MONITORING USE OF ANTIBIOTICS IN AQUACULTURE

Dr O. Korostynska[1], Dr A. Mason[1], Dr I. Nakouti[2], Dr. W. Jansomboon[3], Prof A. Al-Shamma'a[1]

[1] Faculty of Engineering and Technology, Liverpool John Moores University, Liverpool, **UK**
[2] Faculty of Science, Liverpool John Moores University, Liverpool, **UK**
[3] Faculty of Engineering, Kasetsart University, **Thailand**

ABSTRACT

In the aquaculture industry around the world antibiotics are used for fish disease prevention and treatment. High residual levels of those antibiotics may contaminate natural water resources as well as soil, aquatic animals and plants. Their overuse in human and animal populations can lead to the development of resistant microbial strains, posing a dire threat to global health. Use of antibiotics in aquaculture and its impact on the environment is a growing concern amongst scientists, yet quantifying the amount of use and how much is being disseminated into the environment is very difficult. As with the use of antibiotics in food production more generally, there is a need for better data.

To prevent the improper use of antibiotics in aquaculture and to assist the food safety law enforcement, this paper reports on assessing the feasibility of a bespoke electromagnetic wave sensing method for real-time in situ monitoring of residual antibiotic concentrations in water samples. For the first time the antibiotics solutions were tested in contact with planar sensor with interdigitated electrode pattern on a number of substrates, including Rogers®, FR4 and flexible polyimide substrates.

Specifically, this paper communicates the experimental results of using bespoke microwave planar type sensors for the determination of Quinolones, in particular Enrofloxacin (ENR) and Norfloxacin (NOR) antibiotic concentrations. Reflected power signals were analysed in GHz frequency range and these were dependent on both: the type of antibiotic present in water and on its concentration.

Keywords: water ecosystem, electromagnetic waves; planar microwave sensor; antibiotics detection; aquaculture monitoring.

INTRODUCTION

Screening of food products from animal origin for the presence of antimicrobial residues started soon after the introduction of antibacterial therapy in veterinary medicine. Initially it mainly concerned process monitoring in the dairy industry to prevent problems in fermentative dairy production, but from the early 1970s regulatory residue screening in slaughter animals and later in fish also became more commonly introduced across the European Union.

In the aquaculture industry around the world antibiotics are used for fish disease prevention and treatment. High residual levels of those antibiotics may contaminate natural water resources as well as soil, aquatic animals and plants. Their overuse in human and animal populations can lead to the development of resistant microbial

strains, posing a dire threat to global health. The antibiotics used in fish feed can remain in the aquatic environment for an extensive period of time, through excretion, exerting selective pressure and spreading rapidly through water systems. Some suggest 70-80 % of antibiotics given to fish are excreted into water.

For example, large use of Quinolones has been linked with an increase of antimicrobial resistance in foodborne pathogens which can be passed on to humans. Exposure of excess levels of antibiotic residues in food can cause allergic reactions in some hypersensitive persons and may affect the human immune system. Similarly, drug resistance in pathogens in human body can also be a serious problem due to the low-dose poisoning in foodstuffs for a long period [1].

The widespread and indiscriminate use of antibiotics has led to the development of antibiotic resistance in pathogenic, as well as commensal, microorganisms. Resistance genes may be horizontally or vertically transferred between bacterial communities in the environment [2]. Substantial increase of bacteria resistant to quinolones, amoxicillin and oxytetracycline in fish farms employing such antibiotics has been discovered [3].

There are several methods of Quinolones determination in environmental waters, including wastewaters, ground, natural and surface waters, but most of them are lab-based. They require the extraction processes followed by determination and confirmation methods such as variations of liquid chromatography tandem with fluorescence–mass spectrometry [4]. The key challenge is implementing system that can effectively determine Quinolones in real time.

