

Communication

Interference Free HPLC-SERS for the Trace Analysis of Residual Furazolidones in the Aquaculture Sediment

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Abstract: Furazolidone (FZD), a typical highly effective nitrofurantoin antibiotic, has been banned in aquaculture for its carcinogenicity and other adverse health effects, but it is still widely used for its low cost and significant efficacy. Since FZD will be rapidly metabolized in living organisms, the traditional standard mass spectrometry method can quantitatively analyze trace amount of FZD by detecting its derivative. However, a rapid qualitative analysis method is more consistent with market demand in regular monitoring. In this study, high performance liquid chromatography (HPLC) was used to separate and purify FZD from the sea cucumber culture sediment, and the purified effluent was combined with a substrate of gold nanoparticles (Au NPs) for surface enhanced Raman spectroscopy (SERS) detection. The absolute detection limit of FZD by SERS is 1 ng, and the detection limit of FZD in actual sediment samples is less than 1 µg/kg. The cost and period of FZD analysis by HPLC-SERS are greatly reduced for the omission of derivatization compared with the traditional mass spectrometry method, which can better meet the requirements of practical applications.

Keywords: HPLC; SERS; furazolidone; aquaculture sediment



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1. Introduction

Furazolidone (FZD) is a class of broad-spectrum antibiotics which were widely used in livestock and aquaculture to treat enteritis, scabies and ulcer disease caused by *Escherichia coli*, *Salmonella* and *Coccidia* [1,2]. Due to the carcinogenic, teratogenic and mutagenic side effects of FZD and its metabolites, many countries such as the European Union, the United States and Japan have explicitly banned its application in aquaculture [3,4]. The agriculture ministry of China also prohibited the use of FZD in food animal farming in 2002 [5]. However, the illegal usage of FZD in aquaculture is still been repeatedly discovered for its low price and significant efficacy [6].

FZD has a very short half-life and is rapidly metabolized in the organisms so no parent drug can be detected in edible tissues over 12 h after administration [7,8]. Therefore, the traditional standard method for the quantitative detection of FZD is based on its derivatization analysis by liquid chromatography-mass spectrometry (LC-MS), and the detection limit can reach 0.5 µg/kg [9,10]. However, the derivatization process is time-consuming, and the LC-MS analysis is expensive. Most importantly, for chemicals that are not permitted in food such as FZD, detection (yes/no) is far more important than concentration. In other words, qualitative or semi-quantitative detection is appropriate if achieved in a timely manner [11].

In practical application, the aquaculture substrate is a good information carrier which can load historical culture information [12–14]. FZD can exist stably in parent form in sediment, so the qualitative monitoring of the FZD in aquaculture environment can efficiently supervise the overuse of FZD. With the start point of zero tolerance for drug abuse, the development of a simple, rapid and low-cost analytical technique of FZD in aquaculture sediment was urgently in need.

Surface enhanced Raman scattering (SERS) is a phenomenon in which the Raman signal of the substances is significantly enhanced on the surface of noble metal nanomaterials [15–17]. SERS has proven to be a very valuable technique for molecular vibrational spectroscopy, not only in terms of speed (in the range of seconds to minutes), but also in terms of sensitivity (up to single molecule level) [18–22]. However, the activity of noble metal nanomaterials is easily interfered with by proteins, organic matter and other substances, so SERS is difficult to directly apply to biomass samples [23]. In addition, the high-performance liquid chromatography (HPLC) has attracted considerable attention due to its efficient separation performance for mixtures with different polarities [24–26]. Commercial HPLC detectors such as the ultraviolet visible detector (UVD) provided the absorption spectra and the corresponding retention time of each component. Unfortunately, some components are silent in UV spectroscopy, and the poor structural information from UV spectra brought critical difficulties in the identification of unknown components [27].

In this study, the complex sea cucumber culture sediment was separated and purified by HPLC, and the effluents were loaded onto gold nanoparticle (Au NPs) substrates for SERS measurements. The continuous rapid analysis of FZD was achieved with the separation efficiency and detection sensitivity of each technology. The results demonstrated that the HPLC-SERS hyphenated system exhibited the complementary capability of the significant separation and structural identification of illegal additives in real samples. The detection limit of FZD in actual sediment samples is less than 1 µg/kg, and the efficiency and accuracy regarding the separation and identification of complex samples were higher than those of the individual HPLC or SERS technology. It is believed that the HPLC-SERS hyphenated system would be developed as a promising technique for the separation and identification of complex multi-component mixtures.

