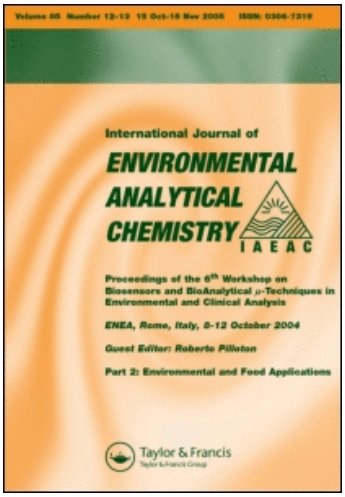

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DETERMINATION OF *PROTEUS MIRABILIS* AND ANALYSIS OF ANTIMICROBIAL PROPERTIES OF TEA WITH A BAW-IMPEDANCE SENSOR

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A bulk acoustic wave (BAW)-impedance sensor has been successfully applied to determine the number of *Proteus mirabilis (P. mirabilis)* in the range of $2.77 \times 10^2 \sim 9.53 \times 10^6$ cells ml^{-1}, based on a linear relationship between the frequency detection time (FDT) and the logarithm of the *P. mirabilis* concentration in this paper. The antimicrobial properties of various teas on growth of *P. mirabilis* have also been studied. The method is simple and rapid. Factors that might affect the determination such as pH value and temperature are discussed in detail. A comparison between the proposed method and the traditional pour plate count (PPC) method has also been investigated.

Keywords: BAW-impedance sensor; *P. mirabilis*; tea

INTRODUCTION

The detection of microbial cells and characterization of their growth and metabolism are very important in environmental science, food technology, toxicology, biotechnology and clinical microbiology.[1,2] *Proteus mirabilis (P. mirabilis)* is a member of the genus *Proteus* within the family Enterobacteriaceae. It occurs widely in human, animals and in the environment, and can be readily recovered from soil, sewage, garden vegetables and many other materials. It is often found as a contaminant of shigellae, appears in the stools of patients recovering from bacillary dysentery, and occurs as a secondary invader in wounds and bedsores. So, the determination of *P. mirabilis* is of practical importance in food hygiene, clinical medicine and environmental monitoring.

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Tea is the most widely drunk beverage in the world and has been shown to have a wide range of beneficial physiological and pharmacological effects.[3] In recent years, much interest has focused on medicinal plants (such as tea) in relation to human health because they are largely free from harmful adverse effects. Several studies have shown that extracts from green and black tea inhibit the growth of many bacterial species and possess anticariogenic properties.[4,5] Specifically, tea tannins have inhibitory or protective effects on cancer.[6] In this paper, a bulk acoustic wave (BAW)-impedance sensor has been suggested for the rapid determination of *P. mirabilis* and the analysis of antimicrobial properties of various teas on growth of *P. mirabilis*.

BAW sensor, attractive as a fast, convenient and sensitive analytical tool, has been widely used in chemical and biological fields.[7,8] Most of the applications employ its highly sensitive mass response property for immunoassays.[9,10] Recently, a BAW-impedance sensor based on a non-mass response has been proposed in our laboratory[11] and used to determine bacteria, such as *Escherichia coliform*[12] and *Staphylococcus aureus*.[13] The proposed method is faster and more sensitive compared with conventional methods such as the pour plate counts (PPC) technique, the most probable number technique, and the turbidity method, all of which are cumbersome and time-consuming.

In this work, the proposed method relies on the fact that the metabolizing bacteria transform uncharged or weakly charged substrates into highly charged end products. These charged substrates cause an alteration in the impedance of the culture medium. The amount of impedance alteration represents the changes in the conductance and permittivity of the growth medium. We use a BAW resonator in series to monitor the change in impedance. Frequency shifts of the sensor are recorded when the concentration of the tested bacteria is in the range of $2.77 \times 10^2 \sim 9.53 \times 10^6$ cells ml^{-1}. The frequency detection time (FDT) is the time required for the initial inoculum to reach the threshold value at which the frequency signal produced can be detected by the instrument and acceleration can be observed in the frequency shift curve. The FDT is linearly related to the logarithm of the initial concentration of bacteria. This is the fundamental principle for *P. mirabilis* determination in this study.