This paper reports on assessing the feasibility of using real-time electromagnetic wave sensing at microwave frequencies with non-thermal energy. Microwave sensors provide the opportunity for a rapid and robust method of materials analysis. The principle of monitoring using microwave sensors, in the context of this work, is based on the interaction of electromagnetic (EM) waves with a sample under test. When this sample is exposed to EM irradiation it alters the velocity of the signal, attenuates, or reflects it. The approach was previously successfully tested on wastewater chemicals detection, in particular NO_3 and COD [5]; for vegetable oil type verification to comply with food labelling regulations, food quality control [6] and in biomedical area [7].

This paper communicates the experimental results of using the microwave sensing approach for the determination of Quinolones, in particular Enrofloxacin (ENR) and Norfloxacin (NOR) antibiotic concentrations in water.

MODERN METHODS OF ANTIBIOTICS DETECTION IN AQUACULTURE INDUSTRY

Most methods currently used for antibiotics detection require the extraction processes followed by determination and confirmation methods such as variations of liquid chromatography (including Ultra Performance Liquid Chromatography (UPLC)) combined with fluorescence–mass spectrometry (MS) [4]. Microbial inhibitions assays were the earliest methods used for the detection of antibiotic residues and they are still widely used. They are very cost-effective and in contrast to, for example, immunological or receptor-based tests, they have the potential to cover the entire antibiotic spectrum within one test. Two main test formats can be distinguished: the tube test and the (multi-) plate test. A tube (or vial, or ampoule) test consists of a growth

medium inoculated with (spores of) a sensitive test bacterium, supplemented with a pH or redox indicator. At the appropriate temperature, the bacteria start to grow and produce acid, which will cause a colour change. The presence of antimicrobial residues will prevent or delay bacterial growth, and thus is indicated by the absence or delay of the colour change.

For example, the determination of erythromycin in medicated salmonid fish feed was done using liquid chromatography and UV spectroscopy (LC-UV method) [8]. This method produced high accuracy, 82-90%, for both salmon and trout feed that represented varied pellet sizes and ingredient amounts. The intraday and interday precisions, at $\leq$6 and 5%, respectively, indicated the method's good repeatability. However, the method is time-consuming as it requires erythromycin to be extracted from feed with acetonitrile and water; then cleaned up by SPE; evaporated to dryness, reconstituted, and only afterwards analysed by LC-UV [8]. Needless to say, an expert is required to perform this analysis and this approach cannot be considered as ready-to-use alternative for instant fish products quality monitoring.

Fast screening immunoassay of sulfonamides in commercial fish samples was reported in [9]. In this approach an indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed in plate to detect three sulfonamide residues (sulfamerazine (SMR), sulfadimetoxine (SDM), and sulfadiazine (SDZ)) in gilthead sea bream (Sparus aurata) samples using different extraction methodologies. The assay detection limits for these antibiotics were lower than 100 $\mu g\ kg^{-1}$ (maximum residue level established by the European Union). Notably, this approach is yet to see wide-scale commercial implementation, not least due to matrix effects, since a standard addition calibration curve in fish extract is necessary for quantification purposes.

There are some attempts to develop an in-situ sensor system for quality monitoring of aqueous media and fish products. For example, rapid automated method for on-site determination of sulfadiazine in fish farming that utilised a stainless steel veterinary syringe coated with a selective membrane of PVC serving as a potentiometric detector in a flow-injection-analysis system was reported [10]. Sulfadiazine is an antibiotic of the sulfonamide group and is used as a veterinary drug in fish farming. Monitoring it in the tanks is fundamental to controlling the applied doses and avoiding environmental contamination. Reportedly, the best performance of this system was obtained for sensors of 1.5 cm length and a membrane composition of 33% PVC, 66% o-nitrophenyloctyl ether, 1% ion exchanger, and a small amount of a cationic additive [10]. It exhibited Nernstian slopes of 61.0 mV $decade^{-1}$ down to 1.0×10^{-5} mol L^{-1}, with a limit of detection of 3.1×10^{-6} mol L^{-1} in flowing media.