2. Materials and Methods

2.1. Chemicals and Materials

Hydrogen tetrachloroaurate (HAuCl₄), trisodium citrate dihydrate, methanol, acetonitrile, dichloromethane, ethyl acetate and ortho-phosphoric acid were purchased from Titan Scientific Co., Ltd. (Shanghai, China). The samples of sea cucumber culture sediment were provided by Beijing Tong Ren Tang Health (Dalian, China) Seafoods Co., Ltd. The pure water was prepared by Nanopure (Thermo, Waltham, MA, USA).

2.2. Instruments

The Elite EClassical 3100 HPLC (Dalian, China) was performed on a SinoChrom ODS-BP-C18 column (200 mm × 4.6 mm × 5 µm) with a column temperature of 35 °C and a UV detection wavelength of 365 nm. The HLB SPE column of 60 mg/3 mL was used for the initial preparation of the extracts. A Raman spectrometer (Pixis-100BR CCD, Acton SP-2500i spectrograph) was used for the final SERS characterization with an excitation wavelength of 633 nm and a laser power of 20 mW.

2.3. Au NPs Preparation

Au NPs were prepared by the trisodium citrate reduction method, which was first proposed by Frens.G [28], and the specific preparation process is as follows: 100 mL, 0.1% chloroauric acid solution was heated to boiling, then quickly added 1 mL, 15% trisodium citrate. After the solution turned red, the solution was kept heating for 15 min with continuous magnetic stirring. Finally, a transparent burgundy Au NPs colloid solution was obtained.

2.4. Standard Solution Preparation

The stock solution of FZD with a concentration of 100 µg/mL was obtained by dissolving 0.01 g FZD into 100 mL acetonitrile/water (4/6) mixture. Then, standard solutions (0.02–1 µg/mL) were prepared by diluting the stock solution with acetonitrile/water (4/6) mixture.

2.5. Extraction Method for FZD in Sediment

The sea cucumber culture sediment was dried, ground and sieved, and then kept in brown bottles for use. Samples of 20 g were separately put into 4 centrifuge tubes, and 10 mL extraction solution was added into each tube. FZD was extracted from the above mixture by vigorous vortexing for 5 min and centrifuging at 4000 r/min for 10 min. The extract process was then repeated twice, and the 120 mL extract was concentrated by rotary evaporation. The dried residue was sufficiently dissolved with 2 mL of acetonitrile, and then concentrated to 200 μ L with nitrogen blowing at 40 °C water bath. The extracts were diluted to 5 mL by 0.1% phosphoric acid and filtered by a 0.45 μ m filter membrane. Then, the above 5 mL of extraction solution was purified by the active HLB solid phase extraction column. Finally, the column was rinsed by 1 mL ultrapure water with vacuum filtration for 20 min and eluted with 2.5 mL methanol. The collected eluate was concentrated to 200 μ L for measurement.

3. Results

3.1. Optimization of Extraction Conditions

The effective extraction of FZD from sea cucumber culture sediment is the most important foundation in this study, so the extraction conditions were optimized first. In most cases, the solvents, such as acetonitrile [29], ethyl acetate [30], methanol [31], dichloromethane [32] and acetonitrile/methanol mixture [33] were used as the FZD extractant. In order to explore the best extractant for the sediment matrix, four kinds of extractants were selected for comparison, including acetonitrile, dichloromethane, ethyl acetate and acetonitrile/methanol (1/1) mixture. The sediment samples were spiked with FZD at the concentration of 100 μ g/kg, then it was extracted and detected according to the steps in Section 2.5.

The recovery rate with different extractants is shown in Figure 1a. It can be seen from the results that the extraction rate with dichloromethane is the lowest, ranging from 28.4% to 32.8%. The ethyl acetate is easiest solvent with which to extract diethyl lipids, which are the metabolites of FZD. Therefore, the extraction rate increased to 70% when acetonitrile and ethyl acetate were used. The highest extraction rate is about 80% to 90% while the acetonitrile/methanol (1/1) was used.