EXPERIMENTAL

Bacteria and Media

P. mirabilis used in our experiments was obtained from Hunan Medicine University, Changsha, Hunan. The composition of the media is as follows: beef extract, 1.0 g; yeast extract, 2.0 g; peptone, 5.0 g; NaCl, 5.0 g; doubly distilled

water, 1000 ml. The media was dispensed into bottles and sterilized by autoclaving at 121°C for 15 min.

Sample Preparation

Two loops of *P. mirabilis* on slant agar were inoculated into a 50-ml sterilized conical vial which contains 20 ml of sterilized media. The bacteria were cultured at 37°C for about 16 hours, then the conical vial was removed from the incubator and stored in a refrigerator. The culture gave an approximate concentration of 9.53×10^7 cells ml^{-1}.

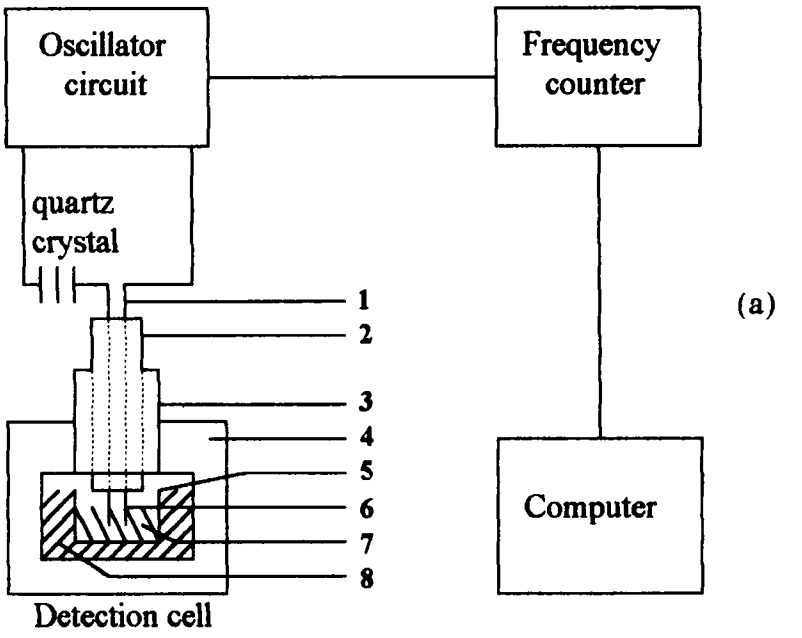
Four different kinds of tea (Green tea, Black tea, Oolong tea and Kudin tea) were supplied by Hunan Agriculture University. 1.5 g dry tea was transferred into 60 ml boiling water for 6 hours, and then 1 ml tea was used for determination.

Apparatus

Experiments were performed with a BAW-impedance sensor shown schematically in Figure 1. The piezoelectric quartz crystals used in this work was an AT-cut crystal (9 MHz resonance frequency, 12.5 mm in diameter). The cell constant of the electrodes was 0.36 cm. The feedback circuit of the oscillator was formed by connecting this crystal and a platinum electrode in series. One terminal of the crystal was connected to the input terminal of the oscillator, the other to the lead wire of a platinum electrode and another platinum electrode was connected to the output terminal of the oscillator. During the experiments, the quartz crystals were sealed in a can which is filled with vacuum. This apparatus was accommodated in a thermostatic chamber maintained at 37 ± 0.5°C. A laboratory-made IC-TTL oscillator was designed to drive the crystal at its resonant frequency, and the oscillation signal was fed to a universal counter (Iwatsu, Model SC-7201).

