



## Optimizing a portable biosensor system for bacterial detection in milk based mix for ice cream<sup>☆</sup>



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### ABSTRACT

One of the primary focuses of the food industry is providing products compliant with safety standards. The microbiological analysis helps in the identification of the presence of pathogen microorganisms in the food. The analysis with Agar Plate is the classic method. This approach guarantees a high accuracy, but it needs a long detection time (twenty-four to forty-eight hours), beyond high costs and skilled technician. In recent times have been proposed many different methods to have a faster response, and between them there is the impedance method. One of its features is that it is fast, in fact it requires between three to fourteen hours to obtain a reliable measurement. The system is accurate, and suitable to be executed automatically. To test this method has been used UHT Ice Cream Mix. A known volume of mix has been inoculated with increasing percentage of cultures of *E. coli*. The measurement of the impedance of the inoculated mix has been done by an electronic board designed for the application, and by applying a sinusoidal voltage to the test tube. The signal was digitally generated by the microprocessor, and supplied externally through a D.A. converter. The signal was then filtered to delete from its spectrum the high frequency components typical of the digitally generated signals. The data obtained from impedance instrument showed a reliable correspondence with those from the plate count. By working in less time compared to traditional methods, this tool is well suited for *in-situ* preliminary analysis in commercial and professional foodservice environment.

### 1. Introduction

For the food industry it is of primary importance ensure quality controlled and approved products. Before reaching the consumer, the product must be subjected to screen in order to identify sources of pollution that may alter the final characteristics. Microbiological testing is essential to ensure a correct safety for the product, be it a mechanical product, biomedical, food or otherwise. Specific laws govern the limits on the presence of pathogenic or potentially pathogenic microorganisms, within which the different products must stay to be considered safe. For example, a maximum microbial concentration of  $10^5$  cfu/ml is the limit imposed from the regulations for the ice-cream industry, as described, for example, in the (CE) N. 1441/2007. In particular, in the section of the regulation for milk and dairy products, the Enterobacteriaceae and *E. coli* are mentioned as quality markers of the production process. Microbiological tests are performed to screen the ice-cream mix to detect the presence of dangerous pathogens. Ice-cream machines are able to perform a pasteurization cycle capable of breaking down and reduce the possible presence of contamination, generally

below the limits imposed by law. For this reason the microbiological analysis helps in the identification of possible pathogenic microorganisms in ice cream mix and dairy products.

One of the classic methods of bacterial identification consists in the analysis with Agar Plate. Medium with specific composition are used to obtain a diversified growth of different bacterial species. The standard plate approach [1] has a high accuracy but needs a long detection time (24–48 h). In the last thirty years many different methods have been proposed with the purpose of giving a faster response. These methods are based on bioluminescence [2], amperometry [3], impedance [4], turbidity [5], piezoelectricity [6], optical waveguide [7] and flow cytometry [8]. The detection time of these method is fast (from 20 min to 2 h) but all of them require a series of operations that only a skilled technician may be able to complete carry out only in a laboratory. However, among these methods, the impedance one lends itself to the development of applications which can simplify the analysis operations [9,10]. In fact, this method is very suitable to be developed in automated forms and easy to use even for an unskilled user.

The Impedance Technique (IT) is based on monitoring the changes

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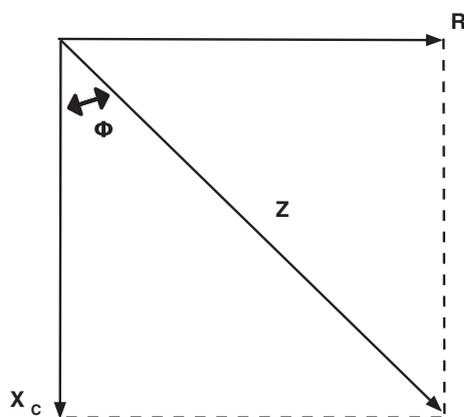


Fig. 1. Vectorial representation of resistive component (R) and reactive component ( $X_c$ ) of impedance.

