Rapid Limit Test of the Eight Quinolones Residue in the Food Based on TLC-SERS

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Article

Rapid Limit Test of the Eight Quinolones Residue in the Food Based on TLC-SERS

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Abstract: Numerous studies have shown that quinolones residues in the foods exceeding the MRL is harmful to the human health. There are some limitations in the existing methods for the residues, so we developed a new limit test method (TLC-SERS) to rapidly determine following residues: enrofloxacin (A), ciprofloxacin (B), ofloxacin (C), fleroxacin (D), sparfloxacin (E), enoxacin (F), gatifloxacin (G) and nadifloxacin (H). The residues can be preliminarily separated by TLC. Labeling the tested compounds' position on the thin layer plate by their relative R_f under 254 nm ultraviolet, and an appropriate amount of nanometer silver solution should be added to the position. The silver on the plate should be irradiated with 532 nm laser to obtain SERS of the compounds. The results showed a significant differences of the 8 quinolones' SERS; the LOD of the H, A, D, E, C, G, F and B were orderly 9.0, 12.6, 8.0, 19.0, 8.0, 8.4, 9.0 and 12.6 ng/mL, and the RSD ≤ 4.9% for SERS of each quinolones.The limit test results of the 20 samples was consistent with that determined by the UPLC–MS/MS. The above results indicate that the TLC-SERS is specific, sensitive, stable, and accurate, providing a new reference for the rapid limit test of harmful residues in foods.

Keywords: TLC-SERS; quinolones; residues; aquatic products; animal foods

1. Introduction

In the past two decades, quinolones, as veterinary drugs with antibacterial effects, have been widely used in animal husbandry and aquatic products industries, mainly including enrofloxacin (A), ciprofloxacin (B), ofloxacin (C), fleroxacin (D), sparfloxacin (E), enoxacin (F), gatifloxacin (G) and nadifloxacin(H), etc [1–5]. If the quinolones in aquatic products and other animal foods exceeds the maximum residue limit (MRL), it will cause serious harm to consumers' health [6]. In fact, the phenomenon of the quinolones exceeding their MRL in the foods is not uncommon. For example, according to relevant literature reports in 2020, enrofloxacin, ciprofloxacin, and ofloxacin were found to exceed their MRL in some aquatic products, eggs, and pork [6–9].To prevent this phenomenon from occurring, it is necessary to establish a rapid limit test method for the residues in these foods.

In China's national food standards, the MRLs of the eight quinolones (A, B, C, D, E, F, G, H) in these foods are specified as 100.0, 100.0, 2.0, 5.0, 5.0, 5.0, 5.0, 5.0 μg/kg, respectively, and these residues are usually determined by UPLC-MS/MS.Despite its salient advantages of high sensitivity and strong specificity in quantitative and qualitative analysis, the method (UPLC-MS/MS) still require complicated sample pretreatment, as well as hours or even days for the completion of the whole analysis process [2–6].

Traditional thin-layer chromatography (TLC) is a fast and convenient method for separating chemical components from complex matrices.With the help of auxiliary means such as chemical color development and ultraviolet light irradiation, this method can only reflect the chemical structural

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characteristics of a certain functional group of the testing component, and its sensitivity is also relatively low [10–13], so it cannot be directly used for limit test of the residues in the food.

Analysis technology based on Raman spectroscopy is a new method that can be used for chemical composition rapid detection. The Raman spectroscopy can show the inelastic scattering phenomenon of compound molecules after being irradiated by laser, so the fingerprint structure information of the compounds can be reflected by the spectroscopy, and the spectroscopy is almost not affected by water and silica gel under experimental conditions. Although this method has high specificity, its sensitivity is relatively low. In order to meet the analysis of some trace components, the sensitivity can be increased by more than 10,000 times through surface enhanced Raman spectroscopy (SERS) [10–13]. In recent years, it was reported that components in complex matrix were separated rapidly by TLC, and the separated component was detected specifically by the SERS [14–20].

The purpose of this study was to develop a new limit test method by combining the TLC with the SERS, abbreviating it as TLC-SERS. The eight quinolones residues in the foods can be rapidly sepseparated and specifically detectd by the TLC-SERS, so it will provide a new reference basis for the rapid analysis of the harmful residues in the food.

2. Materials and Methods

2.1. Materials

All reagents were analytical grade and were bought from Merck Drugs and Co, Ger-many in Germany. The reference substances of A (98%), B (82.1%), C (99.7%), D (99.2%), E (99.4%), F(91.5%), G (97.2%) and H (97%) were purchased from China Food and Drug Control Institute, and anhydrous ethanol (99.5%) was used to dissolve these quinolone compounds. There were ten batches of real samples of aquatic products which were supplied by five different manufacturers (China), and the ten real samples of other animal foods were supplied by the other manufacturers (China). Anhydrous sodium sulfate was used to remove protein from the food, and acetonitrile (99.5%) was used to extract the quinolone residues from the food. Dichloromethane (99.5%) and methanol (99.5%) were used as developing agents in the TLC. Silver nitrate and sodium citrate were used to prepare the SERS active substrates.

The TLC could be obtained by a thin-layer plate (Merck KGaA, Darmstadt, Germany) that is composed of high-performance silica gel and fluorescing additive F_{254}. The plate is called GF_{254} thin-layer plate for short, the layer thickness is 0.2 ± 0.03 mm, the particle size is 8 ± 2 μm and the carrier is aluminum. The micro injector (10 μL) used for spotting on thin-layer plates was purchased from Zhenhai Glass Instrument Factory, Ningbo, China.

