JEMAT 2025, Vol 13, No 1, 1-8
https://doi.org/10.54987//jemat.v13i1.1058

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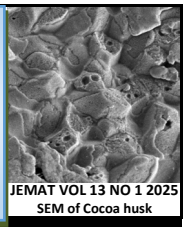

Strategies for Eliminating Matrix Effects in QCM Analysis

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HISTORY

Received: 28th May 2025
Received in revised form: 8th July 2025
Accepted: 30th July 2025

KEYWORDS

C. jejuni
QCM
Matrix effect
Immunosensor
Chicken

ABSTRACT

Effective monitoring and risk assessment of pathogen contamination in foods, such as *Campylobacter jejuni*, rely heavily on reliable detection and enumeration methods. *C. jejuni* is classified as a fastidious bacterium with specific growth requirements, necessitating special handling and careful testing procedures to detect it in food samples. One significant challenge in many pathogen detection assays is non-specific binding, especially when dealing with complex sample matrices found in food. In immunosensor applications, eliminating non-specific binding can be particularly difficult due to the use of microfluidics. Since the detection methods are often indirect or sometimes label-free, the likelihood of false positives increases. The optimization of chicken samples preparation steps is needed in order to reduce the non-specific binding caused by the chicken matrix. In this study, several common additives specifically bovine serum albumin (BSA), the surfactant Tween-20, and sodium chloride (NaCl) were evaluated as potential agents to minimize non-specific binding in the QCMA-1 immunosensor-based system for detecting *C. jejuni* in chicken samples. When the non-specific binding issues is successfully removed, the limits of detection (LOD) obtained using a sandwich assay with signal amplification from antibody-conjugated gold nanoparticles (AuNPs) in both phosphate buffered saline (PBS) and in chicken samples as matrices were developed and compared. The results demonstrated that adding 100 mM NaCl in the chicken samples effectively reduced non-specific binding while maintaining an acceptable binding response. Additionally, the LOD obtained using a sandwich assay with signal amplification from antibody-conjugated gold nanoparticles (AuNPs) developed in PBS showed increased sensitivity, yielding an LOD value of 1.5×10^2 CFU mL^{-1}. In contrast, the same assay conducted with chicken samples resulted in a higher LOD value of 1.1×10^3 CFU mL^{-1}.

INTRODUCTION

Campylobacter jejuni is a bacterium that causes an illness known as campylobacteriosis in humans. It is the most frequently reported foodborne illness in European Union (EU) countries, with over 214,268 human cases reported annually. *C. jejuni* is classified as a fastidious bacterium with specific growth requirements, necessitating special handling and careful testing procedures to detect the bacterium in food samples. This bacterium primarily colonizes chickens, thriving in the microaerophilic environment of their intestines, where the optimal temperature for growth ranges from 41 to 42 °C [1]. Intestinal colonization is the main source of contamination in poultry products and is often widespread in many poultry processing plants [2]. Effective monitoring and risk assessment of *C. jejuni* contamination depends heavily on the availability of reliable detection and enumeration methods. Efficient and robust sampling methods for *C, jejuni* are important for several reasons. Firstly, the bacterium to be detected might be at low concentrations as the infective dose level of *C. jejuni* is at 5×10^2 CFU mL^{-1}.

Detection at this low level will also be challenging with the presence of complex sample matrices of various food samples [3]. Secondly, the sample matrices can be incompatible with the developed sensor method. Successful removal of matrix interferences could allow for the development of high-throughput analysis, which could screen thousands of samples with high

reliability. Further, the sample matrix could clog the microfluidics system in numerous sensor instruments. Finally, the volume of samples utilized in numerous immunosensor methods is small, and the presence of interferences could mask analyte detection. This necessitates efficient sample preparation before assay development, including processes such as centrifugation, filtration, pH adjustments, the inclusion of non-specific binding additives, and even sample dilutions [4].

Even though an immunosensor device is sensitive, sample matrix interference during the application of real samples can be problematic, which reduces its sensitivity and selectivity. Milk, chicken and beef samples are amongst the most difficult samples as these items have high contents of interfering materials such as lipids, nucleic acids and proteinaceous materials that could interact with the bioreceptor or analyte, leading to non-specific binding or false positive signals. Juice samples such as apple and orange are also problematic as they are acidic, and can interfere with pathogen binding to the bioreceptor [5].