of the electrical characteristics induced by the metabolism of the bacteria present in the mix sample [9]. The IT technique works as follows [11,12]: the sample is incubated at a temperature which fosters bacterial growth (for mesophilic bacterial the best growing temperature is in the range 30 °C–40 °C), and at regular intervals the electrical parameters, namely  $|Z|$  (Impedance) and  $\text{Arg}(Z)$  are measured. As discussed elsewhere [14,15], the system composed by the sample under test and a couple of electrodes stimulated with a low frequency sinusoidal voltage can be modeled by means of the series of a resistance (accounting for the conductivity of the medium and of the electrode-electrolyte interface) and a capacitance (due to the double layer present at the electrode-electrolyte interface). After the time needed for the electrode-electrolyte system to stabilize, both the  $|Z|$  resistive and reactive components (Fig. 1) remain essentially constant (at a “baseline” value) until the bacterial concentration in the sample reaches a threshold value (in the order of  $10^7$  cfu/ml), which is sufficient to induce appreciable changes in the sample's electrical characteristics. When such a condition is reached, both the Z components begin to decrease. The choice to monitor the Z resistive or reactive component depends on the chemical composition of the sample, since normally one of the components provides more reliable results than the other.

Since the time needed for the electrical parameter to deviate from its baseline value, hereafter called Detect Time (DT), is linearly related to the logarithm of initial bacterial concentration, this value can easily be worked out. The main drawback of the IT is that, since bacterial concentration is calculated by measuring the time needed for the microbial population to reach a threshold concentration, the measurements time depends on the time needed for a cell to duplicate the bacterial strains of interest. Thus the requirement to correctly choose the incubation temperature needed in order to minimize differences in generation times of different microbial species possibly present in the sample under test. Another paper [13] describes a portable biosensor system for bacterial concentration detection based on the IT, that is fully automated and requires no particular knowledge of microbiological techniques, thus making it particularly suitable for microbial screening in industrial or commercial environments. Moreover, the use of low-cost electronics makes it highly competitive in terms of cost (few hundreds US Dollars).

This paper, instead, describes both an optimized method and a new embedded portable biosensor system for bacterial concentration measurement in milk based mix for ice cream. The Sample Under Test (hereafter called SUT) is always maintained at a constant temperature (generally in the range 30 °C to 40 °C) suitable for efficient bacterial growth. At regular intervals, the electrical parameters, Impedance (Z) and Phase Shift ( $\Phi$ ) by which the current lags the voltage ( $\text{Arg} Z$ ), are measured by a couple of electrodes immersed in the mix. Z and  $\text{Arg} Z$  remains almost constant until the bacterial concentration reaches a

threshold level which depends on the species and on the substrate, and it is believed to coincide with the achievement of the concentration of  $10^7$  cfu/ml. When this bacterial concentration is reached in the sample, Z and Resistance (R) begin to decrease, while Capacity (C) increases. Then if the degree of bacterial contamination of the sample analyzed increases, the resistance values drop and the capacitance values grow faster over time. In this work, the parameter used to find the bacterial concentration of the sample is the angle between current and voltage (“ $\Phi$ ” value).

## 2. Materials and method

### 2.1. Media and organism

*Escherichia coli* ATC11229 was subcultured aerobically at 37 °C for 24 h in Tryptic Soy Broth. Mac Conkey Agar and Plate Count Agar were used for isolation and enumeration of *E. coli* and total bacteria, respectively. All the media were provided by BD Difco, Sparks, USA.

### 2.2. Chemicals

Saline solution was prepared (NaCl, 0.9% P/V) and autoclaved at 121 °C for 15 min. Culture media (BD Difco) used for isolation of bacteria was prepared.

MacConkey Agar was prepared for the isolation of *E. coli* (typical formula g/l: Pancreatic digest of gelatin 17.0; Peptones (meat and casein) 3.0; Lactose monohydrate 10.0; Sodium Chloride 5.0;

Bile Salts 1.5; Agar 15.0; Neutral Red 0.03; Crystal Violet 0.001; pH  $7.1 \pm 0.2$  at 25 °C).

Plate Count Agar (typical formula g/l: Tryptone 5.0; Glucose 1.0; Yeast Extract 2.5; Agar 15.0; pH  $7.0 \pm 0.2$  at 25 °C) was prepared to verify the total bacterial count of the samples.

The culture media were suspended in distilled water. Heated until completely dissolved. Autoclaved at 121 °C for 15 min.

### 2.3. Ice-cream mix

The Soft ice-cream mix used for the test was UHT Milk based Ice Cream Mix.

Ingredients: Skimmed Milk, Cream, Sugar, Whey Powder, Corn Syrup, Natural Flavor, Stabilizers. Nutrition Facts: Protein 3.6%, Carbohydrate 23%, Fat 3.6%, Saturated Fat 2.4%, Sugars 20%, Sodium 0.14%.

