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The SPR detection of Salmonella enteritidis in food using aptamers as recongnition elements

W.T. Di[1], X.W. Du[2], M.F. Pan[1] and J.P. Wang[1]*

[1]Key Laboratory of Food Nutrition and Safety, Ministry of Education, Tianjin Key Laboratory of Food Nutrition and Safety, Tianjin University of Science and Technology, Tianjin 300457, China
[2]Dalian Nationalities University, Dalian 116600, China

Abstract. In this experiment, a fast, accurate, non-destructive, unmarked and simple-operation detection method for *Salmonella enteritidis* in food was established by the BI-3000 plasma resonance biosensor (SPR). This article establishes a method of using nucleic acid aptamer as immune recognition element in SPR which can be employed to detect *Salmonella enteritidis* in food for the first time. The experimental conditions were screened and the experimental scheme was validated and applied. The best flow rate was 5μL/min, the best concentration of the aptamers was 180mM, and the best regenerating solution was the 20mM NaOH. This method had almost no cross-reactivity. Besides, we established a standard curve of *Salmonella enteritidis* and SPR signal, with the detection limit of 2 cfu/mL. Finally, we tested the samples of chicken, pork, shrimp and fish purchased from supermarkets. The method has the advantages of short time, low detection limit and easy operation, which can be used for a large number of food samples.

1 Introduction

Foodborne diseases have not only seriously damaged human health, but also caused a significant impact on the economy. They have become one of the most prominent public health problems in the world. According to statistics in the world, food poisoning caused by Salmonella is at the top of the list. In recent years, we have paid too much attention to Salmonella typhimurium and Salmonella paratyphoid; however, we have ignored the *Salmonella enteritidis*. According to relevant reports, in Japan, the United States and other developed countries, 40% to 80% of the food poisoning occurred have been caused by *Salmonella enteritidis* [1]. It has become an important issue relating to the international public health. Therefore, it is imminent to establish a rapid, sensitive and real-time method for the detection of *Salmonella enteritidis*.

Although the traditional method of detecting Salmonella has a very good reliability, but the sample pretreatment is complex, and the whole process takes at least 7 days to get a clear diagnosis. With the development of detection technology, there have been a number of immunological methods to detect Salmonella, including ELISA immune antibody, polymerase chain reaction (PCR), immunoradiometric labeled antibody, latex agglutination, Chromatography, etc [2]. But it is still difficult to use these methods for the detection at low levels of bacterial content; meanwhile, labeled antibodies and complex sample pretreatment are required to manipulate complex, sophisticated and highly trained analysts. In recent years, in order to overcome these shortcomings, the biosensor method with fast speed, simple operation and high sensitivity is explored and applied to bacteria detection.At present, the ap-

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plication of biosensors in bacterial detection has been explored [3]. SPR-based biosensor has the advantages of high sensitivity, simple operation, no labeling, fastness and low detection limit (S.D. Mazumdar, 2007); [4].

The surface plasmon resonance principle is that a polarized laser is incident on a metal film (such as Au or Ag) and the reflection of the laser is detected. Light in the prism and the metal film on the surface of the phenomenon of total reflection occurs in the optical media to form evanescent wave, while it generate a certain plasma wave in the metal film and prism interface. When the evanescent wave and plasma wave resonance occur, the instrument can detect that the intensity of reflected light will be greatly weakened. When molecules are present on or near the surface of the metal, they change the effective refractive index of the test medium, causing the resonance angle to move. Since the shift of the resonance angle is proportional to the amount of molecules adsorbed on the surface, the angular displacement of the resonance angle can be monitored for accurate measurement of the molecules bound to the sensor surface.

In the past few years, most of the studies used the antibodies as immunological recognition elements [5]. However, antibodies had the shortcomings of high price, and long acquisition time. At the same time, aptamers (which is a DNA or RNA single-stranded nucleic acid sequence) with high specificity, wide target molecules, easy synthesis and modification, strong stability, easy storage and so on have aroused everyone's attention. Aptamers are currently used in analytical chemistry [6], proteomics [7], clinical medicine [8], disease diagnosis [9], food safety [10] and other fields. So aptamer which is used as an immunological recognition element in SPR biosensor to detect *Salmonella enteritidis* in food, will gain more important research significance [11].

