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Optimization and Validation of Europium-Sensitized Fluorescence Method for Determination of Tetracycline Antibiotics in Water from Fish Farms

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Abstract: Sensitized europium fluorescence was used for simple, fast and efficient determination of tetracycline residues in water from fish farms. Tetracycline antibiotics: oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) were extracted from water samples using polymeric hydrophilic–lipophilic balanced cartridges. After evaporation and pre-concentration, tetracyclines form a complex with europium and citric acid as coligand at pH 8.5. The complex formed has a wide absorption spectrum at 388 nm and a narrow emission maximum at 619 nm resulting from the $5D_0$ - $7F_2$ transition within the europium ion. The complex is stable with intensive fluorescence and linear in the concentration range of 5-2500 µg/L for tetracycline and oxytetracycline and 5-1000 µg/L for chlortetracycline. The detection limit was 0.68 µg/L for OTC, 1.29 µg/L for TC and 0.65 µg/L for CTC, respectively. The proposed method is very sensitive and particularly applicable to samples where low concentrations of tetracycline antibiotics are expected.

INTRODUCTION

Nowadays, fish farming represents a significant percentage of the population's food supply. Intensive fish farming is related to the increased usage of antimicrobial agents applied for the treatment and prevention of bacterial fish (Troell, Naylor, Metian*et al.*, 2014). Currently, due to the countries' differences in distribution and registration systems, the determination of the quantities of antimicrobials applied worldwide in aquaculture is very challenging. In each country, various legal restrictions regulate the usage of medicines in aquaculture, and compliance monitoring also varies from country to country (Miranda, Godoy, Lee, 2018; Quesada, Paschoal, Reyes, 2013; Heuer, Kruse, Grave *et al.*, 2009). In aquaculture, therapeutic doses of antibiotics are administered orally for a short time, mixed with specially formulated food or added directly into the water in the form of therapeutic baths. As fish farms are most commonly located in rivers or lakes, residues of directly added antibiotics, unconsumed feed pellets and toxic feces are distributed throughout the entire ecosystem. It is estimated that 75% of the antibiotics administered through feed are excreted into water (Kemper, 2008). The tetracycline antibiotic, oxytetracycline (OTC) is one of the most commonly used antibiotics in fish farms. Due to the low intestinal absorption of fish, which results in slow excretion of large amounts of this antibiotic, its application has to be followed by high doses of 100-150 mg per kg of fish per day for 10-15 days. (Capone, Weston, Miller, 1996). It is estimated that 70–80% of OTC in feces is intact (Miranda, Godoy, Lee, 2018). Although expected, the presence of OTC's in surface waters or sediments is not frequently confirmed. A possible explanation could be based on the fact that OTC undergoes photolysis and hydrolysis, which makes it difficult to detect in water. In addition, OTC binds easily to cations such as calcium and magnesium or binds to proteins as well. Detection of antibiotics is more challenging due to the complexes' environmental immobility and significant differences in properties (Gothwal, Shashidhar, 2014; Havelkova, Beklova, Kovacova, 2016). In order to determine the extent to which the ecosystem has been exposed to OTC, but also

to other tetracyclines, it is of crucial importance to identify and quantify them in surface and groundwater, sludge and sediments (Chen, Chen Y, Ding, *et al.,* 2015; Ahmad, Zhu, Sun, (2021). In addition to OTC, which was the first antibiotic approved by the Food and Drug Administration (FDA) for use in fish farms, tetracycline (TC) and chlortetracycline (CTC) can also be used in aquaculture (Olatoye, Basiru, 2013). To ensure the safety of food for human consumption, the Food and Agriculture Organization (FAO) has defined an acceptable daily intake of 0-30 μg/kg for these three antibiotics (FAO, 2015).

Microbiological method enzyme-linked immunosorbent ELISA (Shahbaz, Ahmadi, Karami, 2015; Aga, Goldfish, Kulshrestha, 2003), UV/VIS spectroscopy, thin layer chromatography (TLC), liquid chromatography (LC), (Bečić, Imamović, Dedić, 2014; Alanazi, Almugbel, Maher *et al.,* 2021; Pérez-Rodríguez, Pellerano, Pezza, 2018), liquid chromatography/mass spectrometry (LC/MS), liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods (Zhu, Snow, Cassada *et al.*, 2001; Cháfer-Pericás, Maquieira, Puchades, 2010; Kang, Lee, Shin *et al.*, 2018) were used for the screening and determination of tetracycline antibiotics residues. Sensitized lanthanide fluorescence is a very sensitive method based on energy transfer from a ligand to a lanthanide ion with a characteristic emission at the lanthanide emission wavelength. This phenomenon has been used to determine tetracycline antibiotics OTC, TC and CTC in various samples e.g. milk, blood plasma, serum (Hongliang, Yang, 2012; Shtykov, Smirnova, Bylinkin, *et.al*., 2005; Arnaud, Geoges, 2001) where tetracycline is used as a ligand. The present study evaluated the possible application of europium ion fluorescence as an indicator for the tetracycline antibiotics determination in the fish farms water and other surface water.