2.2. Apparatus and Conditions

SERS or Raman spectra of the quinolone components were obtained by use of a DXR™ xi Raman Imaging Microscope (Thermo Fisher Scientific, Waltham, MA, USA) with a laser excitation wavelength of 532 nm, a resolution of 5.0 cm^{-1}, and a 10× long working distance microscope objective. The excitation power was 10 mW, the integration time was 0.5 s, and the number of scans was 20. The scan range was 3300–100 cm^{-1}, with a 50 μm confocal pinhole DXR532 full range grating (400 line/mm). The detector was a TE-cooled electron-multiplying CCD (EMCCD). Point scanning was chosen as the scanning mode.

Ultraviolet analyzer (YOKO-2F, Wuhan YOKO Technology Ltd., in China) was used to mark principal spot on the TLC under 254 nm. Ultraviolet Visible spectrophotometer (T_6, Beijing Puxi General Instrument Co., Ltd, in China) was used to detect the ultraviolet absorption spectrum of the SERS active substrates. Transmission Electron Microscope (HT 7700, Beijing Shengjiachen Ke & Trade Co., Ltd, in China) was used to characterize the particle appearance of the substrates. Nanoparticle size analyzer (Nicomp 380 ZLS, Shanghai of meijia Co., Ltd, in china)was used to measure the particle size and the particle Zeat potential.

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Ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was operated on a Dionex UltiMate 3000 ultra-performance liquid chromatography—TSQ quantum mass spectrometer system (ULTivo G6465A, Agilient.com.USA). The limit test results of the residues in real samples by the TLC-SERS were verified by the UPLC-MS/MS, and A ,B, C, D, E, F, G, and H were separated by gradient elution using a Kromasil C_{18} column (100 × 2.1 mm × 1.8 μm) with a mobile phase at a flow rate of 0.2 mL/min. The elution procedure was as follows: 0~3 min, 78%A, 20%B, 2%C; 3~6 min, 75%A, 20%B, 5%C; 6~8 min, 70%A, 20%B, 10%C; 8~13 min, 40%A, 20%B, 40%C; 13~13.1 min, 40%A, 20%B, 40%C; 13.1~16 min, 78%A, 20%B, 2%C; 16 min, 78%A, 20%B, 2%C (A: 0.1% formic acid solution containing 5.0 mmol/L ammonium acetate; B: methanol; C:acetonitrile).The column temperature was the same as the room temperature. ESI positive-ion in MRM mode was used to monitor the precursor ion→product ion transitions m/z 360→316 (A), 332→288 (B), 362→318 (C), 370→326 (D), 393→349 (E), 321→303 (F), 376.2→332.2 (G) and 361→343.2(H).

2.3. Solutions Preparation

The SERS active substrates : in order to obtain SERS of eight quinolones, it is needed that a SERS active substrates was prepared, it is also called as nanometer silver solution or surface enhancer. When 56 mg of silver nitrate was dissolved in 150 mL of water , a mixed solution was obtained by adding evenly 4 mL of 1% sodium citrate solution to the silver nitrate solution, and the nanometer silver solution was prepared by heating the mixed solution in a microwave oven until it boiled for 5 minutes, cooling it to room temperature. This solution was stored at 4 ℃ to ensure its stability.

Reference substance solution: for example, the preparation method of solution Enrofloxacin(A) is to dissolve an appropriate amount of reference substance A in anhydrous ethanol to a concentration of 1.00μg/mL. With the same method, the solutions of the other quinolones (B, C, D, E, F, G and H) were also prepared to the concentrations of 1.00μg/mL.

Mixed reference substances solution 1：in order to detect the degree of separation of the eight quinolones on the TLC, a mixture solution was prepareded by taking respectively 1.00 mL above reference (A, B, C, D, E, F, G and H) solution into the same container. After the mixture solution was dryed in a water bath (85 °C), it was redissolved by the 1.00 mL anhydrous ethanol to obtain the mixed reference substance solution 1.

Mixed reference substances solution 2: according to the MRL of the quinolones in the food, the mixed reference substances solution was prepared by dissolving an appropriate amount of A, B, C, D, E, F, G and H together in the same portion of anhydrous ethanol. In the solution, the concentrations of various substance (A, B, C, D, E, F, G and H) were in order of 400.0, 400.0, 8.0, 20.0, 20.0, 20.0, 20.0 and 20.0 ng/mL.

The sample solutions: in order to accurately detect SERS of the quinolones in the food, it was need that 2.00 g the food and 10 g Na_2SO_4 powder and 10 mL of anhydrous ethanol were placed in the same centrifuge tube, the residues was extracted from the food substrate by sonicate method for 15 min. After centrifugation under 4000 rpm/min for 5 min, the supernatant that may contain the quinolones was obtained in the centrifuge tube. Passing the supernatant through the filter membranes (0.22 um), a filtrate was obtained. Concentrating the filtrate in a water bath (85 °C) to approximately 1 mL, transferring it to a chromatographic vial. Evaporating all the solvent in the vial in a water bath, the residues of the food was redissolved by 500.0 μL anhydrous ethanol to obtain the sample solution.

2.4. The TLC

TLC is a simple and fast separation technique. The eight quinolones could be preliminarily separated by the following methods: The 8.0 μL of the reference substance solutions and the mixture reference substance solution 1 were spotted on a GF_{254} thin-layer plate (10 cm × 10 cm) at a distance of 1 cm from the bottom, separately. When the plate were eluted to a distance of 8 cm by dichloromethane- methanol (5:1) in a glass container, it should be removed from the container, and the agent R_f on the plate should be naturally evaporated. Irradiating at 254 nm, the main spots on the

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thin layer chromatogram could be observed. Using fleroxacin (D) as object of reference, the relative R_f of A, B, C, D, E, F, G and H could be measured by these spots, respectively.