Notably, detection of antibiotics using carbon nanotube (CNT)-based sensors was attempted in [11]. CNT-based sensor transducers were functionalized with the single-chain variable-fragment (scFv) of antibodies that can selectively bind to a specific antibiotic or the certain family of antibiotics. These CNT-based sensors were functionalised with A_2scFv and F_9scFv and exhibited the specific detection of enrofloxacin or the family-selective detection of fluoroquinolone-based antibiotics, respectively, in a real-time manner. In another alternative approach to detect antibiotics, a high-density optical microarrays based on molecularly imprinted microsphere sensors that directly incorporate specific recognition capabilities detected enrofloxacin [12].

This work focuses on the detection of Enrofloxacin and Norfloxacin. Enrofloxacin is an antibiotic widely used for both human and veterinary applications. Novel cost-effective approach is reported, in which non-ionising, athermal electromagnetic waves are used as a sensing and transducing mechanism of monitoring the concentration of antibiotics in aqueous solution in real-time.

EXPERIMENTAL PROCEDURE

Stock antibiotic solutions of Enrofloxacin and Norfloxacin were prepared by dissolving 10.0 mg of each individual antibiotic in 1,000 mL of deionised water to achieve a final concentration of 10 mg/L (10 ppm). Each working antibiotics solutions were prepared by appropriate dilution of aliquots of the stock antibiotic solutions with deionised water.

The experimental setup used in this work with Rohde and Schwarz ZVA24 vector network analyser (VNA) connected to a microwave cavity with antibiotic-containing test water sample is illustrated in Fig. 1. A bespoke planar sensor structure on Rogers® substrate with dielectric constant of 2.2 is detailed in Fig. 2 (a), while Fig. 2 (b) demonstrates planar IDE sensor on a flexible polyimide substrate that was used for the detection of antibiotics in water. Standard edge-mount type SMA connector was used for the planar sensor to allow minimum possible signal interference when loading the test sample and during the sample handling. Edge-mount connection also makes it more convenient to separate the sensing and signal processing parts of the system for portable applications.

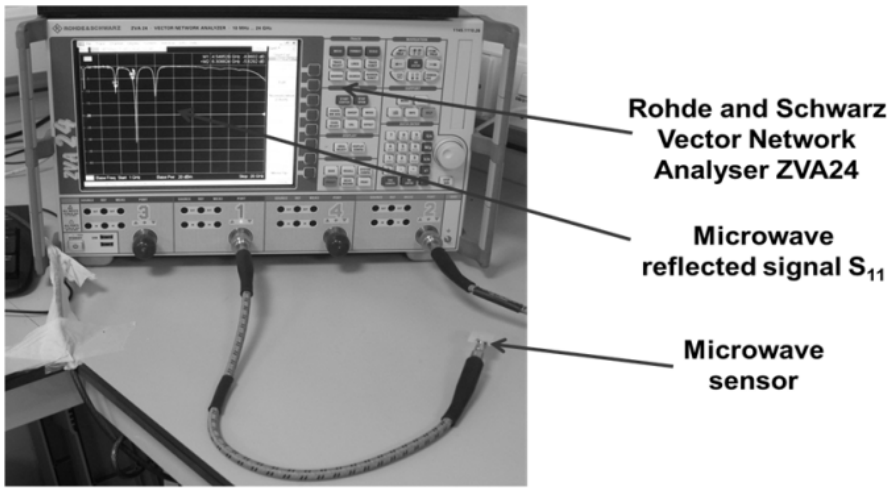


Fig. 1. Measurement setup showing VNA and a microwave sensor connected via coaxial cable.