### 2.4. Inoculum preparation

A known volume of Ice Cream Mix UHT was inoculated (increasing %, vol/vol) with cultures of *E. coli* diluted in saline water. Increasing concentrations of *E. coli* were obtained by adding a known quantity of bacterial culture volume to a known volume of ice cream mix.

### 2.5. Analysis of samples

The ice cream mix samples inoculated with *E. coli* were analyzed in parallel by both the technique plate count and by the measurement with the biosensor. In particular, in the plate count technique the samples were serially diluted in saline solution, and 100  $\mu$ l of opportune dilution was plated in duplicate on MacConkey Agar and Plate Count Agar and incubated for 24–36 h at 37 °C.

### 2.6. The Peltier cell

The Peltier cell is a solid state active heat pump capable of generate or extract heat depending on the direction of the current applied.

The electronic board which manages the Peltier cell is able to maintain the temperature of the thermal chamber to the set value with

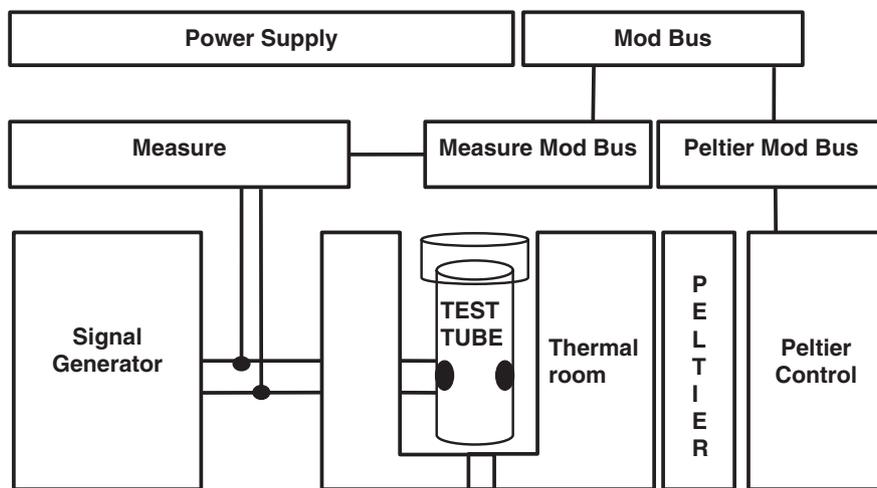


Fig. 2. Schematic representation of the biosensor system represented in this work. The system is composed of an impedance measurement board, a thermoregulation board and an incubation chamber containing the sample under test.

an accuracy of  $\pm 1$  °C. This is possible by using two PT1000 temperature sensors, which measure respectively the temperature of the thermal chamber and the ambient temperature. The control of the Peltier cells is done by a bridge of 4 metal oxide semiconductor (MOS) transistors, driven directly by the microprocessor through pulse-width modulation (PWM). The control algorithm is a proportional-integral-derivative (p.i.d.), which uses as feedback signal the temperature of the heating chamber, while the ambient temperature is used to prevent any thermal shock that might impact the structure of the measuring chamber.

### 3. Biosensor experiments

The biosensor instrument (Fig. 2) has a 5 ml incubation chamber, where the sample is poured, and where the sensors are inserted for the impedance measurement. The thermal chamber and the sensors compose the test tube, which is inserted into the biosensor base to make the tests. The chamber is kept at a constant temperature of 37 °C by a Peltier device, with an accuracy of  $\pm 1$  °C. Two PT1000 sensors measure the temperature of the thermal chamber and the thermal room. Three independent experiments were carried out. The samples inoculated with different amount of *E. coli* were analyzed in parallel and managed by an electronic board. The electronic board is composed by two separate parts, the first used to manage the temperatures and the second to generate the impedance signals. These two parts, as mentioned, are independent each other and configured to be “slave” devices belonging to a single Modbus network, which could be managed by an external master device capable of scheduling the measurement, collect the data and display them through a User Interface.