Non-specific binding can occur under several conditions such as the antigen which could bind indiscriminately to the sensor surface (instead of specifically to the intended capture antibodies), other molecules may bind to the capture antibody, or the other molecules may bind to the antigen, which then prevents it from binding to the capture antibody (usually through steric hindrance). Other than that, non-specific binding can also result from electrostatic or non-electrostatic interactions. In the quartz crystal microbalance (QCM) machine, non-specific adsorption by electrostatic interaction is a major problem and is generally due to the electrostatic attraction between positively charged sample components and the negatively charged surface layer of the sensor chip. This is common when sample components have a pI higher than the pH of the running buffer (PBS, pH 7.4). In addition, as the bioreceptor is a polyclonal antibody, its accurate pI cannot be ascertained, and sample components could also bind to the polyclonal antibody through electrostatic interactions [6,7].

Chicken samples were selected for this work as recent studies have shown that the prevalence of *C. jejuni* in retail chicken products ranges from 90 to 100% in several countries in 2015 [8]. In this work, several additives comprising bovine serum albumin (BSA), a surfactant such as Tween-20, and salt such as NaCl were tested in order to reduce the non-specific binding caused by the chicken samples [6,7] in a fully automated QCMA-1 biosensor platform. Only chicken samples that are free from *C. jejuni* were utilized to avoid any unnecessary background signals obtained from the bacteria that can interrupt the investigation of non-specific binding response from the chicken matrix.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibody against *C. jejuni* was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. Mouse IgG was from a commercial source (Abcam Ltd., Cambridgeshire, UK). N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were sourced from Thermo Scientific, Paisley, UK. 11-mercaptoundecanoic acid (11-MUDA), 40 nm gold colloidal (AuNPs), sodium acetate, ethanolamine hydrochloride, PBS (phosphate buffered saline tablet: 0.0027 M potassium chloride, 10 mM phosphate buffer, and 0.137 M sodium chloride, pH 7.4), Tween-20, and bovine serum albumin (BSA)were sourced from Sigma-Aldrich, Dorset, UK.

Preparation of chicken samples

A 25 g of boneless chicken samples were bought from a local retail outlet in Milton Keynes, UK. The samples were then added to a sterile Baxfilter™ bag from Interscience (France) containing 225 mL of Bolton broth. The bag with the contents was vigorously shaken by hand for 1 min. From each of the Baxfilter™ bags, 100 mL of the filtered chicken rinse samples was transferred to sterile dilution bottles for further analysis. The rinse was analysed for the presence of *C. jejuni* by plating 100 µL aliquots of the rinse in triplicate on Campy Cefex agar. Plates were incubated in an anaerobic jar under microaerophilic conditions at 42 °C for 48 hours. The presence of *C. jejuni* was observed through the colony and morphological properties obtained on the agar. Only negative samples were utilized for the non-specific binding test to avoid any unnecessary background signals obtained from the QCMA-1 sensor. The samples were stored at -20 C and thawed prior to use.

Apparatus

A fully automated QCMA-1 biosensor system (Sierra Sensors GmbH, Hamburg, Germany) was employed in this work for the the detection of *C. jejuni* in PBS and chicken samples. The instrument uses a gold coated QCMA-1 sensor chips (20 MHz) equipped with two sensing arrays. The system enables the simultaneous measurement of active and control sensor surfaces. or. All data produced by the QCMA-1 sensor were analysed with the Analyser R2 Software from Sierra Sensors and with Microsoft Excel.

Bacterial strains

The preparation of bacterial cells in this chapter, which were *Campylobacter jejuni* subsp. *jejuni* ATCC® 33291.

Preparation of QCM sensor surface

The gold sensor surface was activated with an EDC-NHS solution for a total duration of 3 min at a flow rate of 25 µL min^{-1}. Next, 70 µg mL^{-1} of the capture antibody (rabbit polyclonal antibody against *C. jejuni*) were injected (75 µL) over the active and control spots on the sensor for 3 min. The subsequent procedure involved surface blocking with BSA to prevent non-specific binding, followed by capping of unreacted NHS esters using ethanolamine (1 M, pH 8.5) for 7 minutes to stabilize the immobilized antibody layer and reduce background signal during detection [9].