Procedure

In an inoculation chamber, two aliquots of 10 ml sterilized medium was taken into each of two sterilized cells with inserted platinum electrode, then *P. mirabilis* was inoculated into one cell for testing while the other cell received sterilized distilled water for blank comparison. The two cells were connected individually to the oscillator through a double-pole, double-throw switch. At 37°C, the resonant frequency was measured every 10 min. If the frequency in



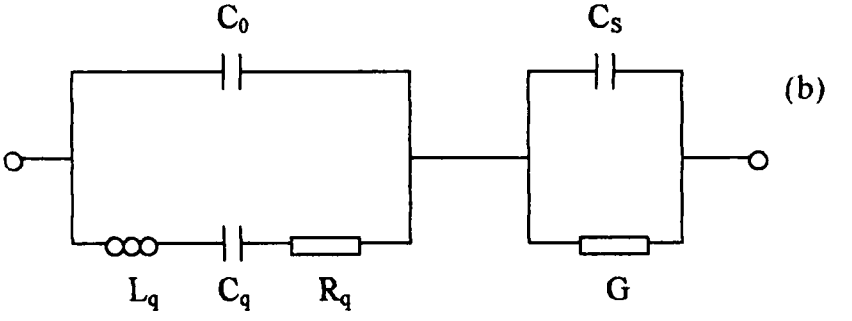


FIGURE 1 (a) Schematic diagram of the BAW-impedance sensor. (1) lead wire, (2) glass tube, (3) cotton plug, (4) thermostatic jacket, (5) sample cell, (6) platinum electrode, (7) sample, (8) water (b) The equivalent circuit of the BAW-impedance sensor. C_o, L_q, C_q and R_q are the static capacitance, motional inductance, motional capacitance and motional resistance of the quartz crystal in air, respectively. R_s and C_s are the resistance and capacitance in solution.

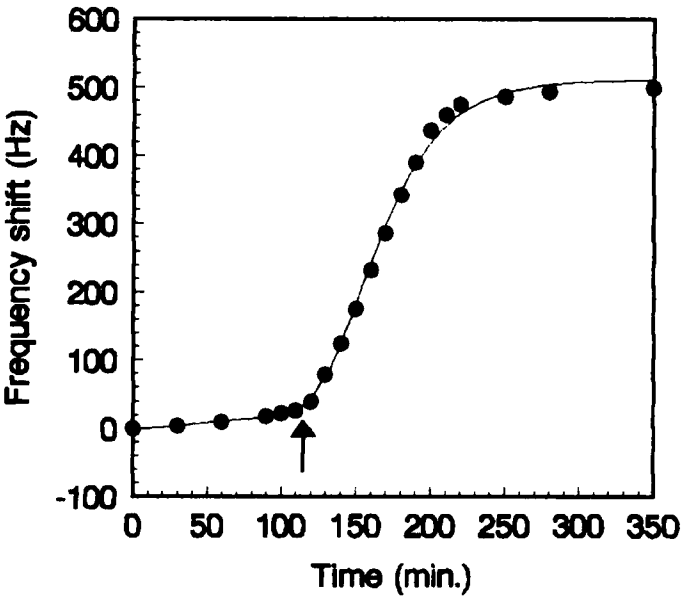


FIGURE 2 Typical frequency curve for *P. mirabilis*. The arrow indicates FDT. (Concentration of *P. mirabilis* 9.53 × 10^5 cells ml^{-1}, pH 7.4 and 37°C)

the sample cell and reference cell are denoted F_1 and F_2, respectively, the frequency difference between the two cells was determined by $\Delta F = F_2 - F_1$. Using a ΔF vs time plot, the FDT is obtained.

RESULTS AND DISCUSSION

As microorganisms metabolize they create new end products in the medium. Generally, uncharged or weakly charged substrates are transformed into highly charged end products, for example, proteins are metabolized to amino acids, carbohydrates to lactate and lipids to acetate, which increase the conductivity of the solution. So, the conductivity changes by increasing microbial numbers and this changing can reflect the growth characteristics of bacteria. Since BAW-impedance sensor can sensitively response to the conductivity change,[11] we use it to determine the number of *P. Mirabilis*.