2 **Materials and methods**

2.1 *Reagents and apparatus*

Bacteria used in this study is the *Salmonella enteritidis* (ATCC14028). In the cross-reactivity texts,the escherichia coli, the staphylococcus aureus and the listeria monocytogenes are all cultured in our laboratory of microorganism research group. The sequence of the aptamer which can identify the *Salmonella enteritidis* specifically is ATAGGAGTCACGACGACCAGAAAGTAATGCCCGGTAGTTATTCAAAGATGAGTAGGAAAAGATATGTGCGTCTACCTCTTGACTAAT [12].

Nutrient lysogeny broth and Salmonella selective medium were purchased from the Bei-Jinglangbridge technology company (BeiJing, China). EA (ethanolamine), NHS (N-hydroxysuccintnide) and EDC (1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide) are purchased from Sigma AldrichAldrich(St. Louis, MO, USA). Phosphate buffer saline(PBS) is the moving phase,which is constituted by Na2HPO4•12H2O, NaH2PO4•2H2O, Nacl, PH≈7.4 [13]. It's concentration is 10mM. Other chemicals are on the analytical grade, and are purchased from China National Pharmaceutical Group Corporation(Shanghai). The water in the experiment is Milipore ultrapure water.

The instrument is the BI-3000 SPR,which is boughet from Biosensing Instrument(USA).The bare Au chips used to standardedized the instrument and the Carboxymethylated dextran CM5 chips which were used as the experiment chip is purchased from Biosensing Instrument(USA).

2.2 *Preparation of standard bacterial samples*

A single colony of the standard *Salmonella enteritidis* from the SS agar culture medium plate was picked out and inoculated into lysogeny broth (LB) and grown for 4 h at 37 °C with shaking at 200 rpm. The cultures containing bacteria were centrifuged at 3,500 rpm for 5 min and washed with phosphate-buffered solution (PBS) (10 mM, pH 7.4) for three times [14]. These *Salmonella enteritidis* were dispersed in sterile 1mL PBS to obtain purified bacteria samples. Remove 100 mL from the bacterial solution and dilute it by a 10-fold dilution. 100 mL of each bacterial strain was taken and uniformly spread on SS agar medium for counting.So a bacterium concentration between 10^1cfu/mL to 10^8 cfu/mL was obtained for subsequent experiments.

2.3 *The description of the detection method*

We can put the CM5 chip into the SPR instrument after debugging .After stabilization of the baseline, 200 μL of a mixed solution of NHS and EDC was injected into the instrument for activation. Then, 200 μL of aptamer was injected as an immunological recognition element, and a large amount of carboxyl group activated by dextran immobilized on the surface of the CM5 chip was immobilized on the surface of the chip by an amide bond [15]. The unused carboxy site was blocked with EA solution and then 200 μL bacteria were injected into the instrument. When the target bacteria flowed through the chip surface, the nucleic acid aptamers would be specific to its capture; and then, the SPR signal changes were employed in the target bacteria after the capture of records, so as to achieve the purpose of testing the target bacteria.

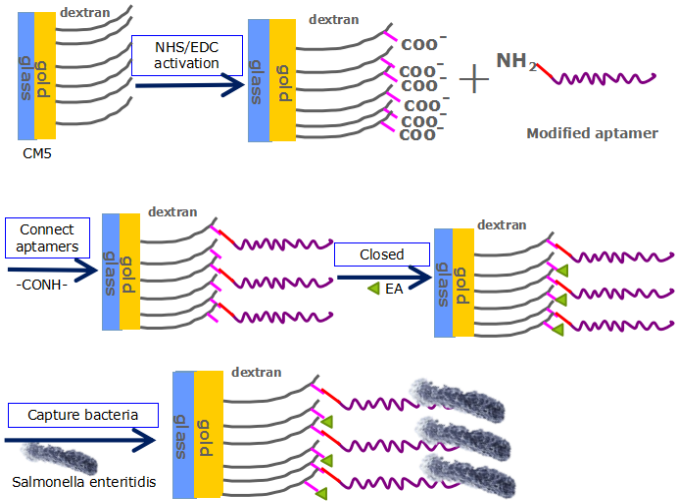

Figure 1: Schematic diagram of the experiment

2.4 *Screening of experimental conditions*

As shown in the experimental flow chart, the experiment is divided into several steps, including activation, aptamer fixation, non-binding site closure, sample detection and chip regeneration. The experimental conditions in almost every step need to be screened, such as the determination of the carboxyl activation time on the CM5 chip surface, the fixation time of the carboxy-immobilized aptamer, optimization of the optimal fixed concentration of the aptamer for a specific time, cross-reaction analysis of common strains, and screening of regeneration reagents.