Investigation on the chicken samples dilution

An investigation on the effect of chicken matrix for the detection of *C. jejuni* was carried out with the preparation of several dilutions of chicken rinse prepared in PBS (10 mM, pH 7.4) and tested through a direct assay format using the QCMA-1 instrument. A set of different chicken sample dilutions was tested, including a 1:1 sample (one volume from the undiluted chicken sample prepared in Bolton broth and mixed with one volume of PBS), 1:2, 1:3, and 1:4.

The undiluted Bolton broth solution (control) and the undiluted chicken sample were also included in this experiment. The experiment was started by injecting 75 µL (3 min) of Bolton broth solution simultaneously over the active and control spots, which were immobilized previously with a capture polyclonal antibody. The surface was then regenerated by the injection of a regeneration solution consisting of 100 mM HCl (1 min, 25 µL). The experiments were then continued in an ascending order of the other chicken rinse dilutions. The entire experiment was repeated in triplicate.

Effect of different additives in the running buffer
In this experiment, PBS (10 mM, pH 7.4) and PBS containing several different additives such as; (i) 0.1% Tween-20 (PBS-T), (ii) 0.5% Tween-20 (PBS-T), (iii) 0.2% BSA, (iv) 0.5% BSA (v) 100 mM NaCl, and (vii) 300 mM NaCl were tested. A 75 µL (3 min) of chicken rinse (1:1 dilution) as the control sample was simultaneously injected over the active and control spots of QCMA-1 sensor for 3 min (75 µL), which were immobilized previously with a capture polyclonal antibody. The surface was then regenerated by the injection of a regeneration solution consisting of 100 mM HCl (1 min, 25 µL). After that, 75 µL of chicken rinse (1:1 dilution) containing 1×10^9 CFU mL^{-1} of *C. jejuni* cells was injected to assess the effect of each running buffer on the *C. jejuni* binding. The experiments were then continued by priming the sensor system with other running buffer additives.

Effect of bovine serum albumin (BSA) as the sample additive
Four different percentages of BSA: 0.5, 1.0, 3.0, and 5.0% were tested. Each of the BSA percentages was mixed vigorously with the undiluted chicken rinse to give the final concentrations of 0.5, 1.0, 3.0, and 5.0% and a 1:1 dilution of chicken rinse. A 1:1 dilution of chicken rinse diluted in PBS without the addition of BSA was included as the control sample. The optimum percentage of BSA resulting in the least non-specific response of the chicken samples was then tested for its effect on *C. jejuni* binding in the QCMA-1 sensor. In this experiment, four different samples were prepared, which were (i) 1:1 chicken dilution (control), (ii) 1:1 chicken dilution containing 1×109 CFU mL^{-1} *C. jejuni*, (iii) 1:1 chicken rinse containing 5.0% BSA and (iv) 1:1 chicken dilution containing 5.0% of BSA and 1×10^9 CFU mL^{-1} *C. jejuni*.

Effect of Tween-20 as the sample additive
Three different percentages of Tween-20, 1.0, 3.0, and 5.0% were tested. Each of the Tween-20 percentages was mixed vigorously with the undiluted chicken rinse to give the final concentrations of 1.0, 3.0, and 5.0% and a 1:1 dilution of chicken rinse. A 1:1 dilution of chicken rinse diluted in PBS without the addition of Tween-20 was included as the control sample. The optimum percentage of Tween-20 resulting in the least non-specific response of the chicken samples was then tested for its effect on *C. jejuni* binding in the QCMA-1 sensor. In this experiment, four different samples were prepared, which were; (i) 1:1 chicken dilution (control), (ii) 1:1 chicken dilution containing 1×10^9 CFU mL^{-1} *C. jejuni,* (iii) 1:1 chicken dilution containing 5.0% Tween-20 and (iv) 1:1 chicken dilution containing 5.0% of Tween-20 and 1×10^9 CFU mL^{-1} *C. jejuni*.