2.5. The TLC-SERS

In the study, we focused on developing a rapid and specific limit test method by the TLC-SERS to control the eight quinolone residues in the food. According to the above TLC, the 8.0 μL of the fleroxacin (D) reference solution and the mixed reference solution 2 were dropped onto the same thin layer plate separately. Under the ultraviolet rays irradiation at 254 nm, there was a very obvious spot on the thin layer chromatograph of the fleroxacin. Due to this fact that the concentration of the eight quinolone components in the mixed reference solution 2 was lower than the LOD of the TLC, so any spot could be not observed in the chromatogram of the mixed solution. Therefor, the eight components' position should be separately marked by their relative R_f using the fleroxacin as object of reference to obtain their SERS. In addition, the blank position which R_f was same as the eight quinolones should also be marked to identify the Raman signal of SERS active substrate itself. When an appropriate amount of the nanometer silver solution was added on these marked position, and silver on the position was irradiated by the laser at 532 nm, the SERS of the A, B, C, D, E, F, G, and H should be obtained respectively, but there should be no obvious Raman signal from the blank position.

When the sample solustion was dropped onto the thin layer plate , these positions should also be marked by the same method to obtain SERS of the residues in the food. Comparing their Raman shift and relative peaks intensity, if the SERS of the tested component is consistent with the that of the corresponding reference substance, we can determine that there are corresponding quinolones residues in the foods. Based on this, if the characteristic peaks of the sample solution is higher than that of the corresponding reference substance solution, we can determine that the residual quinolones in the food have exceed its MRL.

It must be emphasized here that the MRL of ofloxacin (C) in the food is only 2 μg/kg, which is equivalent to 8 μg/mL according to the preparation method of the sample solution, so the sensitivity of the above TLC-SERS could have some limitations, did not meeting the requirements for the limit test of the ofloxacin in the food. That was to say, the LOD of the ofloxacin may be higher than its MRL in the method. In order to make the LOD ≤8 μg/mL (2μg/kg), a in-situ enrichment of the ofloxacin must be performed with anhydrous ethanol at the marked position that may contain the ofloxacin on the on the thin layer plate to concentrate the compound, then an appropriate amount of nanometer silver solution should be added to the concentrated compound to obtain its SERS. Only through such TLC-SERS could the characteristic SERS of the trace ofloxacin be obtained.

3. Results and Discussion

3.1. Characterization of the SERS Active Substrates

The special nature of the SERS active substrates was characterized as follows: diluting the active substrates to 14 times, the ultraviolet absorption spectrum was obtained by detection of the diluted solution, and showing the maximum absorption peak at 424 nm (Figure 1a). At the same time, the appearance of the substrates was characterized as ball shape (Figure 1b). Particle size and zeta potential of the substrates were measured respectively at different times (0～21 date), showing the most of the particle size at 41.40～43.14 nm (Figure 1c,d), and the zeta potential at 31.14～35.80 mv(Figure1e). It was confirmed that the SERS active substrates had a uniform distribution and good stability.

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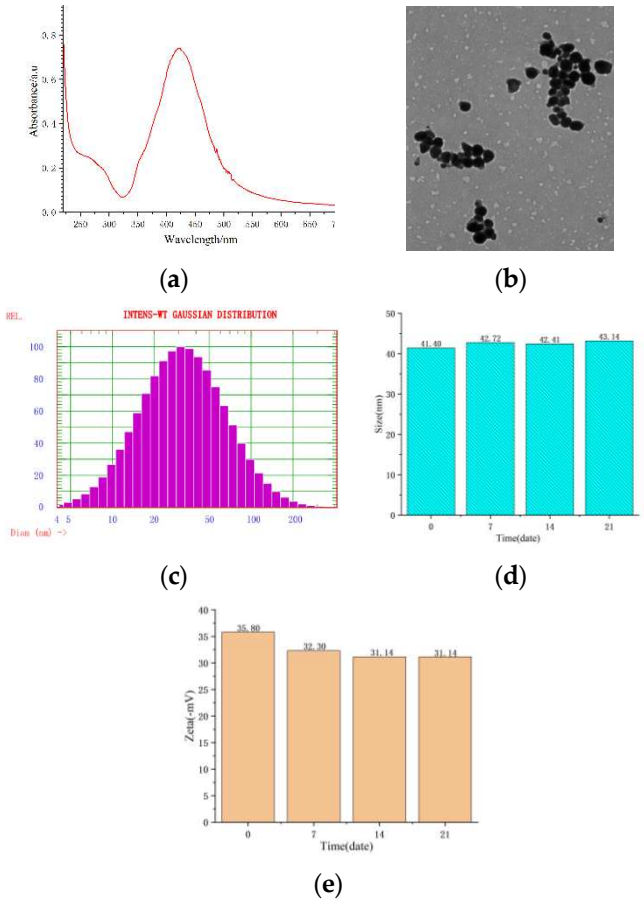

Figure 1. Special nature of the SERS active substrates. a: the ultraviolet absorption spectrum; b: the appearance; c-d: the particle size ; e: the zeta potential.

3.2. Relative R_f by the TLC

According to the TLC, these quinolones could be effectively separated except for C and E in the mixed reference substance solution 1 (Figure 2a). Using fleroxacin as object of reference, the relative R_f from high to low was 1.22 (H),1.16 (A), 1.00 (D), 0.90 (E), 0.89 (C), 0.74 (G), 0.56 (F) and 0.44 (B) in order. When an appropriate amount of the above reference substances solution was placed into aquatic products without any quinolones, the solution to be tested was obtained by referring to the sample solution preparation method. The above TLC was repeated by the solution, displaying the same chromatogram and the relative R_f (Figure 2b). When the aquatic product was insteaded of the other animal food, the same result was also obtained. It indicated that the matrix in the food had no effect on the relative R_f of the quinolones.