#### 3.1. Generation of the measurement signal

The measurement of the impedance of the ice-cream mix is done by applying to the test tube a sinusoidal voltage signal. The signal is digitally generated by the microprocessor, supplied externally through a Digital Analog (D.A.) converter and then filtered to delete from its spectrum the high frequency components typical of the digitally generated signals. The signal chosen for the measurement is a sine wave with a frequency of 200 Hz, and its amplitude is controlled by the microprocessor in order to optimize the measurement error, which is not possible to avoid. This choice has been done to reduce as much as possible the effects of electrolysis caused by direct current (DC). The type of the signal and its frequency to be used in the measure may be set from the device that controls the entire system through the modbus interface.

#### 3.2. Measure phase shift

The measurement of the impedance of the analyzed sample is obtained by applying Ohm's law. The system applies a voltage signal at known frequency to one of the electrodes in direct contact with the ice-cream mix sample to be measured. On the other electrode the current is then measured (Fig. 3).

This process allows the system to calculate the impedance value (1), as follows:

$$Z = V_{in}(t)/I_{out}(t) \tag{1}$$

When the values are measured it may then be estimated the impedance absolute value, while the phase shift is calculated as the time difference between the zero-crossing of the input voltage and the zero-

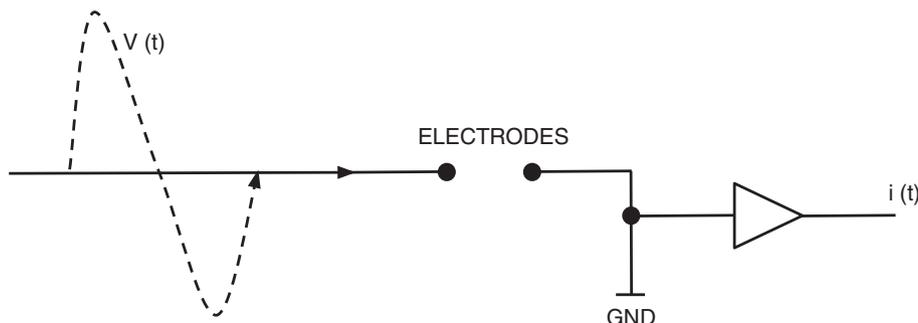


Fig. 3. Schematic representation of impedance measure between the two electrodes.

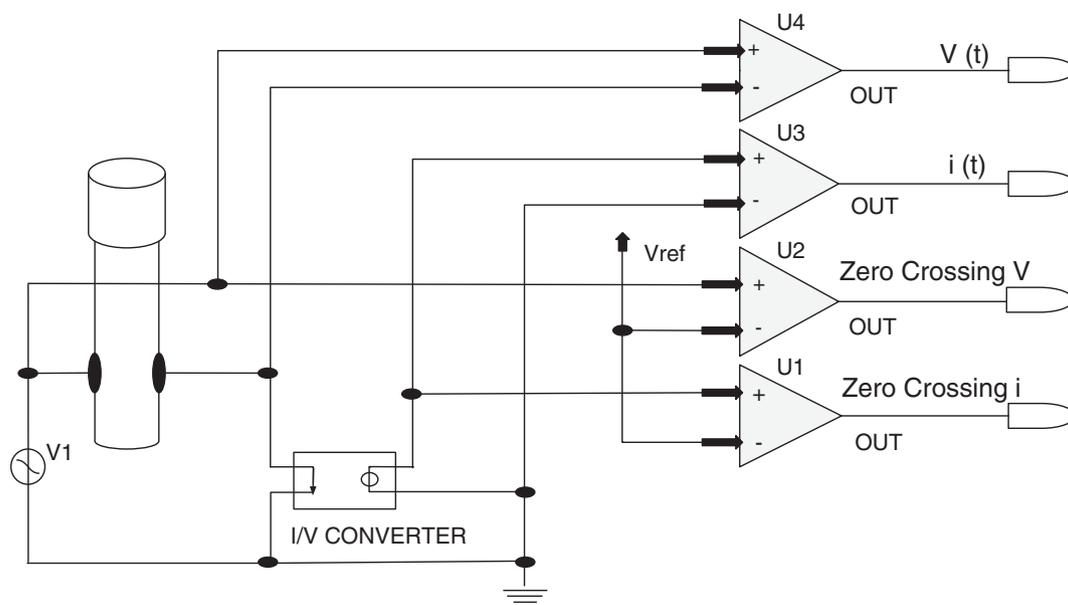


Fig. 4. Schematic representation of the electronic used to manage the measurement.

crossing of the output current (Fig. 4). The precision of the micro-processors which measures this time, allows us to estimate the phase shift ( $\Phi$ ) with great accuracy.