Typical Response Curve

A typical frequency curve for *P. Mirabilis* detected by a BAW-impedance sensor is shown in Figure 2. It can be seen that no significant change was observed in the frequency curves before 116 min. When it was at approximately 116 min.,

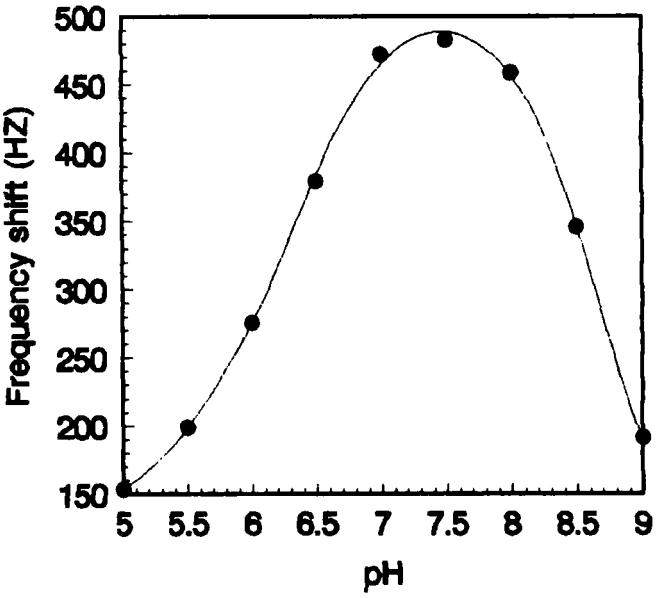


FIGURE 3 Effect of pH value on frequency changes. (Concentration of *P. mirabilis* 9.53×10^5 cells ml^{-1}, 37°C)

there had an abrupt acceleration in the frequency curve. This acceleration point (in min.) is defined as the FDT. FDT is a function of the density of microbials and the number of microbials can be detected through the FDT value obtained by the frequency curve. It is obvious that FDT can be determined easily and accurately from the frequency curves with low drift and sharp slope.

Influence of pH Value and Temperature

Since many biochemical processes in microbe are sensitive to the ionization state of the molecular species concerned, pH is an important parameter to control. During the growth of microorganism, the pH of a culture medium is altered by conversion of carbohydrates into small molecules such as lactic acid, succinic acid, acetic acid, or through the conversion of amino acid to ammonia and bicarbonate. In the range of pH 5.0 ~ 9.0 at 37°C, an investigation has been made to examine the optimum pH for *P. mirabilis* determination and the result is shown in Figure 3. (cell concentration is 9.53×10^5 cell ml^{-1}). The sensor has an maximum frequency response near pH 7.4. When pH value is > 7.8 or < 7.0, the frequency decreases rapidly. So, pH 7.4 was selected for subsequent experiments.