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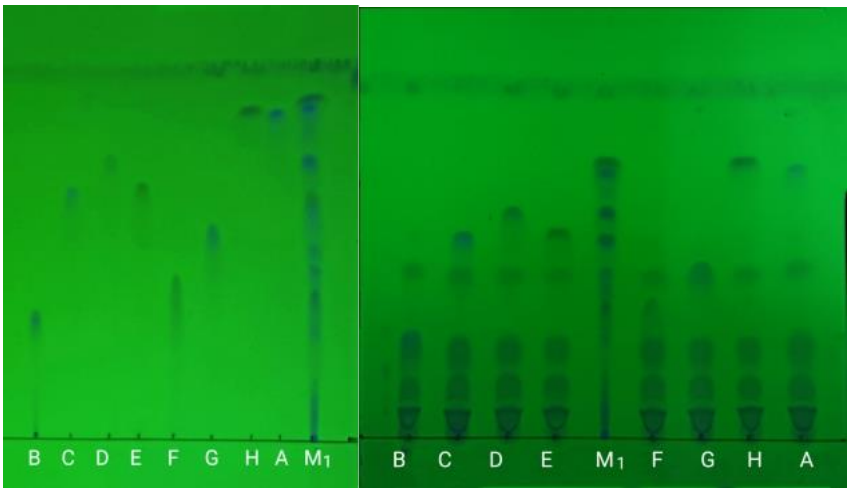
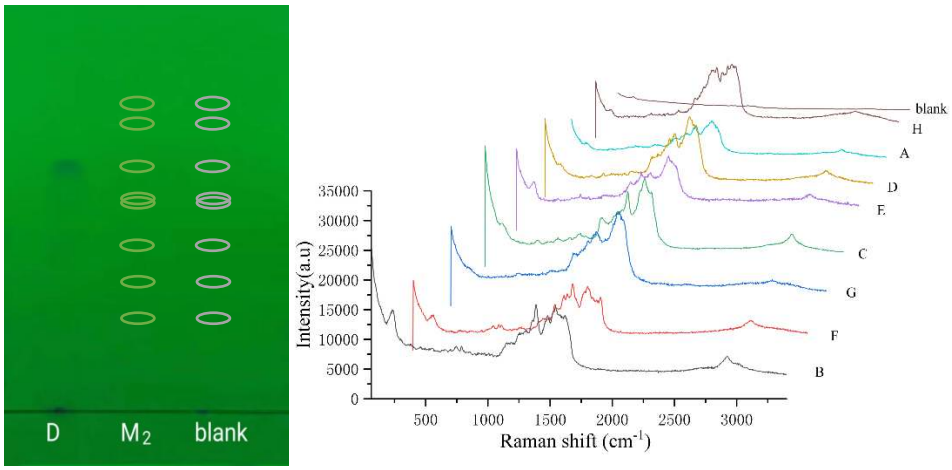


a b

Figure 2. The thin-layer chromatogram of the eight quinolones. A ,B ,C, D, E, F, G, H and M_1 represents the eight quinolones reference substance solution and the mixed reference solution 1, respectively.

3.3. The SERS by the TLC

According to the TLC-SERS, it was shown by the Figure 3a that there was a very obvious spot on the thin layer chromatogram of the fleroxacin reference substance solutions, and the R_f was measured as 0.70. At the same time, there was no spot in the chromatogram of the mixed reference substance solution 2, so the position of the eight quinolones should be marked by their R_f , respectively. This R_f could be calculated through their relative R_f and the R_f of the fleroxacin, so this R_f was 0.85 (H),0.81 (A), 0.70 (D),0.63 (E), 0.62 (C), 0.52 (G), 0.39 (F) and 0.31 (B) , respectively. It was shown by the Figure 3b that SERS of the eight quinolones (H, A, D, E, C, F, G, F and B) was obtained from the chromatogram of the mixture solution 2, but almost no Raman signal was observed from the corresponding blank positions, it indicated that the nanometer silver solution had no effect on the SERS of the quinolones.



a. The TLC b. The SERS

Figure 3. The TLC and the SERS of eight quinolones as their concentration at the MRL. According to the thin-layer chromatogram , D represents the fleroxacin solution, and the spot was from the fleroxacin; M_2 represents the mixed reference substance solution 2, and there were eight quinolones (H, A, D, E, C, F, G, F and B) in the marked positions on the chromatogram ,respectively; blank represents the anhydrous ethanol , there were no quinolones in the marked position on the chromatogram. According to the SERS diagram, A, B, C, D, E, F, G, and H represents the different quinolones in the mixture solution 2 respectively; the blank meaning as above.

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3.4. Comparison between the Raman Spectroscopy and the SERS

According to the aforementioned apparatus and conditions, the Raman spectra of the eight quinolones reference substance powders could be detected directly, showing significant differences in the eight spectra except for the blank with no signal (Figure 4). The SERS of the powder was shown in Figure 3b. When the SERS of the quinolones was compared with its Raman spectra, the results showed that the shape and relative intensity of the main characteristic peaks had obviously changed, but the Raman shift (cm^{-1}) of the peak was similar. In addition, number of characteristic peaks in the SERS is less than that in the Raman spectrum (Table 1). This result indicated that the SERS of the quinolones had an obvious correlation with their Raman spectra. In other words, the SERS could reflect some structural information of the quinolones. Therefore, the TLC-SERS can be used as a specific analysis method for the quinolones.