At regular intervals (typically every 300 s) a small sinusoidal current (Fig. 5) is supplied through the sample to be analyzed and the current-voltage phase shift at the output of the sample is then calculated.

The point-by-point analysis of the values obtained may be unreliable, this is why we based the evaluations on the curve obtained by combining all the measured points approximate with a polynomial interpolation function. To minimize the maximum of the magnitude of the error of the curve with the polynomial approximation, a high degree polynomial is used (typically 9th). If “n” is the degree of the polynomial, interpolation will only start if at least “n + 1” points are collected. For each new sample the system calculates the coefficients of the polynomial of degree n that best approximates the “linear” curve that would result from the union of the acquired points. By calculating the derivative of the polynomial point by point the system can find the shape of the curve over time, and automatically evaluate when it changes its slope. When the decrease of the slope exceeds a threshold value, the system detects it as the “Detect Time”, which in fact corresponds to the time when the electrical parameters begin to deviate from their baseline value. Given that there is a linear relationship between the DT value calculated and the logarithm of the bacterial concentration, it is possible to determine the level of bacterial concentration present in the sample. More precisely, the DT is inversely proportional to the logarithm of the initial microbial concentration: the higher is the microbial concentration the shorter is the DT. Since the bacterial

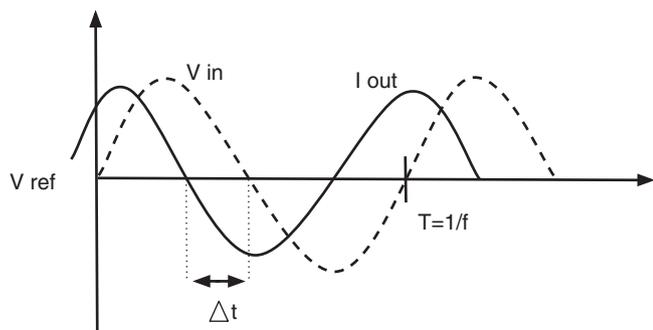


Fig. 5. Representation of voltage and current trends.

concentration is calculated by measuring the time required for the microbial population to reach the threshold concentration, the measurement time depends on how long it takes a bacterial cell to replicate. For each sample, the measurement is repeated in three different tests in order to validate the data. However, for each test an initial time of about 45 min is required for the stabilization of the parameters, before the measure can start.

#### 4. Results and discussion

The biosensor system based on impedance measurement created by Carpigiani [13–15], was used to determine the bacterial load in the ice-cream mix. At first the ice-cream mix without inoculum, then samples inoculated with known bacterial loads of *E. coli* ATC11229 ranging from  $10^1$  to  $10^{10}$  cfu/ml, were analyzed with the portable biosensor in order to find the electric response of the sample. Impedance was monitored and recorded over a time span ranging between 2 h and 16 h, applying the same voltage signal at known frequency.

Data were collected with a sampling time of ten minutes, and then recorded and saved by the software. The ice-cream mix not inoculated didn't show any change in the shift value measured during the whole time of the measurements. On the contrary, where the inoculum was added to the ice-cream mix, a drop of the PHI curve was detected, with an increase of the DT in decreasing the bacterial load. DT ranged between 2 h for  $10^{10}$  cfu/ml and 16 h for  $10^1$  cfu/ml, without taking into account the 45 min necessary to reach the constant temperature required and setting up data acquisition (Fig. 6). Once reached the DT, data acquisition continued until a constant value was reached.

In Fig. 7 is presented the DT as a function of the charge of the inoculum. A linear relationship between the DT value calculated and the logarithm of the bacterial concentration can be extrapolated, making possible to determine the order of magnitude of bacterial concentration present in a test sample. Indeed, DT is inversely proportional to the logarithm of the initial microbial concentration. Since the bacterial concentration is calculated by measuring the time required for the microbial population to reach the threshold concentration, the measurement time depends on how long it takes a bacterial cell to replicate.