Effect of NaCl as the sample additive
Three different percentages of NaCl: 100, 300 and 500 mM were tested. Each of the NaCl concentrations was mixed vigorously with the undiluted chicken rinse to give the final concentrations of 100, 300, and 500 mM and a 1:1 dilution of chicken rinse. A 1:1 dilution of chicken rinse diluted in PBS without the addition of NaCl was included as the control sample. The optimum percentage of NaCl resulting in the least non-specific response of the chicken samples was then tested for its effect on the *C. jejuni* binding in the QCMA-1 sensor. In this experiment, four different samples were prepared, which were; (i) 1:1 chicken rinse (control), (ii) 1:1 chicken dilution containing 1×10^9 CFU mL^{-1} *C. jejuni,* (iii) 1:1 chicken dilution containing 100 mM NaCl and (iv) 1:1 chicken dilution containing 100 mM of NaCl and 1×10^9 CFU mL^{-1} *C. jejuni*.

Standard curve for the detection of *C. jejuni* in PBS
The standard curve for sandwich detection assay of *C. jejuni* using antibody-conjugated AuNPs in PBS system was conducted by preparing a set of six different concentrations of *C. jejuni* cells (1×10, 1×10^2, 1×10^3, 1×10^4, 1×10^5) including control (PBS replaces *C. jejuni* cells). The test begins with the injection of the control sample (3 min, 75 µL) simultaneously over the active and control spot on the QCMA-1. This was followed by a 75 µL injection of rabbit polyclonal antibody-conjugated AuNPs for 3 min to allow binding. The injection of a regeneration buffer of 100 mM HCl (1 min, 25 µL) was utilized to regenerate the surface. The entire experiment was repeated in triplicates.

Standard curve for the detection of *C. jejuni* in chicken samples
The standard curve for sandwich detection assay of *C. jejuni* in chicken samples using antibody-conjugated AuNPs was conducted by preparing a set of six different concentrations of *C. jejuni* cells from undiluted chicken samples to give the final concentrations containing 10, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6 CFU mL^{-1} and 100 mM of NaCl in a 1:1 dilution of chicken rinse. A 1:1 dilution of the chicken rinse containing 100 mM NaCl without *C. jejuni* cells was included in the experiment as the control.

The test begins with the injection of the control sample (3 min, 75 µL) simultaneously over the active and control spot on the QCMA-1 sensor. This was followed by a 75 µL injection of rabbit polyclonal antibody-conjugated AuNPs to allow binding. Finally, an injection of a regeneration solution of 100 mM HCl (1 min, 25 µL) was carried out to regenerate the surface and remove the bacteria from the immobilized capture antibody on the sensor chip. The experiment was then continued with other samples. The entire experiment was repeated in triplicates.

Limit of detection (LOD) calculation method for direct and sandwich assays
Calibration curves were fitted with a non-linear regression using four parameter logistic equations (Karpinski, 1990) as follows;

$$y = \frac{(a-d)}{(1+({}^{x}/_{c})^{b})} + d$$

where y is the response signal obtained (Hz), a and d the maximum and minimum signal response (Hz) of calibration curve respectively, c is the concentration of bacterial cells (Log CFU mL^{-1}) that produced a 50% signal response (EC50) value and x is the bacterial cell concentration (Log CFU mL^{-1}) and b is the slope-like parameter (Hill coefficient), respectively. The limit of detection (LOD) was calculated as the average value of absorbance at a blank concentration of bacteria at three standard deviations (SD). LOD and regression analysis were calculated using four-parameter logistics model available from PRISM non-linear regression analysis software from www.graphpad.com.

Statistical analysis
Data analyses were carried out using GraphPad Prism version 5.0 available from www.graphpad.com. A one-way analysis of variance with post hoc analysis by the Tukey's test or a Student's t-test was utilized for between groups comparison. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Chicken matrix is amongst the most difficult samples as it contains materials such as lipids, nucleic acids, and proteinaceous materials that could interact with the bioreceptor or analyte, leading to non-specific binding or false positive signals. The effect of non-specific interactions could contribute to the false positive signal in a sensor. The investigation of the matrix effect or non-specific binding caused by the chicken rinse on the QCMA-1 sensor was carried out by injecting several different dilutions of chicken samples, which were 1:1, 1:2, 1:3 and 1:4 (diluted in PBS), including the undiluted samples and Bolton broth solution simultaneously over the active and control spots on the QCMA-1 sensor.