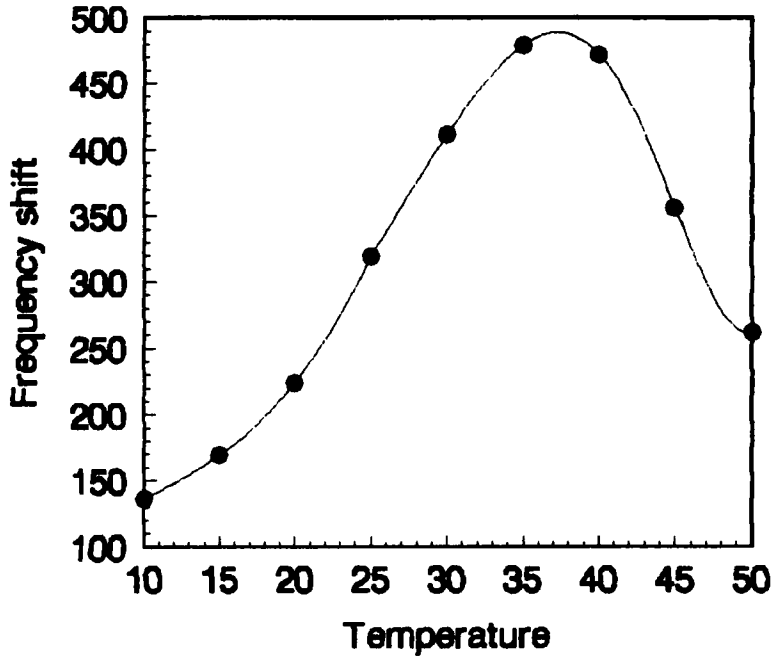


FIGURE 4 Effect of temperature on frequency changes. (Concentration of *P. mirabilis* 9.53 × 10^5 cells ml^{-1}, pH 7.4)

Temperature is another important factor due to its obvious effect on growth of microbe. Under the same experimental conditions as above (cell concentration is 9.53 × 10^5 cell ml^{-1}, pH 7.4), the effect of temperature on *P. mirabilis* determination is also studied. From Figure 4, we can see that the sensor yields the maximum frequency shift at 37°C, which is also the optimum temperature for bacteria growth. Therefore, 37°C was chosen throughout the experiments.

The Calibration Curve

In order to estimate the number of bacteria by the BAW-impedance sensor, a calibration curve should be established. Experiments have been done to show the relationship between bacteria concentrations and FDT. Results are listed in Figure 5. It can be seen that FDT at higher concentrations of *P. mirabilis* is shorter than those at lower concentrations. In Figure 6, a linear relationship between the FDT and the logarithm of the *P. mirabilis* concentration was obtained in the range of 2.77 × 10^2 ~ 9.53 × 10^6 cells ml^{-1}. The regression equation is described as follows:

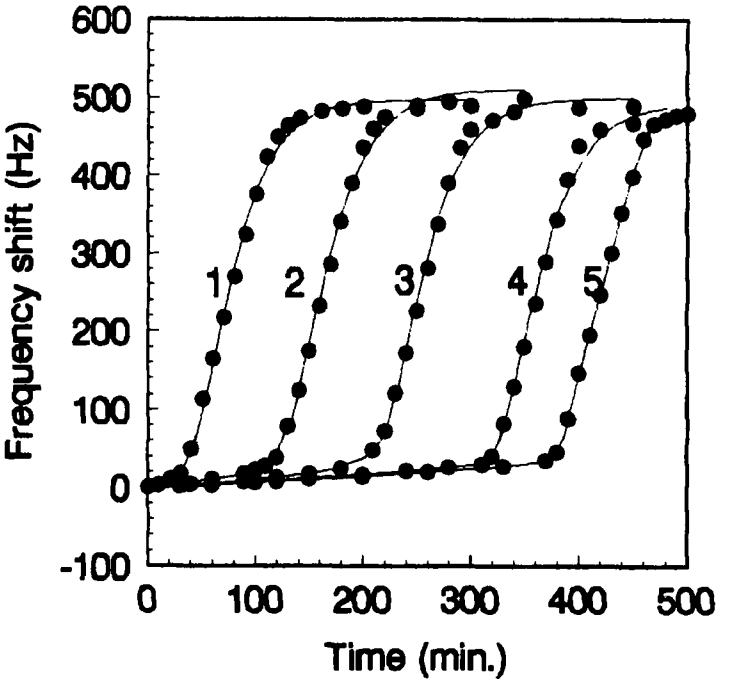


FIGURE 5 Frequency curves of different concentrations of *P. mirabilis* (cells ml^{-1}). (1) 9.53×10^6, (2) 9.53×10^5, (3) 9.53×10^4, (4) 9.53×10^3, (5) 9.53×10^2

$$\log C = -0.0113FDT + 7.346 \quad (1)$$

where C is the initial concentration of *P. mirabilis* in cells ml^{-1} and FDT is the frequency detection time in min. The correlation coefficient is $r = -0.98$ ($n = 10$) and the standard error of the regression line is $S = 0.288$. When bacteria concentration is $> 10^8$ or $< 10^2$ cells ml^{-1}, a non-linear relationship is found between FDT and log C.