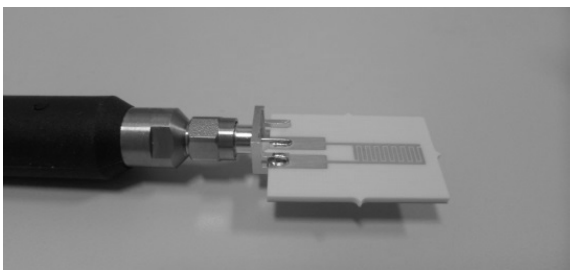
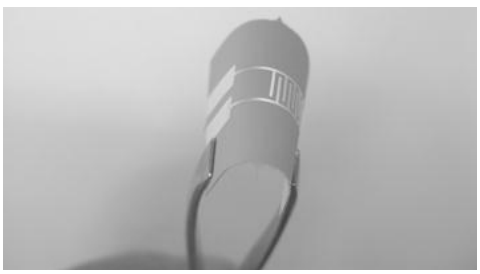

(a)

(b)

Fig. 2. Planar IDE sensor on (a) Rogers® substrate; and (b) flexible polyimide substrate.

RESULTS AND DISCUSSION

The change in the optical absorption with increasing concentration of ENR was recorded using UV-Vis Spectrophotometer. This set of measurements served as a benchmark for validating concentrations of antibiotics and for comparison of sensitivities of standard optical and novel electromagnetic wave sensing approaches. The optical measurements were performed in 190 nm – 1000 nm wavelength range. However, Fig. 3 illustrates the change in the differential optical absorption with concentration at 321 nm wavelength, as it provides better illustrations of the linear trend, with R^2=0.995.

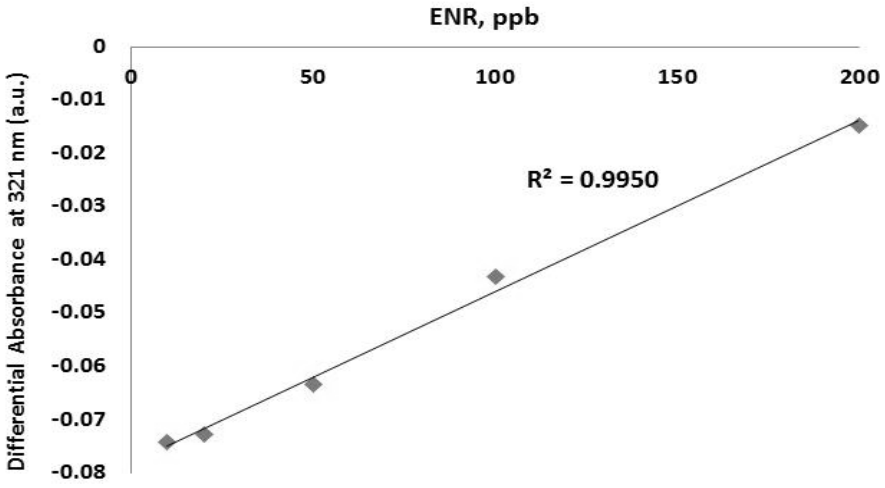


Figure 3. Absorbance change with ENR concentration at 321 nm.

The experimental approach reported in this paper was using microwaves at non-ionising intensity, with a low power output of approximately 1 mW (0 dBm). Microwaves have a good penetration depth and associated equipment can be portable for real-time use at the point-of-control in aquaculture applications. The multi-parameter nature of wide band microwave analysis can provide unique signal spectrum signatures. Typically these would be in the form of a reflected signal S_{11} signal for a single-port system. The spectral response is influenced by tested water samples parameters such as conductivity and permittivity. Permittivity relates to a material's ability to transmit an electric field and is a complex value which varies with frequency, and accounts for the energy stored by a material (ε') as well as any losses of energy (ε") which might occur.