EXPERIMENTAL

ll solvents and chemicals used were p.a. and analytical reagent grade (Panreac, Italy; Merck, Germany). The ultrapure water was from a Sartorius purification system. $EuCl_3 \times 6H_2O$ (Sigma Aldrich, St. Louis, MO, USA) was used to prepare a standard solution of Eu^{3+}($1{,}6 \times 10^{-3}$ M). Tetracycline hydrochloride, Stock standard solutions of oxytetracycline hydrochloride and chlortetracycline hydrochloride (Sigma Aldrich, Germany) (500 μg/mL) were prepared by weighing accurate quantities of the standards, dissolved in 1 mL of ultra-pure water and diluted with acetonitrile. For the calibration curve, the stock solutions were diluted with acetonitrile in the concentration range 5–2500 μg/L for OTC and TC and 5-1000 μg/L for CTC.

For analysis, solutions of 1 mM citric acid, oxalic acid and tartaric acid in ultra-pure water were prepared. Tris and borate buffer solutions pH 6-9 were prepared according to Ph. Eur. Procedure (European Pharmacopoeia, 10th Edition, 2020). All prepared solutions were stored at 4°C. A Shimadzu RF-5301-PC spectrofluorometer (Kyoto, Japan) with Panorama fluorescence 1.1 software was used to measure fluorescence intensity

Sample preparation (water samples). Water samples from two different fish farms were taken in two-liter amber-colored glass bottles and used for analysis. All collected samples were filtered through Whatman filter paper to remove suspended matter and then filtered through a 0.45μm membrane filter. The samples were stored in a refrigerator at +4°C until solid phase extraction (SPE) and further analysis. Before extraction, the total concentration of calcium and magnesium was determined. An appropriate amount of EDTA was added to prevent the binding of the antibiotics to calcium and magnesium.

Blank sample matrix preparation. Water samples used as blanks were collected in amber colored glass bottles, upstream from the fish farms. These samples were extracted in the same way as spiked water samples and environmental samples to detect possible endogenous interferences and method selectivity .

Spiked water samples. The spiked water samples were prepared with an appropriate amount of tetracycline standard solution in the blank sample matrix. The concentration of tetracycline antibiotics added was 50 μg/L, 500 μg/L and 1000 μg/L.The recoveries were measured by the optimized and validated method described below.

Environmental samples. Environmental samples *w*ere collected from two fish farms, extracted and measured as the blank sample matrix and spiked water samples.

SPE extraction

Extractions of 250 mL acidified (pH 3) matrix sample, spiked water samples and environmental samples were performed on a hydrophilic-lipophilic balance (Oasis, HLB, Waters) 6 mL/500 mg cartridge at a flow rate of about 3 mL min^{-1} with Supelco vacuum manifold system connected to the vacuum pump. The cartridges were pre-conditioned with 5 mL methanol and 5 mL ultrapure water pH 3. After extraction, the cartridges were washed with 2 mL of ultrapure water to remove EDTA residues and dried by vacuum for 5 minutes to remove excess water. Tetracycline elution was performed with 3 mL of methanol. The filtrates were evaporated in a nitrogen stream after which the residues were dissolved in 1 mL of acetonitrile (Bečić, Imamović, Dedić, 2014).

Fluorescence assay

Fluorescence measurements for the calibration curves were performed as follows: one milliliter of each solution: europium ($5x10^{-5}$M), OTC or TC (5-2500 μg/L) or CTC (5-1000 μg/L) standard solutions, Tris (0.1M, pH 8.5) and citric acid (1 mM) were mixed, stirred vigorously and left for 10 minutes at room temperature. After 10 minutes, fluorescence was measured at an excitation wavelength of 388 nm and an emission wavelength of 619 nm. After SPE extraction, 1 mL of spiked water and environmental samples were used instead of standard antibiotic solutions to measure the fluorescence of these samples.

RESULTS AND DISCUSSION

Due to the high fluorescence sensitivity of the europium - tetracycline complex, it is essential to optimize the influencing parameters (Kaczmarek, 2020).