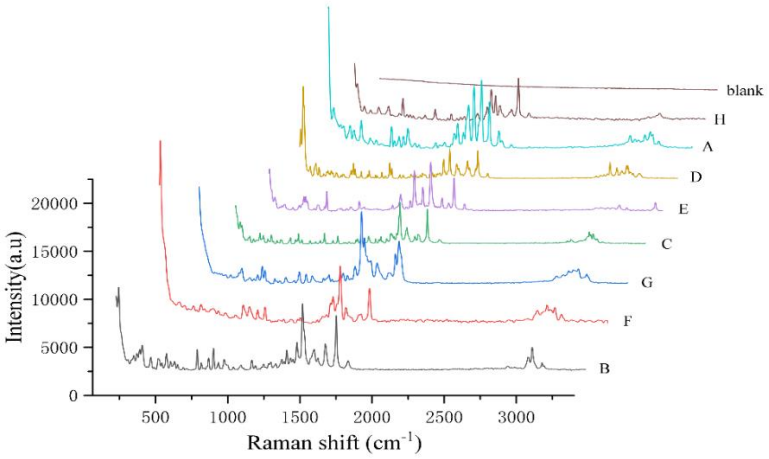
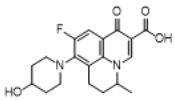
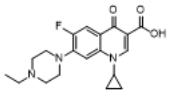
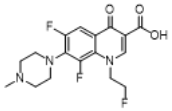


Figure 4. Raman spectra of the eight quinolones powder. (H, A, D, E, C, G, F, and B represents Nadifloxacin, Enrofloxacin, Fleroxacin, Sparfloxacin, Ofloxacin, Gatifloxacin, Enoxacin, and Ciprofloxacin, respectively.).

Table 1. The Assignments of the characteristic peaks of the eight quinolones.

Chemical Structure	Raman Shift (cm^{-1}) of the Podwer/Relative Peak Intensity	Raman Shift (cm^{-1}) by the TLC-SERS/ Relative Peak Intensity	Assignments	Relative R_f
Nadifloxacin(H) 	2964~2861 (2 peaks)	2991~2846 (1 peak)	common peak: $\nu_{=CH}$,ν_{-CH2},ν_{-CH3}	1.22
	1721~1147 (9 peaks)	1610~1157 (8 peaks)	characteristic peak:	
	1721/0.28	1610/1.01	$\nu_{C=O}$	
	1621/1.33	1587/1.03	$\nu_{C=C}$ from phenyl rings	
	1363/1.00	1398/1.00	$\nu_{C=C}$ from phenyl rings	
	1324/0.46	1257/0.57	β_{CH2}, β_{CH3}	
	1147/0.16	1157/0.32	ν_{C-N}	
Enrofloxacin(A) 	3094~2833 (5 peaks)	2995~2823 (1 peak)	common peak: $\nu_{=CH}$,ν_{-CH2},ν_{-CH3}	1.16
	1743~1129 (9 peaks)	1600~1173 (4 peaks)	characteristic peak:	
	1743/0.08	1600/0.94	$\nu_{C=O}$	
	1629/0.29	1552/1.14	$\nu_{C=C}$ from phenyl rings	
	1400/1.00	1389/1.00	$\nu_{C=C}$ from phenyl rings	
	1350/0.71	1281/0.77	β_{CH2}, β_{CH3}	
	1129/0.12	1173/0.65	ν_{C-N}	
Fleroxacin(D) 	3057~2800 (6 peaks)	3037~2806 (1 peak)	common peak: $\nu_{=CH}$,ν_{-CH2},ν_{-CH3}	1.00
	1791~1147 (7 peaks)	1595~1255 (5 peaks)	characteristic peak:	
	1791/0.21	1595/1.12	$\nu_{C=O}$	
	1634/0.94	1535/1.32	$\nu_{C=C}$ from phenyl rings	
	1387/1.00	1379/1.00	$\nu_{C=C}$ from phenyl rings	
	1332/0.65	1298/0.77	β_{CH2}, β_{CH3}	
	1147/0.21	1255/0.64	ν_{C-N}	
Sparfloxacin(E)	3095~2835 (6 peaks)	2997~2823 (1 peak)	common peak: $\nu_{=CH}$,ν_{-CH2},ν_{-CH3}	0.90
	1721~1179 (9 peaks)	1621~1176 (5 peaks)	characteristic peak:	