The results in **Fig. 1** show that the undiluted sample expectantly gave a significantly higher ($p<0.05$) non-specific binding at 403.82 Hz whilst the 1:4 dilution gave the lowest non-specific binding at 202.2 Hz. Dilutions of 1:1 to 1:3 gave a mild decrement of non-specific binding from 302.8 Hz to 243.3 Hz, representing a 20% decline in non-specific binding, with the decrement being observed to be significant between the two values ($p<0.05$), whilst a 1:4 dilution gave a 50% decline in binding response compared to the 1:1 dilution. Although other dilutions, especially 1:4, gave the lowest non-specific binding, 1:1 dilution was chosen for further studies as further dilutions of chicken samples would also dilute the *C. jejuni* cells that were intended to be determined in the samples.

Another important consideration in the experiment is that the chicken samples were prepared previously in Bolton broth, which is a requirement for the ISO method. The result showed that Bolton broth contributes minimal responses to the chicken sample in relation to non-specific binding (**Fig. 1**). This suggests that the non-specific binding observed from the chicken sample comes from the complex matrix existing in the chicken sample itself and not from the Bolton broth solution.

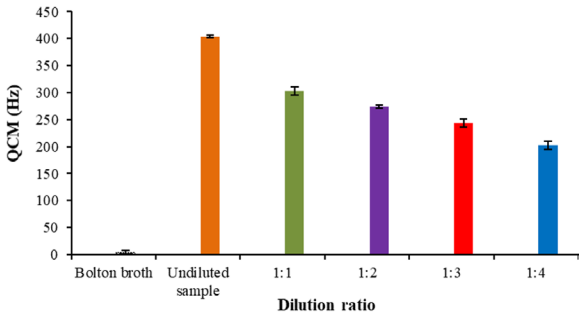

Fig. 1. Binding responses obtained from the Bolton broth solution, undiluted chicken samples, several dilution ratios of chicken samples diluted in PBS through a direct assay using the QCM sensor. Error bars represent the average ± standard deviation of triplicates.

The use of the undiluted sample is also not preferred as the non-specific binding is considered too high, which can lead to false positive results and ultimately a wrong interpretation of the results [10]. Thus, a 1:1 sample dilution was utilized in the assay development to ensure assay uniformity.

A case in point is the detection of pathogen in milk, where certain reports utilized undiluted samples [11,12], while a four times dilution for the detection of *L. monocytogenes* was reported [13], and dilutions from 10 [14–16] to 100 times dilution [17] have also been reported due to the high non-specific response caused by milk samples. There appears to be non-uniformity for sample dilutions utilized in the development of an immunosensor. The standard preparation of chicken sample according to the ISO method is through the mixing of 25 g chicken sample in 225 mL of buffer followed by homogenisation in a stomacher and the final solution obtained is considered as undiluted sample and was employed directly in several immunosensor development for the detection of bacteria on the piezoelectric devices including QCM [18–20], evanescent wave sensor including SPR [21] and including the detection of *C. jejuni* in poultry products such as turkey and chicken using quantum dots and nanobeads based fluorescent immunoassay [22].

A slight deviation to the ISO method was employed in the SPR-based detection of *C. jejuni* in chicken samples where a 50 g of chicken sample was stomached in 200 mL of PBS for 1 min [23], and in an electrochemical immunosensor-based on screen-printed interdigitated microelectrode for the detection of *E. coli* O157:H7 and *S.* Typhimurium where 4.5 kg of chicken was stomached in 400 mL of 0.1% buffered peptone water [5].

Effect of additives in the running buffer

The results obtained in the previous work revealed that the chicken samples caused a major non-specific binding problem in immunosensor work. When the signal from the non-specific binding of the food sample is high, especially when the signal is about one-third of the signal for bacterial or analyte bindings, the addition of additives to reduce the non-specific binding is strongly suggested to avoid false positive results [24]. The addition of additives can be done in several ways, of which the most common is to add additives during sample preparation, to the running buffer or both. The addition of additives must be done judiciously, as the binding of bacterium or analyte to the ligand can be reduced, resulting in a less sensitive assay, with appreciably reduced LOD [4,24,25].