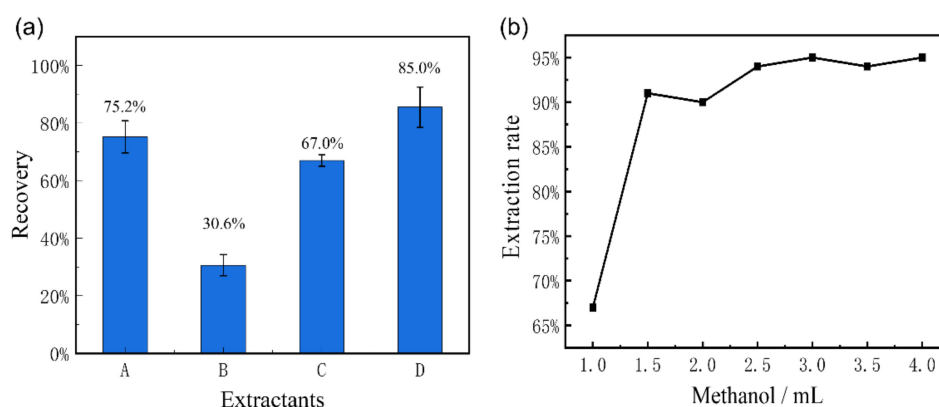


Figure 1. (a) The recovery of FZD with different extractants, including acetonitrile (A), dichloromethane (B), ethyl acetate (C), and acetonitrile/methanol (1/1) mixture (D); (b) the elution curve with the methanol.

The HLB solid phase extraction column was used for the preliminary purification of the extract. It has been reported that the impurities were dissolved less when the N, N-dimethylformamide was introduced in eluents. Therefore, the elution efficiencies of different eluents were compared in this work, including methanol, acetonitrile and gradient concentrations of N, N-dimethylformamide with methanol. It was found that the introduction of N, N-dimethylformamide resulted in an increase in the consistency of the

eluent and a slow speed of elution process. Conversely, the pure methanol showed the best elution performance. In order to achieve the best elution effect, the different volumes of methanol were implemented. The elution curve of the HLB column with methanol was shown in Figure 1b. It can be seen that the methanol can realize 94% recovery rate when the eluent volume increases to 2.5 mL, showing a good elution ability for FZD.

Therefore, the optimized extraction conditions, including the acetonitrile/methanol (1/1) extractant and 2.5 mL methanol eluent, were employed in the following experiments.

3.2. HPLC Analysis and Purification

Since the chemical structural formula of FZD has the basic structure of 5-nitrofuran, an acidic solution is used as the mobile phase to improve its peak shape in HPLC analysis. In this study, the mixture of 0.1% phosphoric acid and acetonitrile was used as the mobile phase, and the mixing ratios were optimized. The mobile phase A is constituted by acetonitrile/0.1% phosphoric acid (4/6), and mobile phase B is the mixture of acetonitrile/0.1% phosphoric acid (6/4). The injection volume of 0.5 µg/mL FZD was 10 µL, and the flow rate was 1 mL/min. The detection results are shown in Figure 2a.

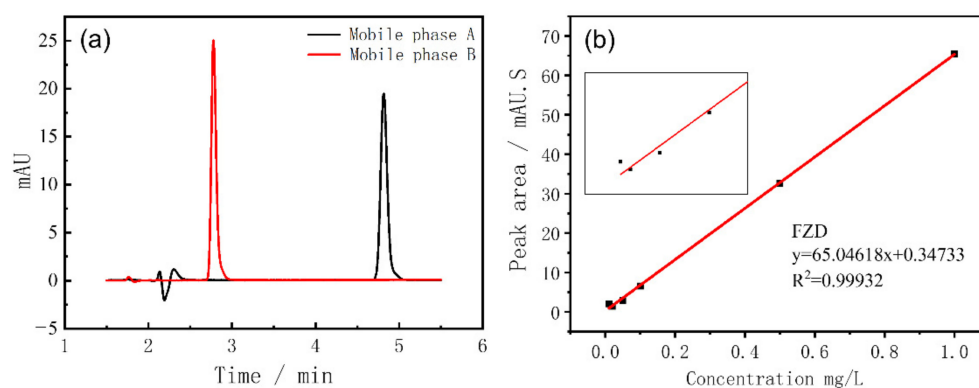


Figure 2. (a) Chromatograms of FZD standard solution with different mobile phases; (b) the FZD calibration curve. A local enlargement of the curve is shown in the inset.

It can be seen that the increasing proportion of acetonitrile will advance the peak position of FZD, which easily leads to the inability to separate the target peak from other impurity peaks. Therefore, the mobile phase A was used in subsequent experiments. The FZD calibration curve was acquired with the above chromatographic conditions to explore the response performance of HPLC to FZD, which was shown in Figure 2b. The linear range is 0.02 to 1 µg/mL and the R^2 is 0.9993. From the calibration curve, it can be seen that there is a poor linear relationship at low concentrations and the lowest response concentration of FZD by HPLC instrument was 0.01 µg/mL.

