the TY could not dissociate into negative divalent acid radical anion which would stop the formation of ion association. When the pH was higher than 5.0, the positive charge of TCs would be decreased so that it could not react with the negative charge of TY completely. Consequently, the optimal pH was selected as 3.5. Different concentrations of TY (3 mmol L^{-1} to 18 mmol L^{-1}) and $CuSO_4$ (0.0375 mmol L^{-1} to 0.45 mmol L^{-1}) were also tested. Initially, the RRS intensity of the ion-association complexes increased obviously as the TY and $CuSO_4$ solution concentrations increased. However, at higher concentrations, the RRS intensity of the ion-association complexes decreased as the concentration increased. The optimal TY concentration was 9 mmol L^{-1} and the optimal $CuSO_4$ concentration was 0.225 mmol L^{-1}.

3.2.2 The length of the reaction tube

The influence of the length of the reaction tube was investigated using tube lengths ranging from 100 cm to 400 cm (Fig. 2). The length of the reaction tube affected the degree of the reaction between the TCs and the TY-Cu(II) probe. The RRS intensity gradually increased as the length of reaction tube increased up to 300 cm. This was because the TCs and TY-Cu(II) could interact completely in a longer tube. However, the RRS intensity decreased if the reaction tube was longer than 300 cm. The result showed that when the length of the reaction tube was less than 300 cm, the reaction time was too short and the formation of ion association was not complete. When the

length of the reaction tube was more than 300 cm, due to the interaction between the association and other drugs, the certain association objects would decrease. Therefore, a reaction tube of 300 cm was selected for subsequent experiments.

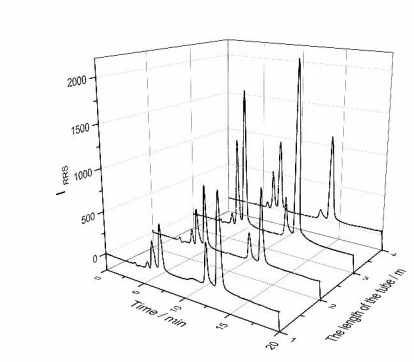

Fig. 2 Influence of the length of the reaction tube on the signal intensity

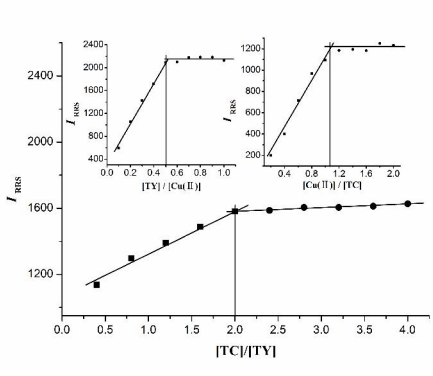

Fig. 3 Composition ratio for the ion-association complex of the TY-Cu(II)-TC system established by molar ratio method

3.3 Composition of the ternary complex and reasons for RRS enhancement

3.3.1 Structure of the complex and reaction mechanism

Taking TC as an example, the composition ratios of the binary chelate for the TC-Cu(II) system and ternary complex for TC-Cu(II)-TY system were established using Job's method of continuous variation and the molar ratio method. Fig. 3 shows that the composition ratio was 1:1 for TC-Cu(II) and 2:2:1 for TC-Cu(II)-TY. Therefore, the ternary complex was $[Cu{\cdot}TC]_2$ TY. The complex structure and reaction mechanism are discussed below.

(1) As reported in the literature [27-29], Cu(II) can react with TC to form 1:1 chelate cation $[Cu{\cdot}TC]^+$. Generally, the binding sites between a metal ion and a TC are at the carbonyl oxygen atom 11 and enol hydroxyl group 12 [30,31]. Therefore, the reaction equation for the formation of chelate cation $[Cu{\cdot}TC]^+$ is shown in Supplementary material, Fig. 4 A.

(2) In pH 3.5 BR buffer solution, the two $-SO_3H$ groups of TY are disassociated completely and TY exists as the negatively charged organic ion TY^{2-}. This reacts with $[Cu{\cdot}TC]^+$ to form a ternary ion-association complex by electrostatic attraction and hydrophobic forces.The structure of $[Cu{\cdot}TC]_2$TY is given in Supplementary material,

Fig. 4 B.