The results obtained in this work showed that the addition of the tested additives into the running buffer (PBS) at all concentrations did not provide any relief from the effect of the non-specific binding when compared to the PBS alone, since both of the non-specific binding obtained due to the chicken matrix and bacterial binding caused a reduction in signals (**Fig. 2**). Furthermore, the addition of the additives into the running buffer gave no significant difference in signal ($p>0.05$) when *C. jejuni* was added, suggesting that the binding of *C. jejuni* was severely compromised with the addition of the additives. Ideally, the signal obtained after the addition of additives should be greater in the presence of the bacterium than in the control sample. However, only the result for the PBS showed the desired effect, which was significantly different ($p<0.05$). The addition of Tween-20 in the running buffer (PBS) caused the most substantial reduction in response for both tests, compared to all the running buffer additives. This implies that both non-specific binding and the analyte binding to the ligand were affected.

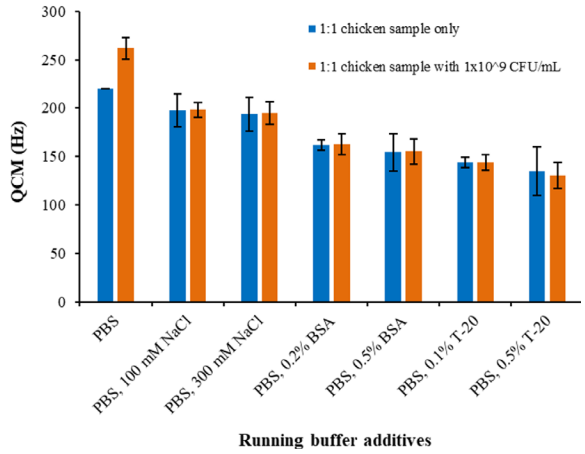

Fig. 2. Effect of the responses obtained from the chicken samples (1:1 dilution) and chicken samples spiked with *C. jejuni* (1×10^9 CFU mL^{-1}) on several sample additives added into the running buffer, tested using the QCM sensor. Error bars represent the average ± standard deviation of triplicates.

Effect of BSA

BSA is able to reduce the non-specific binding as it contains both hydrophilic and hydrophobic sub-groups that can surround proteins or analytes and prevent them from binding to other proteins, analytes, or to the sample container and tubing, especially at low protein concentrations [26]. Normally, BSA is utilized at a concentration of less than 5.0% due to economic reasons. The results show that the chicken sample (1:1 dilution) containing BSA with the percentages from 3.0 to 5.0% were the most optimum in reducing the non-specific binding down to the minimum with 83.01 and 85.52% of reduction, respectively, of which the values were not significantly different to each other ($p<0.05$) compared to untreated chicken sample (control). The poorest performance was at 0.5% of BSA with only a 40.54% reduction of the non-specific binding response, followed by a 66.19% reduction at 1.0% of BSA compared to the control (**Fig. 3a**). The non-specific binding responses for 0.5 and 1.0% are considered too high and unsuitable for assay development, as the rate of false positive results will be very high.

The BSA concentration of 5.0% was then chosen for further testing for *C. jejuni* binding. In the absence of BSA, a high non-specific binding was observed upon bacterial binding, and after subtracting the non-specific binding signal, a binding response for the bacteria of 50.2 Hz was observed (**Fig. 3b**). The binding response of *C. jejuni* at 1×10^9 CFU mL^{-1} was tested in the presence of 5.0% BSA in the chicken sample. The results showed that a dramatic reduction of *C. jejuni* binding response in the presence of BSA occurred at 5.0% BSA, with nearly a 70.71% reduction of binding response from 50.2 Hz to only 14.7 Hz was observed. Although the difference observed compared to control was significant ($p<0.05$), the difference indicates very poor performance by BSA, which reduces non-specific binding but strongly affects bacterial binding at the same time (**Fig. 3b** and **3c**).