We explored the blank and spiked sediment samples, and the results are shown in Figure 3. Even at the level of FZD spiked at 1 µg/kg, the absorption peak was still clearly visible at about 4.8 min. In addition, the blank sample also has a certain signal response at the peak position of FZD. However, due to the influence of other substances in the sediment matrix, the chromatogram baseline is terrible, and it is difficult to determine whether the blank sediment sample contained the FZD. Therefore, the effluent from 4.7 to 4.9 min was collected and concentrated by nitrogen blowing for further SERS analysis.

3.3. SERS Detection

Au NPs colloid solutions were prepared as SERS substrates by the method in Section 2.3. Figure 4a shows the UV-Vis absorption spectra of three batches of Au NPs prepared repeatedly. It can be seen that Au NPs have an absorption peak at around 520 nm, and the high reproduction of the absorption spectra indicates that the Au NPs prepared by this method are very stable. In addition, the transmission electron microscopy (TEM)

characterization of the Au NPs was carried out (Figure 4b), which shows that the Au NPs give rise to the spherical shape and uniform size.

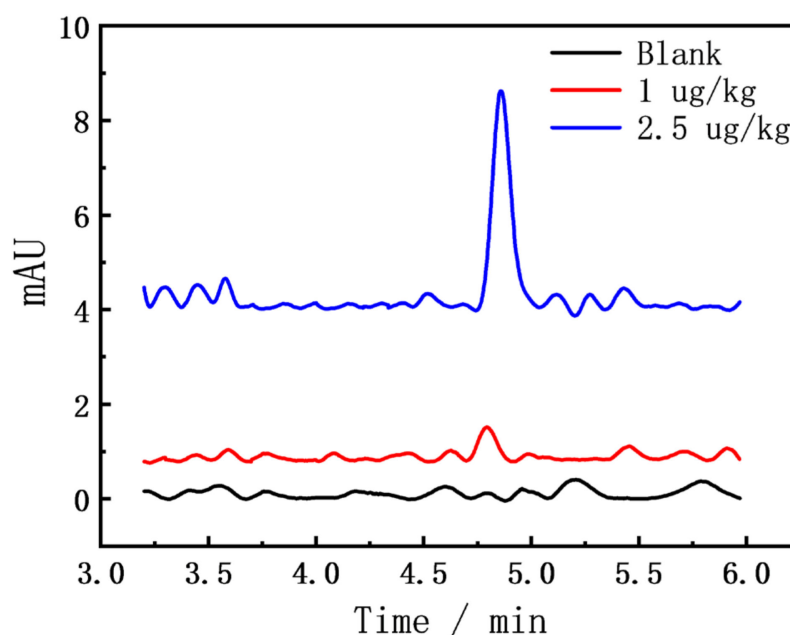


Figure 3. HPLC chromatogram of the FZD in sediments.