#### 5. Conclusion

In this study it has been verified that the measurement of the

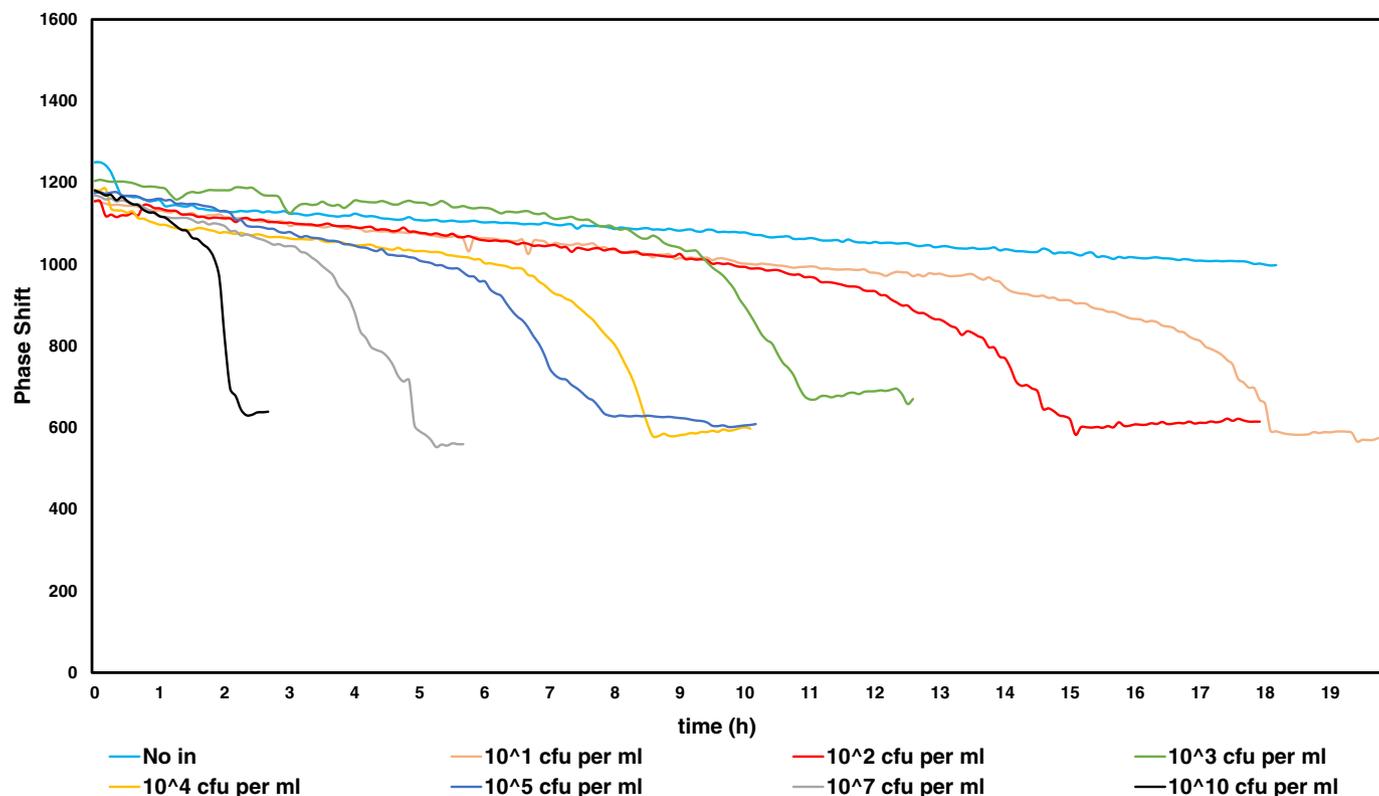


Fig. 6. The image shows the time trend of the measured Phase Shift value for each analyzed concentration of *E. coli*. Increasing the bacterial concentration decreases the time required for the measurement.