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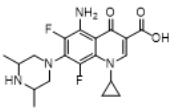
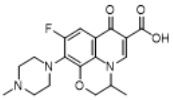
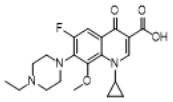
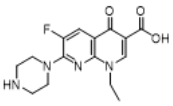
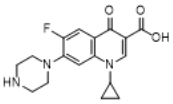
	1721/0.16	1621/1.21	$\nu_{C=O}$	
	1632/0.68	1549/1.48	$\nu_{C=C}$ from phenyl rings	
	1435/1.00	1373/1.00	$\nu_{C=C}$ from phenyl rings	
	1296/0.83	1279/1.03	β_{CH2}, β_{CH3}	
	1179/0.34	1176/0.71	ν_{C-N}	
	3002~2763 (6 peaks)	3003~2810 (1 peak)	common peak: $\nu_{=CH}$,ν_{-CH2},ν_{-CH3}	
Ofloxacin(C)	1720~1147 (9 peaks)	1612~1151 (5 peaks)	characteristic peak:	
	1720/0.16	1612/0.98	$\nu_{C=O}$	
	1632/0.85	1552/1.19	$\nu_{C=C}$ from phenyl rings	0.89
	1401/1.00	1394/1.00	$\nu_{C=C}$ from phenyl rings	
	1329/0.29	1300/0.68	β_{CH2}, β_{CH3}	
	1147/0.23	1151/0.57	ν_{C-N}	
	3081~2846 (6 peaks)	3026~2806 (1 peak)	common peak: $\nu_{=CH}$,ν_{-CH2},ν_{-CH3}	
Gatifloxacin(G)	1620~1185 (9 peaks)	1562~1151 (6 peaks)	characteristic peak:	
	no signal	no signal	$\nu_{C=O}$	
	1620/0.93	1562/1.33	$\nu_{C=C}$ from phenyl rings	0.74
	1350/1.00	1357/1.00	$\nu_{C=C}$ from phenyl rings	
	1328/1.57	1286/0.85	β_{CH2}, β_{CH3}	
	1185/0.24	1151/0.65	ν_{C-N}	
	3055~2878 (4 peaks)	3032~2823 (1 peak)	common peak: $\nu_{=CH}$,ν_{-CH2},ν_{-CH3}	
Enoxacin(F)	1674~1239 (7 peaks)	1649~1161 (7 peaks)	characteristic peak:	
	1674/0.07	1649/0.74	$\nu_{C=O}$	
	1627/0.63	1540/0.93	$\nu_{C=C}$ from phenyl rings	0.56
	1409/1.00	1414/1.00	$\nu_{C=C}$ from phenyl rings	
	1355/0.48	1265/0.60	β_{CH2}, β_{CH3}	
	1239/0.10	1161/0.34	ν_{C-N}	
	3089~2990 (3 peaks)	2995~2823 (1 peak)	common peak: $\nu_{=CH}$,ν_{-CH2},ν_{-CH3}	
Ciprofloxacin(B)	1711~1162 (8 peaks)	1621~1151 (8 peaks)	characteristic peak:	
	1711/0.16	1621/0.85	$\nu_{C=O}$	
	1628/0.82	1543/0.98	$\nu_{C=C}$ from phenyl rings	0.44
	1389/1.00	1386/1.00	$\nu_{C=C}$ from phenyl rings	
	1277/0.31	1307/0.64	β_{CH2}, β_{CH3}	
	1162/0.14	1151/0.47	ν_{C-N}	

ν represents stretching vibration; β represents in-plane bending vibration. The relative intensity of this peak could be obtained by the ratio of itself absolute intensity to that of the reference peak, and the Raman shift of the reference peak had be marked as red.

3.5. Identification of the quinolones by combining SERS with relative R_f

As shown in Table 1, not only was the relative R_f different in the TLC, but also the relative strength and number of the characteristic peaks were different in the TLC-SERS for the same quinolones. This result showed that combining the relative R_f with the SERS could identify the quinolones with higher specificity and accuracy. Here are some examples:

Due to the close relative R_f between nadifloxacin (H) and enrofloxacin (A) (The relative R_f of H and A was 1.22 and 1.16 respectively in Table 1), the separation degree of the two components was relatively small, making it difficult to identify them solely through TLC. Observing carefully H and A at the SERS (Figure 3b) and data of their characteristic peaks (Table 1), it was found that there were 8 peaks (H) and 4 peaks (A) at Raman shifts (1600～1173 cm^{-1}) respectively. At the same time, the relative peak intensities were 0.57 (H) and 0.77 (A) from the β_{CH2} or β_{CH3} respectively; And the relative peak intensities were 0.32 (H) and 0.65 (A) from the ν_{C-N} respectively. This result indicated that although the differences of the two components's R_f was small by the TLC , there was significantly different spectrum by the TLC-SERS. Therefore，a new analytical method could be established by combining the relative R_f of the two compounds (H and A) with their characteristic SERS, and this method would more accurately distinguish H from A.

Due to the almost identical relative R_f between sparfloxacin (E) and ofloxacin (C) (The relative R_f of E and C was 0.90 and 0.89 respectively in the table 1), the two components could not be effectively separated, making it impossible to identify them solely through TLC. Observing carefully E and C at the SERS (Figure 3b) and data of their characteristic peaks (Table 1), it was found that there were very obvious differences. By comparing the chemical structures of the two components, it was found that there is an additional CH_2 in E, which would lead to stronger in-plane binding vibrations

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(β_{CH2}, β_{CH3}) in the SERS, for example, the relative peak intensities of the peak (β_{CH2}, β_{CH3}) was 1.03 (E) and 0.68 (C) respectively (Table 1). In addition, it was found that there was an additional NH_2 in E, which would lead to stronger stretching vibration (ν_{C-N}) in the SERS. For example, the relative peak intensities were 0.71 (E) and 0. 57 (C) respectively. The result indicated that although C and E could not be effectively separated by the TLC, the SERS of E and C could be obtained separately by the TLC-SERS, and the two SERS were completely different. Therefore，a new analytical method could be established by combining the relative R_f of the two compounds (E and C) with their characteristic SERS, and this method would accurately distinguish E from C.

3.6. Experiment of Simulated Positive Sample

When aquatic products without quinolones were used as negative samples, an appropriate amount of the eight quinolones was placed into the negative samples respectively to prepare the simulated positive sample, making the content of the quinolones to be equal to the MRL of the quinolones in the food. This means that the content of the quinolones(A, B, C, D, E, F, G and H) in the simulated positive sample was 100.0, 100.0, 2.0, 5.0, 5.0, 5.0, 5.0 and 5.0 μg/kg , respectively.

According to the preparation method of the sample solution, an appropriate amount of simulated positive samples were added to anhydrous ethanol to prepare a simulated positive sample solution. Prepare the negative sample solution using the same method. Then, the 8 μL of the reference substance solutions, the simulated positive sample solutions, and negative sample solution were deposited onto the GF_{254} thin-layer plate, respectively.The experiment was carried out by the TLC-SERS, and the result was shown in Figure SM_1, indicating that the SERS of the simulated positive samples was in accordance with the corresponding reference substance when no Raman signal was obtained in the negative sample. The result further confirmed that the matrix in aquatic products did not interfere with the limit test of the eight quinolones residues, showing that the TLC-SERS has strong specificity.