Dependence of the S_{11} received microwave signal depending on ENR and NOR concentration was recorded for 0.4 ml solutions placed in contact with planar IDE microwave sensors based on Rogers® material. Differences in the microwave spectra were recorded in 0.01 – 15 GHz frequency range, but some ranges gave more pronounced response with antibiotics concentration. Importantly, these ranges depend not only on the measured analytes, but on the parameters of the electromagnetic wave sensor, including its substrate materials, dimensions and metal electrodes. Fig. 4 shows the difference in the S_{11} received microwave signal response to the presence of pure deionised water, and water with 10 ppm of streptomycin, enrofloxacin, and norfoxacin in contact with planar IDE microwave sensors based on PTFE material. Fig. 5 illustrates the response of the sensor at specific frequency of 1.52 GHz to illustrate that both antibiotic type and concentration can be determined.

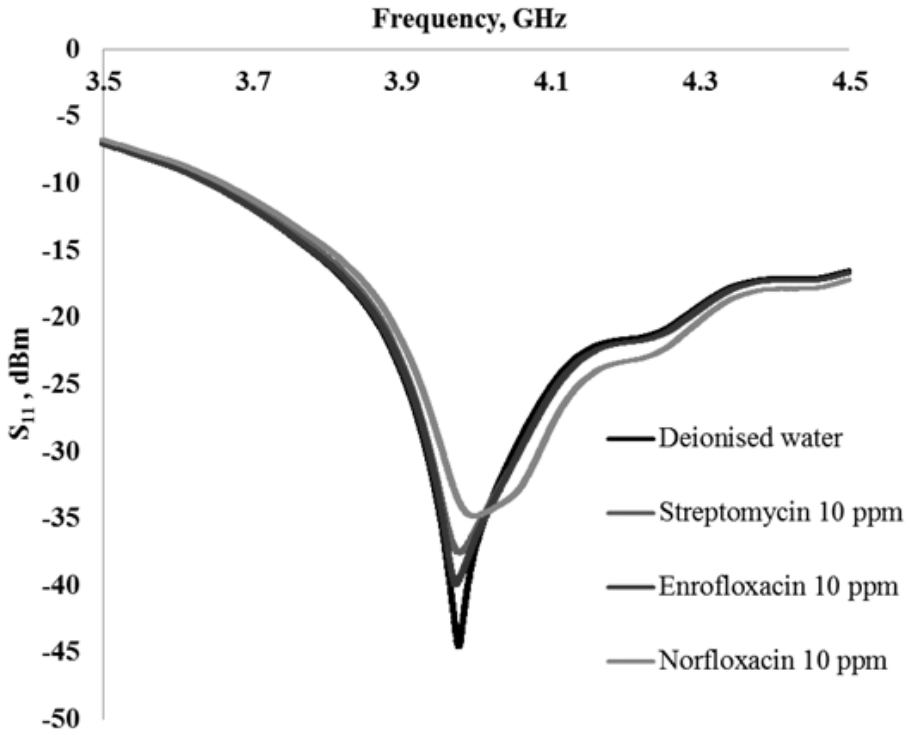


Figure 4. Difference in the S_{11} received microwave signal response to the presence of pure deionised water, and water with 10 ppm of streptomycin, enrofloxacin, and norfoxacin in contact with planar IDE microwave sensors based on PTFE material.