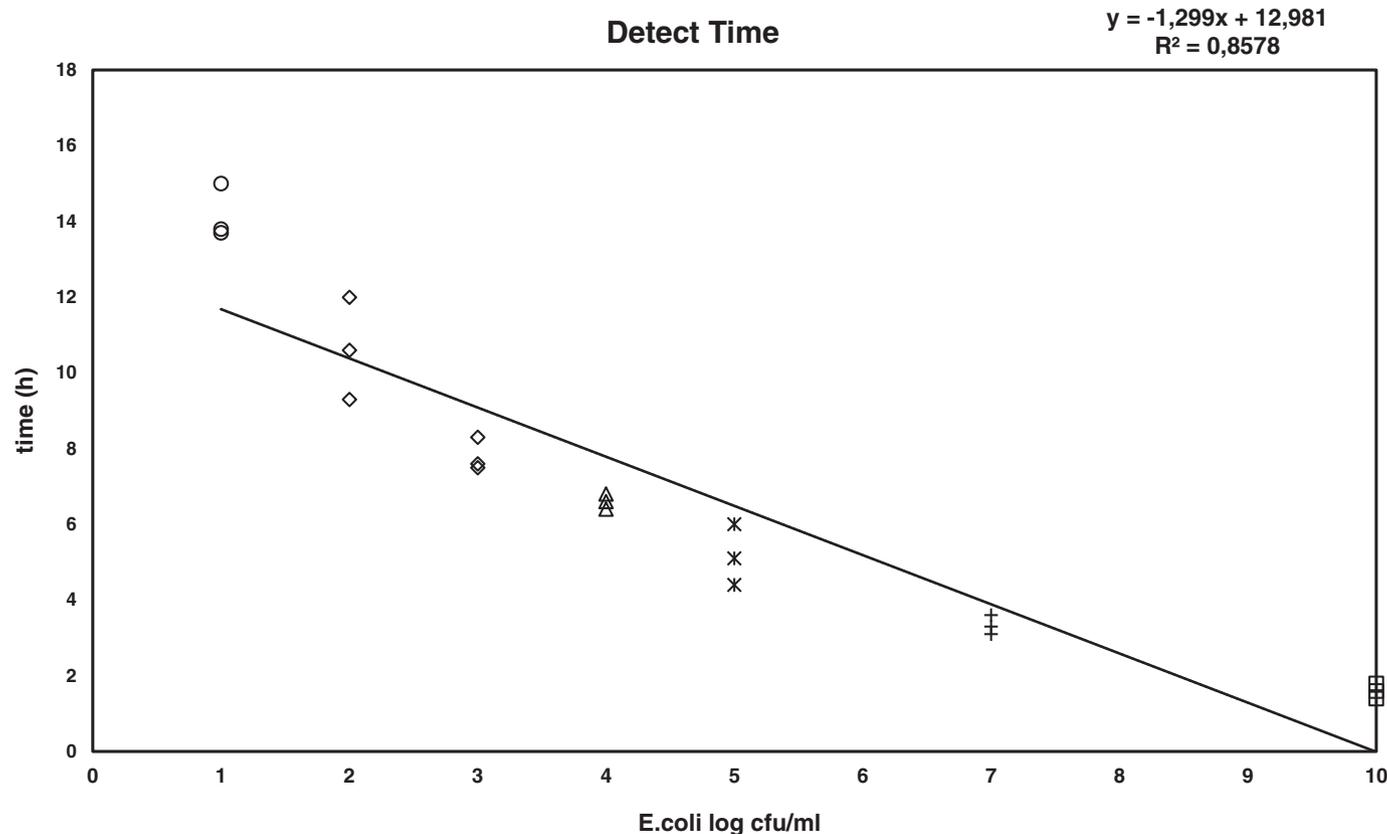


Fig. 7. The image shows the DT measured by the instrument for each concentration of *E. coli* in the UHT milk based mix for ice cream.

impedance angle phase shift  $\Phi$  is a reliable tool to determine the microbial charge of an ice cream mix. The measurement of this parameter was reproducible and reliable. The curves did not show any background noise. The algorithm developed by Carpigiani removed the noise and produced more reliable measures. The use of “ $\Phi$ ” angle as parameter related to bacterial load allowed analysis of ice cream mix samples within a wide range of *E. coli* bacterial concentrations. Previous studies reported two different DT values calculated for the capacity curve and for the resistance curve, respectively, and only in a second time these values were compared to each other [13–15]. The possibility to work with a single curve resulting from the two variables considered in the system allows to have a reliable response and facilitates the final measurement operations. Then, the “ $\Phi$ ” value provides a faster, more precise and more reliable measurement. In addition it helps to automate the measurement method. An intuitive software allowed the operator to manage the measurement using a touchscreen display and to see and verify the measurement results.

The instrument does not aim to be as selective as the analyses in laboratory, but it offers indicators on the status of the sample showing the presence of potentially harmful bacteria and it can be integrated with existing systems.

The device requires a small sample of ice cream mix, is easy to use and portable, and potentially it can be used also for other dairy products, improving the quality control for food industries and environmental monitoring, scalable for distributed control within the food supply chain. It can offer a low cost analysis useful for the customer to plan for example the cleaning operations of the ice cream machine on the basis of bacterial charge.

#### Conflicts of interest

The authors don't have any conflict of interest.

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