3.7. Stability Test

After placing the above simulated positive sample solutions for 0, 1, 2, 4, and 8 hours, respectively, the SERS of the eight quinolones in the solutions at different times were detected sequentially by the TLC-SERS, and the relative standard deviations (RSDs) of the four characteristic peaks height ($\nu_{C=C}$, β_{CH2}, β_{CH3},ν_{C-N}) in the SERS (Table 1) were respectively obtained as 1.9~2.2% (H), 2.6~3.3% (A), 2.2~3.5% (D), 3.5~3.7% (E), 4.1~4.9% (C), 3.6~3.9% (G) , 3.2~3.5% (F) and 3.1~3.4% (B), indicating that the stability of the method was relatively reliable.

3.8. Test of the Limit of Detection

In the method , the concentration range of the solution used to determine the limit of detection (LOD) should be a gradient concentration, including MRL（ng/mL）of the quinolones in the food. For example, the MRL of nadifloxacin (H) in the food is equal to 5.00 μg/kg which is equivalent to 20 ng/mLof the nadifloxacin in the sample solution, so the concentration range of the reference substance (H) could be at 12.5～50.0 ng/mL. Similarly, it was shown in Table 2 that the concentrations range of the reference substance (A, D, E, C, G, F, and B) was at 12.5 ~ 420.0, 12.5 ~ 50.0, 12.5 ~ 50.0, 8.0 ~ 32.0, 12.5 ~ 50.0, 12.5 ~ 50.0 and 12.5 ~ 420.0 ng/mL, respectively.

According to the TLC-SERS, the 8.0 μL of the solution with the different concentrations were deposited onto GF_{254} thin-layer plates respectively, and the corresponding SERS was obtained. The results were shown in Figures S1–S9. When the signal-to-noise ratio was equal to 3:1 (S/N=3:1), corresponding the quinolones concentration was called its LOD. The S/N was calculated by the ratio of the quinolones characteristic peak (ν_{C-N}) heights to that of the blank noise peak, and the Raman shifts of these characteristic peaks was at 1157 (H), 1180 (A), 1237 (D), 1175 (E), 1151 (C), 1151 (G), 1170 (F), and 1151 (B) cm^{-1}, respectively.

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The calibration curves were established by the concentration and the S/N. The results were shown in Figure 5. According to this curve, the LOD of the eight quinolones was equal to 9.0 (H), 12.6 (A), 8.0 (D), 19.0 (E), 8.0 (C), 8.4 (G), 19.0 (F) and 12.6 (B) ng/mL, respectively.

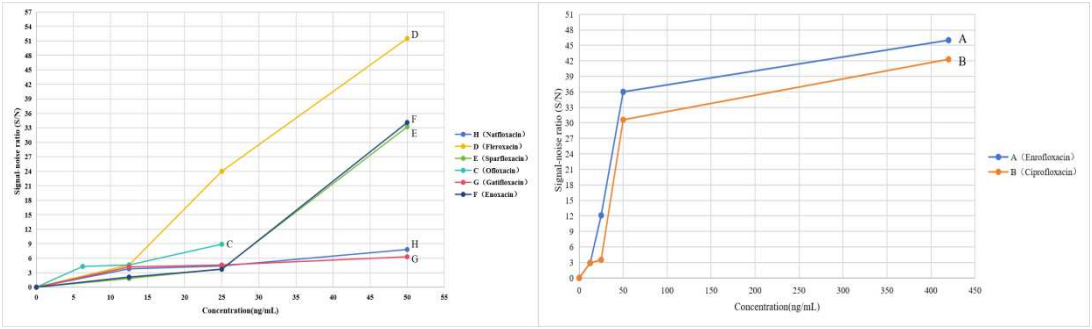

Figure 5. LOD of the eight quinolones by TLC-SERS. H, A, D, E, C, G, F, and B represents Nadifloxacin, Enrofloxacin, Fleroxacin, Sparfloxacin, Ofloxacin, Gatifloxacin, Enoxacin, and Ciprofloxacin, respectively.

Table 2. Comparison between the LOD and the MRL of the eight quinolones residues in the food.

Quinolones	MRL μg/kg	MRL ng/mL	concentration range (ng/mL)	LOD (ng/mL)
Nadifloxacin (H)	5.0	20.0	12.5 ~ 50.0	9.0
Enrofloxacin (A)	100.0	400.0	12.5 ~ 420.0	12.6
Fleroxacin (D)	5.0	20.0	12.5 ~ 50.0	8.0
Sparfloxacin (E)	5.0	20.0	12.5 ~ 50.0	19.0
Ofloxacin (C)	2.0	8.0	8.0 ~ 32.0	8.0
Gatifloxacin (G)	5.0	20.0	12.5 ~ 50.0	8.4
Enoxacin (F)	5.0	20.0	12.5 ~ 50.0	19.0
Ciprofloxacin (B)	100.0	400.0	12.5 ~ 420.0	12.6

According to the preparation method of the sample solution, the quinolones residue was extracted from 2 g (0.002 kg) of the foods and was soluted with 500.0 μL (0.5 mL) of anhydrous ethanol. So the conversion method between the MRL (μg/kg) and the MRL (ng/mL) in the Table 2 is as follows:

$$\text{MRL (ng/mL)} = \text{MRL (μg/kg)} \times 0.002\ \text{kg} \times 1000/0.5\ \text{mL} = 4 \times \text{MRL (mg/kg)}$$

In the Table 2, comparing the LOD with the corresponding MRL (ng/mL), the results showed that the LOD of each quinolones was not larger than its MRL.So the sensitivity of the method (TLC-SERS) can meet the limit test of the eight quinolone residues in the food.