The reproducibility of the BAW-impedance sensor was studied (in Table I). The data were obtained with five different bacteria concentrations and each concentration had been examined five times. Between each measurement, the device was washed back to base in line with distilled water. As can be seen, the sensor plays a good reproducibility and would be most practical as a rapid detecting device for bacteria.

Calculation of Generation Time

The generation time affects the FDT. Generally, a longer generation time results in a greater FDT. Detecting with a BAW-impedance sensor, the generation time (T_{gen}) can be calculated as follows:[14]

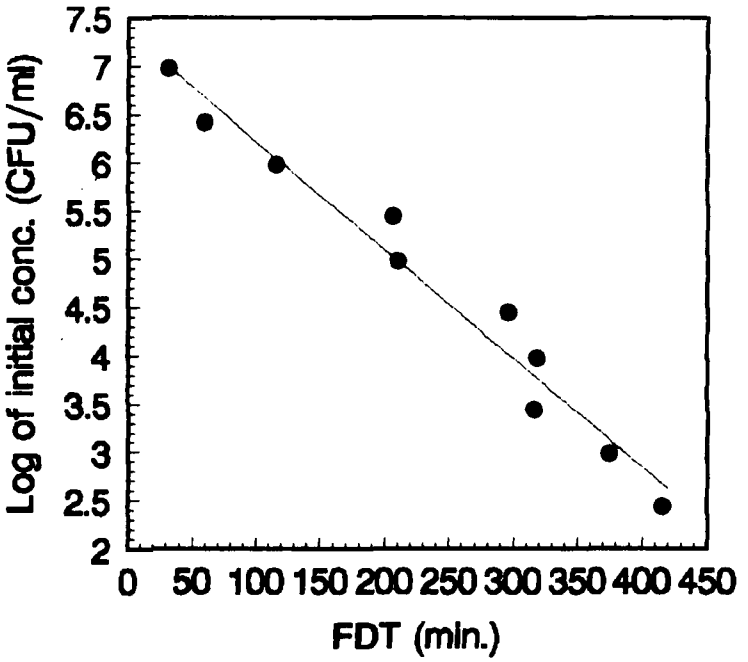


FIGURE 6 FDT calibration graph for the determination of *P. mirabilis*.

$$T_{gen} = \frac{\Delta FDT \times \log 2}{\log N_2 - \log N_1} \quad (2)$$

where, N_1 = *P. mirabilis* number in the 10^{-5} dilution, N_2 = *P. mirabilis* number in the 10^{-3} dilution and ΔFDT = frequency detection time of N_2 minus that of N_1. In the present experiment, $N_1 = 9.53 \times 10^3$ cells ml^{-1}, $N_2 = 9.53 \times 10^5$ cells ml^{-1} and $\Delta FDT = 202$ min, giving $T_{gen} = 30.4$ min.

TABLE I Reproducibility of Calibration FDT by BAW-impedance Sensor

Concentration of P. mirabilis cells ml⁻¹	*FDT (min.) Series No.*					*Mean ± S.D*
	1	*2*	*3*	*4*	*5*	
9.53×10^6	31	32	29	33	28	30.6 ± 2.07
9.53×10^5	116	121	118	119	122	119.2 ± 2.39
9.53×10^4	210	209	214	207	211	210.2 ± 2.59
9.53×10^3	318	306	310	314	308	311.2 ± 4.82
9.53×10^2	375	386	382	378	380	380.2 ± 4.15