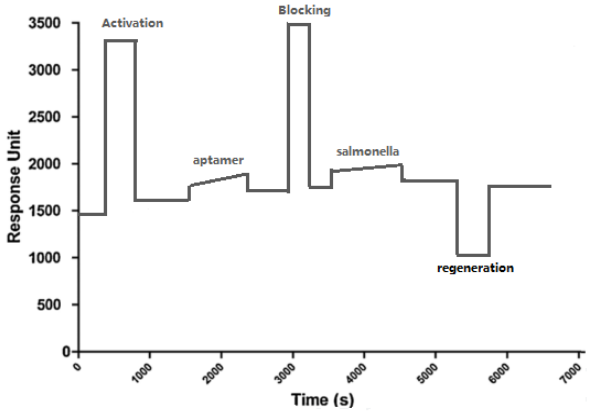

Figure 2: Schematic diagram of the SPR sensing scheme

2.5 *The regeneration of the CM5 chips*

The reproducibility of the chip is an important evaluation parameter to verify whether the established method is practical or not. And the regeneration solution is a reagent which can completely remove the analyte molecules bound with the ligand from the chip surface by a certain method or means without deteriorating the performance of the captured antibody [16]. Regeneration principle is shown below:

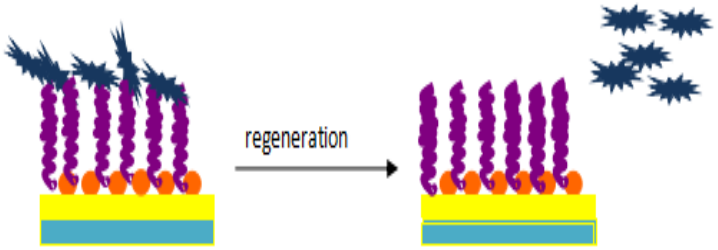

Figure 3: Schematic diagram of regeneration

In this experiment, two regeneration solutions, 0.1mol/L HCl and 20 mM NaOH, were compared with each other. After each bacterial test, two regeneration solutions were injected respectively repeatedly at least 3 times, and the changes of SPR response were observed and compared. The optimum regeneration liquid was selected, and the regenerated solution was used to regenerate the chip for 50 times. The effect of each regeneration was recorded and the comparison chart was drawn to observe the regeneration and reuse of the chip.

2.6 *The standard curve of the Salmonella enteritidis test*

After the several rounds of experimental operation before, the optimum flow rate for the experiment, as well as the fixation time and concentration of the aptamer has been obtained. On this basis, repeated experiments were carried out, respectively, taking 200μL of different concentrations of *Salmonella enteritidis* standard bacteria solution gradient injection, with the concentration of bacteria ranging from 10^8cfu / mL~10^0cfu/mL employed for injection operation, observation and recording baseline. Before and after the change value, we summed up and drew a standard curve about the bacterial concentration and SPR signal changes.

2.7 *The detection of food samples*

Salmonella enteritidis infection in poultry mainly occurs in broilers, eggs and waterfowl. So the fish, shrimp, pork and chicken were chosen as the actual test samples. The content of Salmonella was detected by the designed method.

The samples were purchased from a small supermarket in Tianjin. First, 10 g of solid sample was weighed and put into 90 mL of PBS buffer, after which it was homogenized for 2 min [17]. Then, 10 mL of the supernatant was centrifuged in a sterile centrifuge tube, and it was further centrifuged at 60×g for 10 min at 4°C and discarded. Then the supernatant was transferred to a sterile centrifuge tube and centrifuged at 1000×g for 15 min at 4 °C. The supernatant was discarded, while the sediment was resuspended in 1 mL PBST and washed for three times. Finally, the resulting solution was assayed using the constructed sensor method. At the same time, we could know the accuracy of the method by comparing the results with the traditional counting method.