The spectroscopic properties of tetracycline are influenced by two separate chromophores in the molecule. Depending on the pH, these chromophores are protonated, which enables binding to the europium ion. Increasing the pH value increases the possibility of chromophore protonation. Therefore, it is very important to find an appropriate buffer solution and pH value. In addition, the pH value influences the fluorescence intensity of the complex. At pH values up to 5 fluorescence is very low and reaches a maximum at pH around 9 (Courrol, Samad, 2008; Shtykov, Smirnova, Yu, 2005).

In our previous studies, we have optimized the following parameters: tetracycline and europium concentration, pH and coligands (Bečić, Mušanović, Imamović, 2016).

It is known that a high concentration of Eu (III) can cause quenching of fluorescence upon collision in the singlet excited state of tetracycline (Courrol, Samad, 2008). Therefore, we optimized europium concentration by measuring the fluorescence intensity at different europium concentrations of $1.6x10^{-3}$M-$1x10^{-6}$M while tetracycline concentration was constant. Following the results obtained, europium concentration 5×10^{-5} M was selected. A linear increase in fluorescence intensity was found in the following concentrations of TC, OTC 5-2500 µg/L and CTC 5-1000 µg/L. The influence of pH on the fluorescence of Eu (III)-tetracycline complex was also optimized. The results showed that with an increase in the pH of both used buffers to 8.5, the fluorescence intensity increased. This was expected due to the deprotonation of the tetracycline molecule. (Figure 1.)

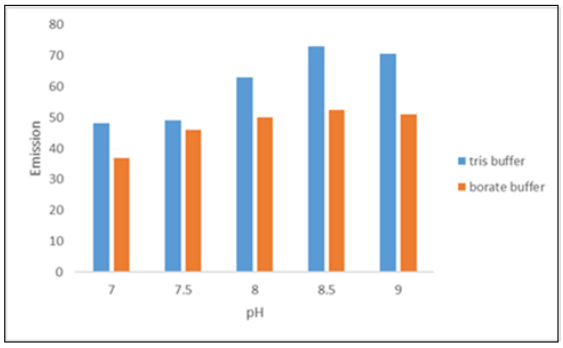

Figure 1. Fluorescence intensity with tris and borate buffer

Due to the possibility of europium hydroxide formation at pH 9, tris buffer pH 8.5 was selected for further measurements.

Citric acid was selected as a coligand, which resulted in a significant increase in fluorescence intensity. Due to the importance of the influence of pH on complex formation as well as the possibility of lanthanide precipitation, the order of addition of reactants was optimized. As seen in Figure 2, the highest fluorescence intensity was demonstrated by the complex formed by the reactants added in the following order: europium - tetracycline – tris – citric acid.

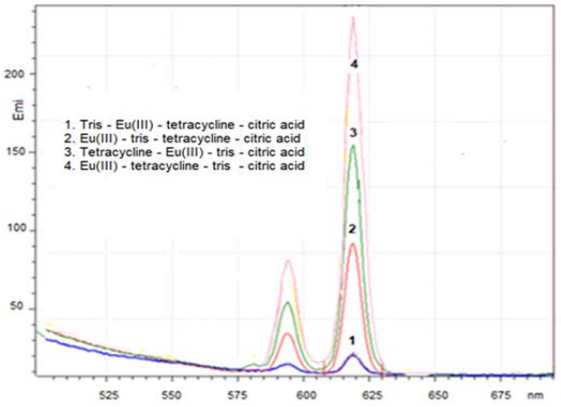

Figure 2. Influence of reactant addition order on the Eu(III) sensitized fluorescence

Method validation

To evaluate and confirm the validity of the method, analytical parameters were measured based on the ICH Guidelines (ICH, 2019).

Specificity

After SPE extracting of the blank sample matrix, specificity was determined and analyzed with endogenous interference. No interference was detected.

Linear dynamic range

The linearity of the method was determined by analyzing six solutions with antibiotic concentrations in the range of 5-2500 µg/L for TC and OTC and 5-1000 µg/L for CTC, respectively. Fluorescence measurements for each concentration were repeated six times. Calibration standard curves were constructed by plotting the mean values of fluorescence measurements against the concentrations of the standard (Figure 3.). Under optimized experimental conditions, good linearity was found between concentration and fluorescence emission with r^2=0.9987 and 0.9986for OTC and CTC and 0.9991 for TC.