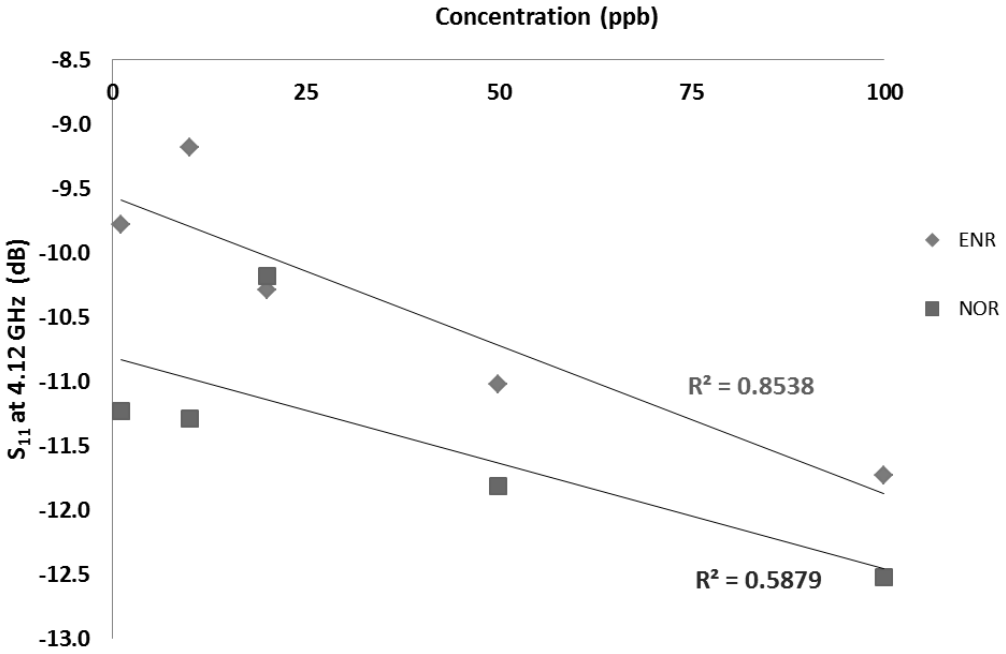


Figure 5. Dependence of the S_{11} received microwave signal on ENR and NOR concentration at 1.52 GHz recorded for 0.4 ml solutions placed in contact with planar IDE microwave sensors based on Rogers® material.

According to the experimental results presented above, which were repeated numerous times, substantial changes in the microwave spectra caused by the solutions with varying antibiotic concentrations were recorded and these can be used as an alternative

to the traditional optical method measure of an unknown analyte composition. The discrepancy between each sample measurement was within 5%, and could be due to imperfection of manual sample loading process. This method is non-destructive, it provides real-time responses and the microwave power used is in the order of 1 mW, which is non-ionising radiation, less than commonly used even in the mobile phones, and thus has negligible deleterious effect on the material being measured. Another advantage of this method is that there are no additional chemicals required to reveal the concentration of the analyte solution. The proposed system can potentially be integrated into an industrial production line or at any aquaculture industry check-point to automatically assess the composition of solutions in real time and manage the processes accordingly.

Notably, the sensors' responses returned to their original benchmark positions, namely air spectra, after each measurement of the analyte solution were performed and the sample was removed from the sensor surface. This confirms that the developed microwave sensor is reliable, re-usable and thus a sustainable solution for precise in-situ method for industrial process monitoring, where real-time information on solutions composition is essential.

The response of the sensor to other analyte solution types and concentrations is being explored and a database of these microwave signature spectra is being compiled, which can later be used for online process control in a broad range of industrial applications in the wastewater industry, chemical and pharmaceutical production lines. Further, the prototype system will be tested in real industrial settings and a challenge of differentiating between multiple water pollutants possibly present in a sample and determining their concentrations would emerge.

Real-time nature of the measurements and portable sensor size makes the suggested approach a valuable alternative to mainly lab-based method of health and safety and quality control in food industry, in aquaculture and environment.

CONCLUSION

In recent years, the intensive use of antibiotics induces the development of antibiotic resistant genes, which is an increasingly critical problem affecting human health, and the potential toxic effects of the ARGs have drawn great attention all over the world. This paper communicates the experimental results of using a bespoke planar electromagnetic wave sensor operating at microwave frequency range for water ecosystem protection. In particular, real-time determination of enrofloxacin and norfloxacin antibiotics concentrations was successfully demonstrated with low power athermal signals. A comprehensive set of complementary experiments using optical and microwave detection methods confirmed the potential of this novel sensing approach to serve as an alternative method of residual antibiotics concentration monitoring in a wide range of applications, including food industry and environmental monitoring. The proposed system can potentially be integrated into an industrial production line or at any aquaculture industry check-point to automatically assess the composition of solutions in real time and manage the processes accordingly.