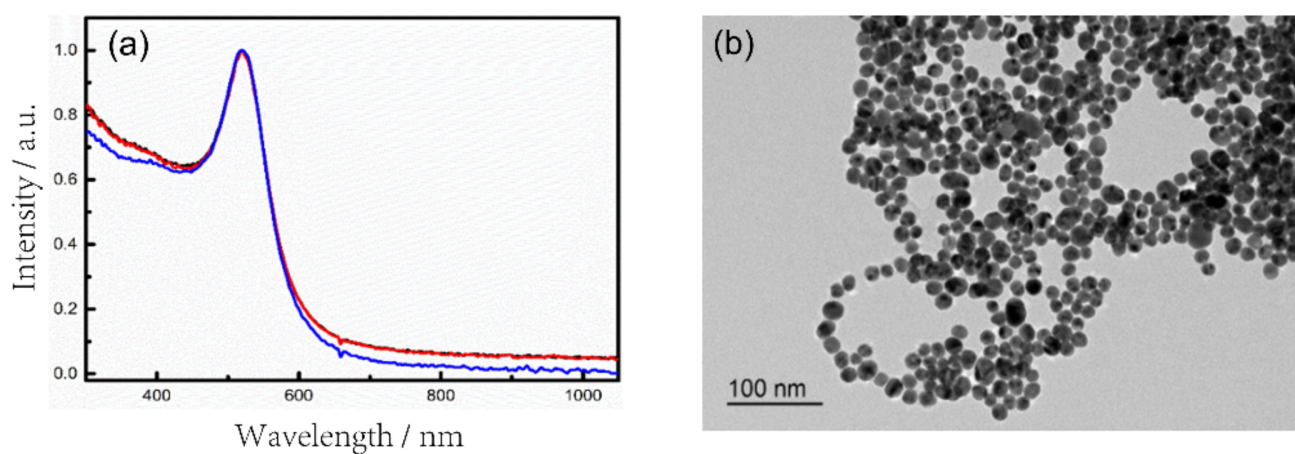


Figure 4. (a) UV-Vis absorption spectra of the Au NPs colloid solution; (b) TEM image of Au NPs.

In order to verify the SERS performance of the Au NPs substrate, 20 μL of the prepared Au NPs were dropped on the aluminum foil, and then 10 μL of the standard FZD solution was dropped on the Au NPs. After the analyte was dried at room temperature, SERS detection was implemented. Each substrate was collected 10 spectra, and three parallel experiments were performed to obtain the final averaged spectrum. The results are shown in Figure 5a. It can be seen that the SERS characteristic signal of FZD can still be obtained with the absolute amount of 1 ng, including the clearly visible characteristic peaks at 1350 cm^{-1} , 1400 cm^{-1} and 1610 cm^{-1} , which show a better performance than the reported enzyme-linked immunosorbent assay method (detection limit, $2\text{ }\mu\text{g/kg}$) [34] and the similar SERS methods [35,36].

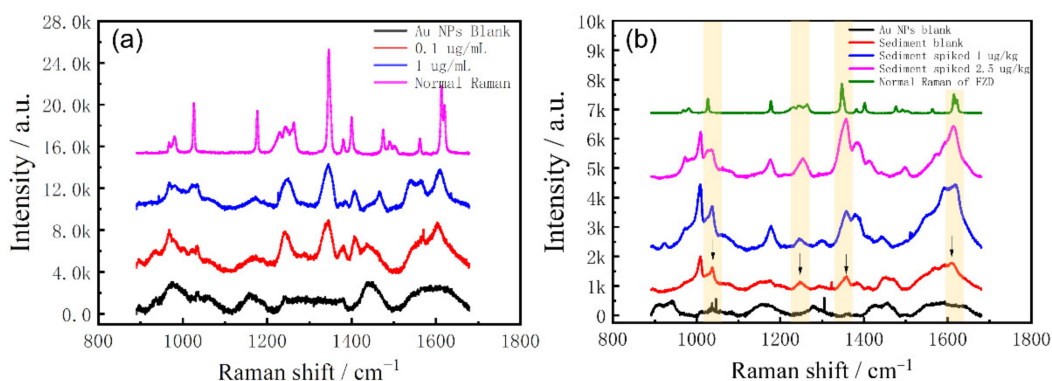


Figure 5. (a) SERS and Raman spectra of FZD; (b) SERS spectra of HPLC effluent from the sediments.

The spiked sediment extracts were tried to directly combine with Au NPs for SERS detection, but the SERS features of FZD could not be detected at all. It should be caused by the masking effect from the complex composition in sediment matrices, so that the extract cannot be used for SERS detection directly.

The HPLC effluent collected from the spiked sediments in Section 3.2 was concentrated, and then combined with Au NPs for SERS analysis. The detection process was the same as the above, and the results are shown in Figure 5b. It can be seen that HPLC realizes the effective separation and purification of the extract, so that the signal of FZD can be detected in the collected effluent. Compared with the normal Raman spectrum of FZD, we found that the characteristic peak of FZD was still obvious with 1 µg/kg spiked, and it was even visible in the blank sediment sample. The blank sediment sample could not be accurately identified as containing FZD by HPLC analysis. However, the contamination of FZD in the blank sediment was confirmed by SERS detection, which further verified the practical application potential of the developed HPLC-SERS analysis method.

4. Conclusions

In this study, HPLC was used to separate and purify the crude FZD extract from the sea cucumber aquaculture sediment, and then the effluent was concentrated and analyzed by SERS to realize the rapid qualitative detection of FZD. The absolute detection limit of FZD by SERS was 1 ng, and the detection limit of FZD in actual sediment samples was lower than 1 µg/kg. Moreover, the doubtful contamination of FZD in the blank sediment in HPLC analysis was confirmed by SERS detection, which further verified the practical application potential of the developed HPLC-SERS analysis method.

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