3 **Result and discussion**

3.1 *Determination of Activation Time of Carboxyl Groups on CM5 Chip Surface*

After placing the CM5 chip in the instrument, we injected 200μL NHS and EDS mixed solution (volume ratio 1: 1) from the injection port to activate the carboxylation of CM5 chip surface at different

flow rates. Compared the change value of the baseline before and after, the results are shown as follows:

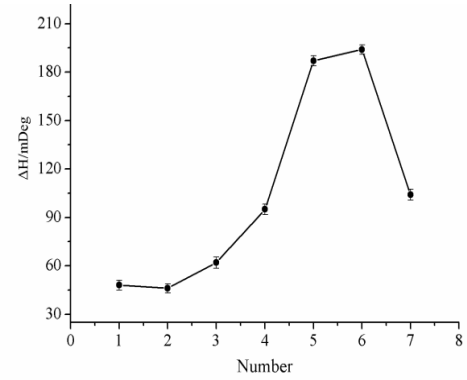

Figure 4: Baseline changes before and after activation at different flow rates

As shown in Figure 4, with the decrease of the flow rate, the injection time increases, so does the baseline height increases. That is, the smaller the flow rate is, the longer the activation time will be, and the better the activation effect will become. It should be activated when the flow rate is 3μL/min. However, with the smaller flow velocity, the impact of the experimental system will be greater by the environment. Besides, there is also much unstableness; because the flow rate is too small, it is easy to have bubble interference with the experiment. In order to keep experiment stable and save time, the final activation flow rate should be 5μL/min, and the activation time is 40min.

3.2 *Determination of the aptamer fixation time*

After completion of the activation of the chip, the surface of the leakage for a large number of carboxyl groups, amino-modified aptamers can be amide reaction, so as to achieve the purpose of aptamer to the chip surface. The aptamers with a concentration of 500nM were injected to determine the fixed time of the aptamers. The changes in the baseline were observed at different flow rates of 40μL/min, 30μL/min, 20μL/min, 10μL/min, and 5μL/min to observe the fixation of the aptamers on the chip surface. The results are shown as follows:

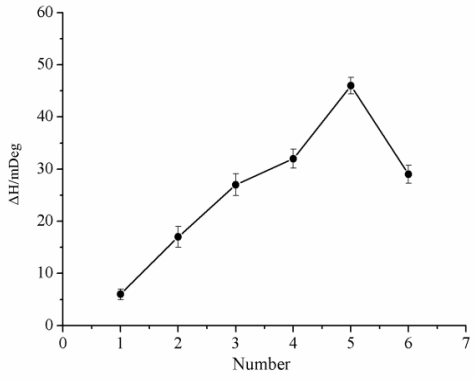

Figure 5: Baseline changes of immobilized aptamers at different flow rates

As shown in Figure 5, as the flow rate decreases and the injection time increases, the baseline height also increases, which is in proportion to the amount of aptamer immobilized on the surface of the chip. Moreover, the flow rate drops to 5 μL/min, and the baseline rises to the maximum. After a comprehensive determination, the final aptamer fixed flow rate should be set at 5μL/min, with the fixed time being 40min.

3.3 *Selection of the Optimum Fixation Concentration of Aptamer*

We selected different concentrations of aptamers employed for gradient injection, comparison and selection under the flow rate of 5ul/min. The optimal concentration of aptamer was 200μL, and the concentration of aptamer was 20nM, 50nM, 80nM, 100nM, 150nM, 200nM, 260nM, 300nM, 350nM, 400nM and 500nM, respectively. The results are shown as follows:

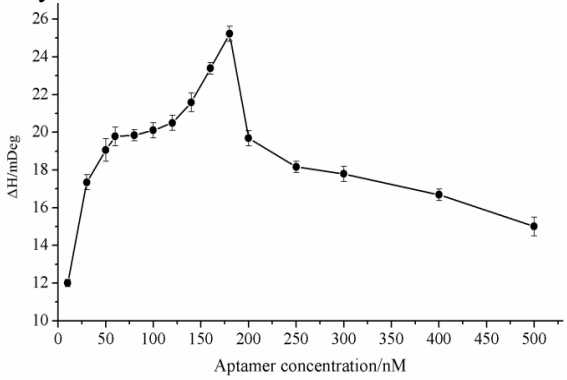

Figure 6: A baseline map of the aptamers with different concentrations

As shown in Figure 6, with the increase of the concentration of nucleic acid aptamer, the increase of baseline showed an increasing tendency first before the decrease, and at 180 nM, the maximum value appeared. It indicates that when the aptamer concentration was 180nM, there would be the best aptamer and the carboxyl surface of the chip combination. When the aptamer concentration is between 10 and 180 nM, the amount of aptamers is small, and the binding to carboxyl groups on the chip surface is not saturated. However, the concentration of aptamer was too large at 180 ~ 500 nM, so that the aptamer could not be well dispersed in PBS solution, and the amino moiety of aptamer could not react well with the carboxyl surface of the chip. In this way, a good fixing effect cannot be achieved.