3.3.2 Reasons for RRS enhancement

The diameter and shape of the formed aggregates were measured by the SEM as shown in Fig. 4. The SEM images revealed the changes in the size of the aggregations after reaction. It was noted that the single drug molecule (Fig. 4a) could only be found at high magnification. Meanwhile, the $[Cu{\cdot}TC]_2TY$ complex (Fig. 4b) could be observed as bigger congeries at low magnification. It made pharmic molecules forming bigger aggregation and RRS was enhanced.

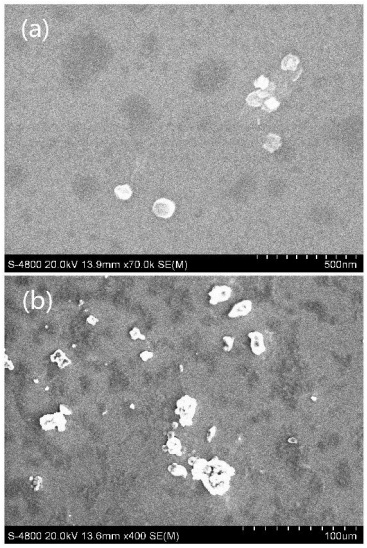

Fig. 4 SEM images of DOTC (a) and $[Cu{\cdot}DOTC]_2TY$ (b).

There are three factors that contributed to the RRS enhancement observed in this study.

(1) Resonance enhanced Rayleigh scattering effect: RRS detection was carried out at 450 nm and this wavelength is located in its absorption band (Fig. 5). This means Rayleigh scattering can absorb light energy and produce a re-scattering process. As a result, the scattering intensity increased because of the resonance enhanced Rayleigh scattering effect [32]. This was the basis for the determination of the four TCs using HPLC coupled with RRS.

(2) Enhancement of hydrophobicity: Cu^{2+}, TY^{2-} and $[Cu{\cdot}TC]^+$ are hydrophilic and easily dissolve in aqueous solutions so that they cannot form an interface with water. When $[Cu{\cdot}TC]^+$ reacts with TY^{2-} to form $[Cu{\cdot}TC]_2TY$, a hydrophobic liquid–solid interface forms because of the presence of the hydrophobic aryl framework of the ternary complex. Formation of the hydrophobic interface enhances the RRS signal [33].

(3) Increased molecular volume: larger molecular volumes result in higher RRS intensities. If the molecular volume is not easy to calculate, it can be substituted by the molecular weight (i.e., I=KCM) [34]. When the binary chelate $[Cu{\cdot}TC]^+$ reacts with TY^{2-} to form the ternary complex $[Cu{\cdot}TC]_2TY$, the molecular weight increases

from 490 ($[Cu{\cdot}TC]^+$) to 1,629.75 ($[Cu{\cdot}TC]_2TY$). This increase in the molecular volume (or weight) is an important factor in the enhancement of the RRS signal.

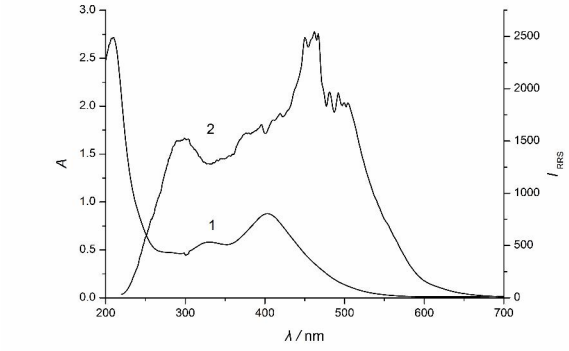

Fig. 5 Comparison of the spectral characteristic for the absorption spectrum (1) and RRS spectrum (2) of DOTC.

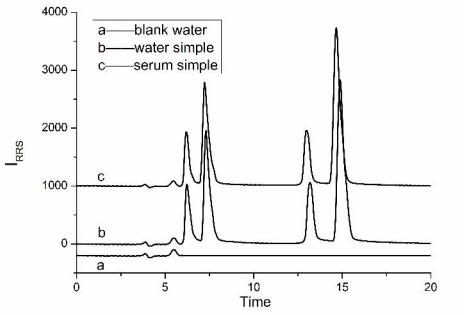

Fig. 6 Chromatograms of blank river water, river water sample spiked with 150 μg mL^{-1} TCs and human serum samples with 150 μg mL^{-1} TCs.