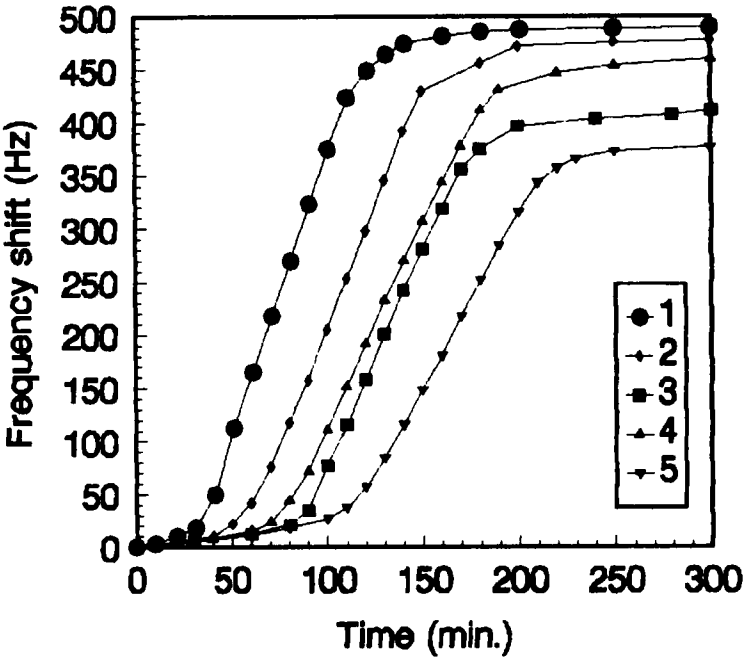


FIGURE 7 Effect of various teas on growth of *P. mirabilis*. (1) Absence of tea, (2) Green tea, (3) Kudin tea, (4) Black tea, (5) Oolong tea

Effects of Various Teas on Growth of P. mirabilis

Effects of four kinds of tea (Green tea, Black tea, Oolong tea and Kudin tea) on *P. mirabilis* growth were examined by the BAW-impedance sensor. 9.53×10^6 cells ml^{-1} *P. mirabilis* was selected for this measurement at 37°C and pH 7.4. Results are listed in Figure 7. The results showed that all of the tea had inhibitory effects on growth of *P. mirabilis* and Oolong tea exhibited the most inhibitory action due to its longest FDT and lowest frequency shifts among them. The reason for the antimicrobial properties of tea is possibly based on the chemical composition of tea. Although the chemical composition of tea is complex and not completely understood, tea tannins are the most important composition in tea and several studies have reported their inhibitory function on bacteria.[4,5]

Comparison of the FDT Method with the PPC Method

In Table II, a comparison of the proposed method with the conventional PPC method for determination of *P. mirabilis* is given. It can be seen that all the PPC values fall inside the 95% confidence area of the FDT predicted concen-

TABLE II Comparison of the bacteria concentration predicted by the FDT method with that obtained from the PPC method

No.	*Concentration predicted by FDT method (cell ml^{-1})*	*Logarithm of predicted value*	*Concentration obtained from PPC method (CFU* ml^{-1})*	*Logarithm of pour plate counts value*
1	3.2×10^2	2.51	3.5×10^2	2.54
2	4.5×10^3	3.65	6.1×10^3	3.79
3	8.6×10^3	3.93	7.7×10^3	3.89
4	3.3×10^4	4.52	3.8×10^4	4.58
5	7.8×10^4	4.89	8.1×10^4	4.91
6	3.7×10^5	5.57	4.5×10^5	5.65
7	7.5×10^5	5.88	7.7×10^5	5.89
8	3.9×10^6	6.59	2.1×10^6	6.32

CFU, colony forming units.

trations. So, the FDT method agrees well with the PPC technique. Taking into consideration the advantages of the BAW-impedance sensor system (rapid determination, simplicity in operation, enough sensitivity, inexpensive and practical), it appears to be an efficient technique for bacteria determination.

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