In summary, whether from the perspective of saving drugs, or from a fixed effect, the choice of 180nM concentration of aptamers can fix the best. In order to achieve a fixed saturation of the chip, the method can be carried out for five consecutive injection methods.

3.4 *Specificity test (cross-reactivity test)*

Binding specificity is a significant difference in the binding strength of aptamers compared to target and non-target species [18]. In this experiment, four kinds of pathogenic bacteria (*Salmonella enteritidis*,Listeria monocytogenes, Escherichia coli and Staphylococcus aureus)were used as the target bacteria to carry on the capture experiment. Each kind of bacteria was also injected for 200ul in the same way after completing the steps such as the activation of the chip, the fixation of the aptamer and the site closure. Read the changes caused by the SPR signal values as follows:

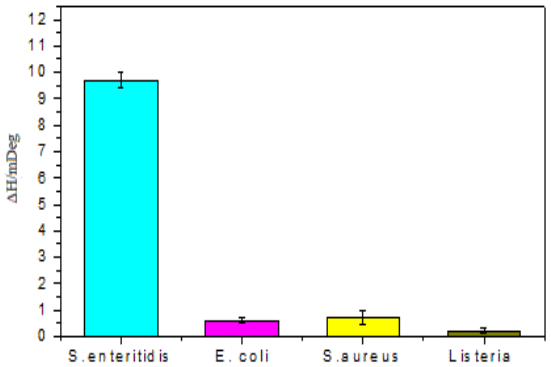

Figure 7: SPR results of the specific test

From Figure 7, we can see that Salmonella caused by the SPR sensor signal was the largest change, indicating that in the experiment, *Salmonella enteritidis* was used as a good chip to capture the surface of the chip, and Salmonella binding of nucleic acid aptamers most. The other three strains caused very weak sensor signal changes, respectively 0.6mDeg, 0.7mDeg and 0.2mDeg, indicating that they is almost no capture by the sensor chip. The data show that the aptamers used in this experiment have high specificity for the target Salmonella, and the application of this program has very good specificity.

3.5 *The regeneration of the chips*

The regeneration solution chosen in this experiment was the 0.1 mol/L hydrochloric acid solution and the 20 mM NaOH solution, namely the two most commonly used in the SPR experiment. In the experiment, the two kinds of solutions were tested and the results were analyzed.

We use the "baseline" and "sample response" two parameters on the chip to reproduce the effect of characterization. The change of the "baseline" position indicates the detachment of the target bacteria from the chip surface. The "sample response" represents the ability of the regenerated chip to recapture the target. The results are shown as follows:

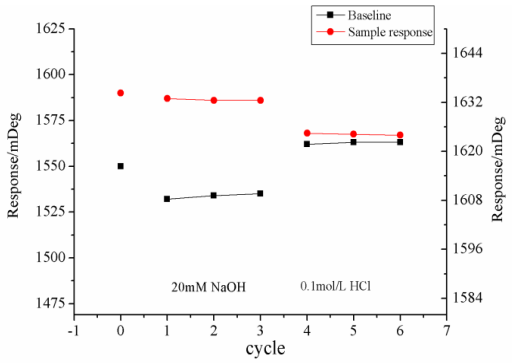

Figure 8: The regeneration of different solution

As shown in Figure 8, the baseline position after the regeneration of the 20 mM NaOH solution was slightly decreased compared with the original baseline position, but remained substantially near the original baseline. However, the baseline position of 0.1 mol/L HCl solution was significantly higher than the original baseline position. It indicates that the bacteria on the surface of the chip were not completely eluted; the regeneration effect was not very good. From the analysis of the sample response, the sample response of the chip regenerated by NaOH solution decreased, but remained almost in the vicinity of the original response value. However, the response of the chip sample after the regeneration of HCl solution was significantly lower than that of the original chip. Above all, 20mM concentration of NaOH solution as the experimental chip regeneration solution is the best choice.