3.4 Method validation

For the TCs tested, TC, OTC, CTC and DOTC were well resolved and eluted within 20 min. Calibration curves were constructed using the peak area (y) and concentrations of the TCs (x, g mL^{-1}). The equations of calibration curves and correlation coefficients for TC, OTC, CTC and DOTC were listed in Table 1. And the detection limits of the assay were 668 μg L^{-1} for TC, 342 μg L^{-1} for OTC, 480 for CTC and 132 μg L^{-1} for DOTC at a signal-to-noise ratio of 3.0. For measurement of the precision and repeatability, six replicate injections of the mixed standard solution were assayed.

	Calibration curves	Range of linearity(μg mL^{-1})	Correlation coefficients
TC	y=50.454x+660.786	11–190	0.9945
OTC	y=66.523x–86.629	22–190	0.9915
CTC	y=63.239x–2362.226	58–200	0.9926
DOTC	y=280.005x–21115.512	80–210	0.9905

Table 1 The equations of calibration curves and correlation coefficients for three TCs.

3.5 Application to water samples and serum samples

The proposed strategy was applied to the detection of TCs in water samples. Water samples spiked with TC, OTC, CTC and DOTC were analyzed under the optimum conditions. The HPLC-RRS provided good separation of TC, CTC, OTC and DOTC in the water samples. Six replicate TC sample solutions were injected consecutively. The results are summarized in Table 2. The accuracy ranged from 97.4% to 102.4%. The relative standard deviation was below 4.0% for intra-day precision and below 5.0% for inter-day precision.

Analytes(n=6)	TC			OTC			CTC			DOTC		
Spiked(μg mL^{-1})	50	100	150	50	100	150	70	120	160	90	120	160
Found(μg mL^{-1})	48.7	101.2	149.7	49.5	98.6	151.6	68.3	117.8	163.8	87.9	121.4	162.6
RSD(%)	3.9	2.7	2.1	4.0	3.1	2.7	2.5	2.3	4.2	2.8	1.7	2.6
Recovery(%)	97.4	101.2	99.8	99.0	98.6	101.1	97.6	98.2	102.4	97.7	101.2	62.5

Table 2 Average recovering results for three TCs.

The described method was also applied to the detection of TCs in water sample taking from river and in human serum samples. Chromatograms of blank river water and river water spiked with TCs at 150 μg mL^{-1} are shown in Fig. 6. It also illustrates the chromatograms of serum spiked with the same concentration. They could be separated and detected in water samples spiked. And no interference from the matrix was observed under the assay conditions. The result implied that the method is feasible under optimum conditions.

4.Concluding remarks

In conclusion, this is the first report of a ternary ion-association complex method for HPLC-RRS. The TY-$CuSO_4$ reacted with TCs to form ion-association complexes that enhanced the RRS signal in the BR buffer solution. Compared with a similar method [35], the method established here is more stable for sensitive and selective detection of TCs. What is more, this work is using the enhancement with addition of Titan yellow (TY) and $CuSO_4$ as probe. Compared with the first reported about the application of probe [22], this method is introduced for the first time of using the ternary system which have two probes. Namely, it makes the two probes and drug to

be detected after they are combined into an aggregation. There is a big advantage in increasing the molecular size and it has certain novelty, also the stability is enhanced. At the same time, this method is applied to the analysis of antibiotics in water environment and provides important basis for subsequent antibiotics management in water environment. Future work should concentrate on increasing the reactor volume to increase the RRS signal and on developing a suitable flow-through cell to improve the HPLC-RRS sensitivity. HPLC combined with RRS could also be used to analyze substances that do not have fluoresce or absorption of UV-Vis light for other detection methods.

5. Acknowledgments

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6. Appendix Supplementary material

The supplementary material include some figures described in this article.

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Supplementary material

Fig. 1 The RRS spectra: (1) TC, (2) OTC, (3) CTC, (4) DOTC, (5) TY, (6) Cu(II), (7) TY-Cu(II), (8) TY-Cu(II)-TC, (9) TY-Cu(II)-OTC, (10) TY-Cu(II)-CTC, (11) TY-Cu(II)-DOTC.

Fig. 2 Effect of the mobile phase flow rate on the signal intensity in HPLC-RRS.

Fig. 3 The influence of pH of the BR on the signal intensity.

Fig. 4 A: The reaction equation for the formation of chelate cation $[Cu{\cdot}TC]^{+}$; B: The structure of $[Cu{\cdot}TC]_2TY$.