REFERENCES

[1] H. C. Wegener, "Antibiotics in animal feed and their role in resistance development," *Current Opinion in Microbiology*, vol. 6, pp. 439-445, 2003.

[2] P. T. Biyela, J. Lin, and C. C. Bezuidenhout, "The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes," *Water Sci Technol*, vol. 50, pp. 45-50, 2004.

[3] H. Q. Wen, J. Shi, H. Xun, and H. P. Deng, "[Distribution, dissemination and removal of antibiotic resistant genes (ARGs) in the aquatic environment]," *Ying Yong Sheng Tai Xue Bao*, vol. 26, pp. 625-35, 2015.

[4] N. Dorival-García, A. Zafra-Gómez, S. Cantarero, A. Navalón, and J. L. Vílchez, "Simultaneous determination of 13 quinolone antibiotic derivatives in wastewater samples using solid-phase extraction and ultra performance liquid chromatography–tandem mass spectrometry," *Microchemical Journal*, vol. 106, pp. 323-333, 2013.

[5] O. Korostynska, A. Mason, M. Ortoneda-Pedrola, and A. Al-Shamma'a, "Electromagnetic wave sensing of NO_3 and COD concentrations for real-time environmental and industrial monitoring," *Sensors and Actuators B: Chemical*, vol. 198, pp. 49-54, 2014.

[6] A. Mason, B. Abdullah, M. Muradov, O. Korostynska, A. Al-Shamma'a, S. Bjarnadottir, K. Lunde, and O. Alvseike, "Theoretical Basis and Application for Measuring Pork Loin Drip Loss Using Microwave Spectroscopy," *Sensors*, vol. 16, p. 182, 2016.

[7] R. Blakey, I. Nakouti, O. Korostynska, A. Mason, and A. Al-Shamma'a, "Real-Time Monitoring of Pseudomonas Aeruginosa Concentration Using a Novel Electromagnetic Sensors Microfluidic Cell Structure," *Biomedical Engineering, IEEE Transactions on*, vol. 60, pp. 3291-3297, 2013.

[8] H. G. De Alwis, L. Girard, P. J. Kijak, and N. Rummel, "Determination of erythromycin in medicated salmonid fish feed by liquid chromatography and UV spectroscopy," *Journal of AOAC International*, vol. 96, pp. 1233-1238, 2013.

[9] C. Chafer-Pericas, A. Maquieira, R. Puchades, J. Miralles, and A. Moreno, "Fast screening immunoassay of sulfonamides in commercial fish samples," Tiergartenstrasse 17, Heidelberg, D-69121, Germany, 2010, pp. 911-921.

[10] S. A. A. Almeida, L. R. Amorim, A. H. Heitor, M. C. B. S. M. Montenegro, J. Barbosa, L. C. Sa, and M. G. F. Sales, "Rapid automated method for on-site determination of sulfadiazine in fish farming: A stainless steel veterinary syringe coated with a selective membrane of PVC serving as a potentiometric detector in a flow-injection-analysis system," *Analytical and Bioanalytical Chemistry*, vol. 401, pp. 3355-3365, 2011.

[11] B. Kim, D. Lim, H. J. Jin, H. Y. Lee, S. Namgung, Y. Ko, S. B. Park, and S. Hong, "Family-selective detection of antibiotics using antibody-functionalized carbon nanotube sensors," *Sensors and Actuators B: Chemical*, vol. 166–167, pp. 193-199, 2012.

[12] S. Carrasco, E. Benito-Pena, D. R. Walt, and M. C. Moreno-Bondi, "Fiber-optic array using molecularly imprinted microspheres for antibiotic analysis," *Chemical Science*, vol. 6, pp. 3139-3147, 2015.