In order to maximize the use of each experimental chip, the same chip for a continuous 50 times of the regeneration test operation was taken respectively, with the diagram drawn as follows:

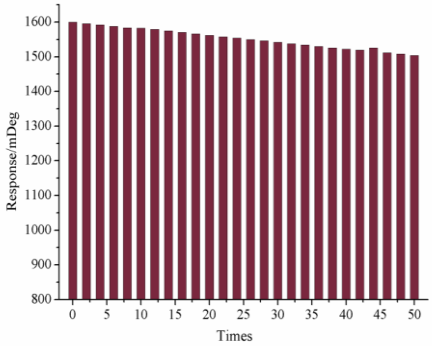

Figure 9: The stability of chip for 50 times' regeneration

As shown in Figure 9, the chip was regenerated with 20 mM NaOH solution for 50 times, and the response value of the sample after regeneration decreased with the increase of the number of times of use; however, the decreasing value was a slight change. And the activity decrease from the 1st to the 50th was only 6.67%. The results showed that the experimental chip had good reproducibility and could be reused repeatedly. On the basis of reducing the experimental cost, the experimental group could also eliminate the experimental group between the errors, thus increasing the accuracy of the experiment.

3.6 *The Standard curve of testing Salmonella enteritidis*

After the screening of the above experimental steps, the chip was activated at a flow rate of 5 μL/min and the aptamer at 180 nM was immobilized. After the vacuoles were blocked, 20 mM NaOH solution was used as the regenerative solution. The concentration of Salmonella standard solution, as the gradient of the sample, ranges from 10^0cfu/mL~10^8cfu/mL in order to observe the instrument response to different concentrations of bacteria solution. The experimental data for the average of the three experimental results are as follows:

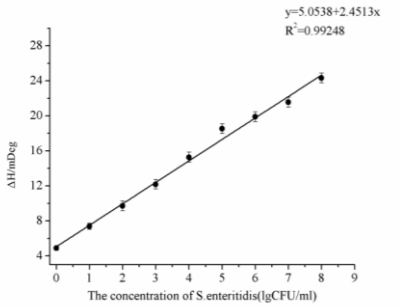

Figure 10: The SPR detection standard curve of *Salmonella enteritidis*

As shown in Figure 10, the number of bacteria can be detected from 2 to million. With the increase of the concentration of the tested bacteria, the SPR signal also kept increasing, and the two had positive correlation. We can see each bacteria concentration from the figure, and there is only one SPR response with a corresponding relationship, which will be applied to the actual food testing according to this relationship.

3.7 *Detection of food sampless*

In this experiment, four kinds of food products (chicken, pork, shrimp, fish) were purchased from a small supermarket, respectively, for simple treatment was dissolved in PBS experimental samples. We injected the sample and read the SPR instrumentation signal changes to determine the *Salmonella enteritidis* concentration in the food by the control standard curve :

Table 1 SPR detection results of food samples

samples	chicken	pork	shrimp	fish
Baseline changes ΔH(mDeg)	2.967	5.395	0.523	7.286
Concentration in curve(CFU/mL)	0	2~10	0	15~20

Based on the SPR signal values obtained in the experiment, we can obtain the bacterial concentration from the standard curve. The results are: *Salmonella enteritidis* concentration is 0 cfu/mL in chicken, 2 to 10 cfu/mL in pork, 0 cfu/mL in shrimp, and 15 to 20 cfu/mL in fish.

In order to verify the SPR test results, the same food samples were employed in the plate count experiments; after cultivation for 18 to 24 hours, the number of colonies on the plate was counted, and the results are shown as follows:

Table 2 Plate Counting Results of Food Sample

Number	1	2	3	4
samples	chicken	pork	shrimp	fish
Number of colonies(CFU/mL)	0	4	0	18

The colony counts of the food samples were 0 cfu/mL in chicken, 4 cfu/mL in pork, 0 cfu/mL in shrimp, and 18 cfu/mL in fish. The accuracy and reliability of the SPR experimental method were verified by the same results.

4 Conclusions

In this experiment, we designed a rapid, nondestructive test program that can accurately detect *Salmonella enteritidis* in food within 20 minutes. Besides, we established a SPR signal standard curve with the concentration of *Salmonella enteritidis* from 2.1×10^{1} cfu/mL to 2.1×10^{8} cfu/mL.The successful validation and application of this protocol provide a reference for the rapid detection of pathogenic bacteria in food by SPR sensor method. In addition to the *Salmonella enteritidis* test established in this experiment, it can be inferred that it can also show good advantages and application prospects in the detection of other pathogens, pesticide residues, melamine and other substances in food. To amplify the SPR detection signal, the use of carbon point, magnetic beads or nano-gold and other materials on the experimental signal amplification, which can be used as future research directions to continue to explore.

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