It is particularly emphasized here that the characteristic peaks height of the SERS would increase with that of its concentration, when the concentration of the quinolones was greater than its LOD (Figure 6). If the quinolone residues are present in the food, we can determine whether the amount of the residue exceeds its MRL by comparison with the characteristic peak heights of the quinolones in the food with that of the corresponding reference in the mixed reference substances solution 2. According to the preparation method of the mixture solution 2, the concentration of the quinolones in the mixture solution was equal to corresponding the MRL (ng/mL) in Table 2.

3.9. Limit Test of Real Samples

Ten batches of different varieties of aquatic products and ten batches of different varieties of other animal foods were taken, and in accordance with the preparation method of the sample solution, the corresponding twenty sample solutions were prepared. According to the TLC-SERS, 8.0 μL of the fleroxacin (D) reference substance solution, mixed reference substance solution 2, and twenty batches of samples solution were dropped onto the same GF_{254} thin layer plate, respectively.

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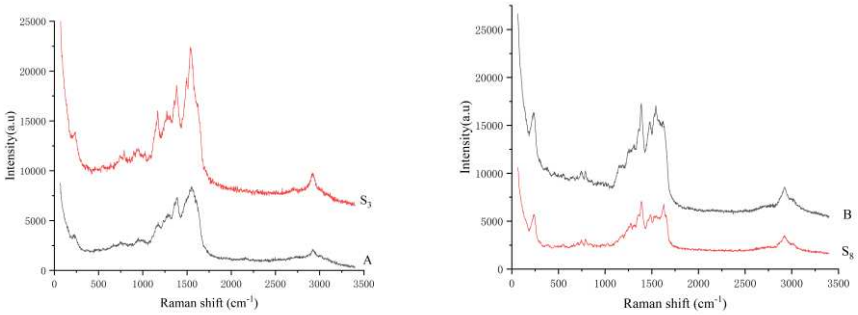
The detection results showed that no SERS signal was observed in other samples except for sample 3 and sample 8, the SERS of the sample 3 and sample 8 were the same as that of the enrofloxacin (A) and the Ciprofloxacin (B) in the mixed reference substance solution 2, respectively. It was shown in the Figure 6 that the characteristic peaks of the sample 3 were higher than that of the reference substance (A), and the characteristic peaks of the sample 8 were weaker than that of the reference substance (B). The information indicated that the content of the enrofloxacin (A) exceeded its MRL (100 μg/kg) in sample 3, and the content of the quinolones (A, B, C, D, E, F, G and H) did not exceeded the corresponding MRL in the other samples. That is to say, according to China's national food standards, among the twenty batches of samples tested by the TLC-SERS above, except for the test result of the enrofloxacin (A) in sample 3, the test results of the eight quinolones in the other samples were all qualified.



sample 3 sample 8

Figure 6. SERS of the sample by the TLC-SERS. S_3: sample 3 from the aquatic products, A: enrofloxacin; S_8: sample 8 from the other animal foods, B: ciprofloxacin.

In order to verify the accuracy of the TLC-SERS, an authoritative analysis method (UPLC–MS/MS) was used to quantitatively determine above the eighe quinolones in the twenty batches of samples, and the data information showed that the results obtained by the two methods were completely consistent, which indicated that the limit test method by the TLC-SERS is accurate and reliable.

4. Conclusions

In this study, a limited limit detection method (TLC-SERS) for the quinolones residues in aquatic products and other animal foods was established, which has high sensitivity, strong specificity, reliable accuracy, and good stability. In addition, compared to existing related methods, the method is indeed simpler and faster.

The results of this experiment showed that the SERS of the quinolones acquired by the TLC-SERS had a good correlation with the Raman spectra of the corresponding references substance powder; by comparing the relative intensity and the Raman shift of their characteristic peaks, the SERS of the different quinolones showed significant differences; by comparing the determination results of the three solutions (the reference substance solution, the simulated positive sample solution and the negative sample solution.), it is shown that the matrix components in the foods did not interfere with the limit test results of the residues; by measuring the relative height changes of the four characteristic peaks （$\gamma_{C=C}$, β_{CH2}, β_{CH3}, γ_{C-N}） of the same sample at different times, RSDs of the SERS for eight quinolones were obtained respectively at 1.9 ~ 2.2% (H), 2.6 ~ 3.3% (A), 2.2～3.5% (D), 3.5～3.7% (E), 4.1～4.9% (C), 3.6～3.9% (G), 3.2 ~ 3.5%(F) and 3.1 ~ 3.4%(B), indicating the RSD ≤4.9% for each quinolone compound; by measuring different concentrations of the quinolones reference substance solutions, the LOD of the eight components were obtained as 9.00 (H), 12.60 (A), 8.00 (D), 19.00 (E), 8.00 (C), 8.40 (G), 19.00 (F), and 12.60 (B) ng/mL, respectively; and the limit test results of the twenty real samples proved that except for enrofloxacin (A) and ciprofloxacin (B) found in two batches of samples, no quinolones residues were detected in the other samples, and only in one batch

of samples, the content of enrofloxacin (A) exceeded its MRL, which was consistent with the results determined by the authoritative analysis method (UPLC–MS/MS).

In conclusion, this method can provide a new reference for the rapid limit test of harmful residues in foods.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figures S1–S9.

Author Contributions: Conceptualization, H.Z.; methodology, L.L.; software, M.Z., J.L. and F.X.; validation, Q.R., Y.W., T.X. and W.D.; formal analysis, L.L. All authors have read and agreed to the published version of the manuscript.

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