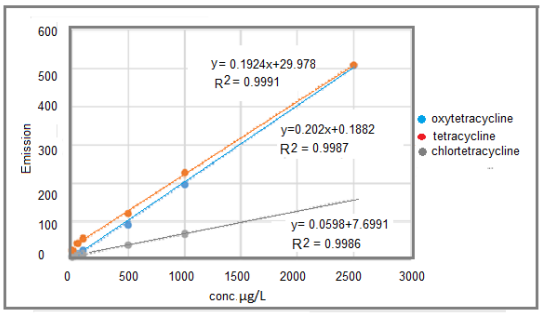

Figure 3. Calibration curve for OTC, TC and CTC

Precision

Precision (repeatability) was tested by analyzing 6 samples of the sample matrix spiked with 500 µg/L OTC, TC and CTC. The coefficient of variation was calculated by the formula:

$$CV\ (\%) = SD \times 100/mean$$

Accuracy

The accuracy of the method was expressed as the average recovery factor (%) calculated at three concentration levels using six replicates for each concentration. The

obtained results for the analysis of spiked sample matrix water containing 50, 500 and 1000 µg/L tetracycline antibiotics were higher than 90% for all compounds (Table 1).

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the blank and for OTC, TC and CTC were found to be 0.68, 1.29, 0.65 µg/L, and 1.61, 4.78 2.03, µg/L, respectively.

Table 1. shows all the parameters of method validation

Table 1. Method validation parameters

Validation parameter	Value
OTC	
Linearity	R^2= 0.9987
Precision	CV (%) = 0.92
Accuracy	Recovery (%)
Spiked sample (50 µgL)	94.46
Spiked sample (100 µg/L)	97.35
Spiked sample (1000 µg/L)	100.97
LOD	0.68 µg/L
LOQ	1.61 µg/L
TC	
Linearity	R^2= 0.9991
Precision	CV (%) = 0.97
Accuracy	Recovery (%)
Spiked sample (50 µg/L)	93.53
Spiked sample (100 µg/L)	96.35
Spiked sample (1000 µg/L)	101.65
LOD	1.29 µg/L
LOQ	4.78 µg/L
CTC	
Linearity	R^2= 0.9986
Precision	CV (%) = 0.91
Accuracy	Recovery (%)
Spiked sample (50 µg/L)	92.15
Spiked sample (100 µg/L)	96.55
Spiked sample(1000 µg/L)	97.22
LOD	0.65 µg/L
LOQ	2.03 µg/L

Analysis of environmental samples

After SPE extraction, water samples taken from the fish farms were tested by the proposed method. No residues of tetracycline antibiotics TC, OTC, CTC were found in the tested samples. A possible explanation for such results could be that the tetracycline antibiotics were photodegraded or that antibiotics were not applied during fish farming. Furthermore, the continuous flow of water can also affect the detection of antibiotic residues, if they are, due to dilution, of low concentration in the fish farm water.

CONCLUSION

The sensitized fluorescence of europium (III) ion can be used for the determination of OTC, TC or CTC residues individually or as the total amount of residues in water from the fish farm or other surface water. This method is simple, with high sensitivity, selectivity and accuracy. Due to its simplicity, it can be used as a rapid screening method to assess environmental exposure to tetracycline antibiotics residues.

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Summary/Sažetak

U radu je optimizirana i validirana metoda u kojoj se pobuđena fluorescencija europijuma koristi za jednostavno, brzo i učinkovito određivanje rezidua tetraciklinskih antibiotika u vodi iz ribogojilišta. Tetraciklinski antibiotici oksitetraciklin (OTC), tetraciklin (TC) i hlortetraciklin (CTC) ekstrahirani su iz uzoraka vode ekstrakcijom na čvrstim fazama. Nakon ekstrakcije i prekoncentracije uzorci vode su pomiješani sa europijumom i limunskom kiselinom kao koligandom pri pH 8,5. Formirani kompleksi imali su maksimum ekscitacijena 388 nm i emisije na 619 nm koji je rezultat prijelaza $5D_0$ - $7F_2$ unutar jona europijuma. Kompleks je bio stabilan s intenzivnom fluorescencijom i linearan u rasponu koncentracija od 5-2500 µg/L za tetraciklin oksitetraciklin i 5-1000 µg/L za hlortetraciklin. Metoda ima limit detekcije za OTC 0,68 µg/L, TC 1.29 µg/L i 0.65 µg/L za CTC. Predložena metoda je osjetljiva i jednostavna. Posebno je primjenjiva na uzorke gdje se očekuju niske koncentracije rezidua tetraciklinskih antibiotika.