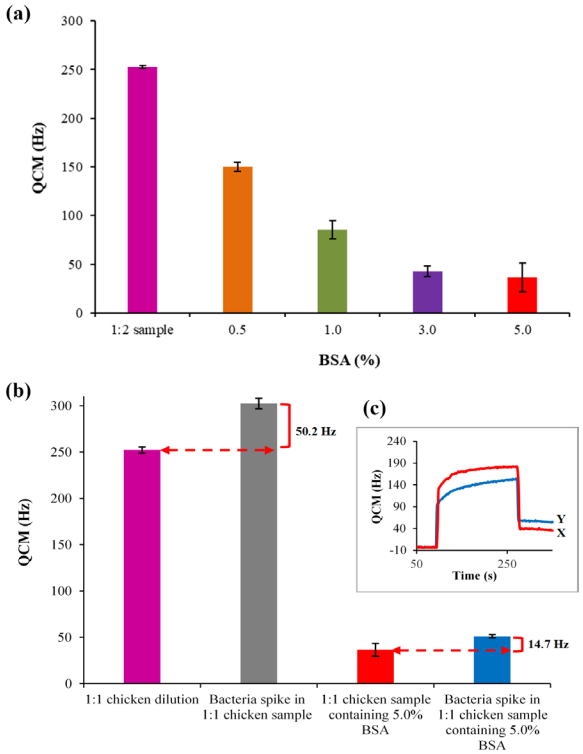

Fig. 3. Binding responses obtained from chicken samples (1:1 dilution) containing (a) several percentages of BSA, (b) the binding responses obtained with and without the addition of 5.0% BSA, and responses obtained in the presence of 1×10^9 CFU mL^{-1} of *C. jejuni* in the samples with the addition of 5.0% BSA, and (c) the sensorgram in the form of an overlay plot demonstrating the responses obtained from the binding of chicken samples treated with 5.0% BSA (insert top right, labelled X), and in the presence of 1×10^9 CFU mL^{-1} *C. jejuni* (insert top right, labelled Y) tested using the QCM sensor. Error bars represent the average ± standard deviation of triplicates.

Sample additives such as BSA, casein, and dry milk can be contaminated with bovine IgG that can react to anti-anti-goat Ig Ab, bovine Ig Ab, and anti-sheep Ig Ab causing an increase in false positive signals, making the addition of BSA ineffective as a non-specific binding remover. In addition, sample additives such as BSA and Tween-20 are generally effective to reduce the non-specific binding due to hydrophobic interaction in samples containing lipids and free fatty acids, and not generally effective to reduce the non-specific binding due to electrostatic interaction [6,7].

Effect of Tween-20

The next potential sample additive to be added to chicken samples is Tween-20, a non-ionic surfactant that may be used to alleviate non-specific binding caused by the hydrophobic interactions between unwanted protein or biological contaminants and the antibody-capturing ligand. The presence of hydrophobic residues on a protein can bind non-specifically to hydrophobic regions of the ligand. Tween-20 shields this region by providing a hydrophobic protective cage as its hydrophobic tail binds to the hydrophobic region, while its hydrophilic end is stabilised in a hydrophilic environment.

If the analyte-ligand binding requires hydrophobic interaction, the use of Tween-20 will result in a reduced binding signal [6,7]. In this experiment, a 1:1 chicken sample containing 1.0, 3.0, and 5.0% of Tween-20 was tested separately. The results showed that the addition of Tween-20 in the 1:1 chicken sample at the concentrations of 1.0, 3.0, and 5.0% was successful in reducing the non-specific response to 55.49, 81.17, and 89.66%, respectively, compared to the untreated control (**Fig. 4a**). The response obtained for 1.0% Tween-20 is considered too high and unsuitable for assay development as the rate of false positives will be very high. The Tween-20 concentrations of 5.0% gave the reduction of non-specific binding that was significantly lower than 3.0%, and hence 5.0% was then chosen for further testing for *C. jejuni* binding. In the absence of Tween-20, a high non-specific binding was observed upon bacterial binding, and after subtracting the non-specific binding signal, a binding response for the bacteria of 49.7 Hz was observed (**Fig. 4b**). The binding response of *C. jejuni* at 1×10^9 CFU mL^{-1} was tested in the presence of 5.0% Tween-20 in the chicken samples. The results showed that a dramatic reduction of *C. jejuni* binding response in the presence of Tween-20 occurred at 5.0% Tween-20, with nearly an 88.15% reduction of binding response from 49.7 Hz to only 5.89 Hz, as shown in **Fig. 4b** and **4c**. Although the difference observed compared to control was significant ($p<0.05$), this indicates very poor performance by Tween-20 which reduced the non-specific response but strongly affected the bacterial binding at the same time. The result was very similar to BSA as an additive.

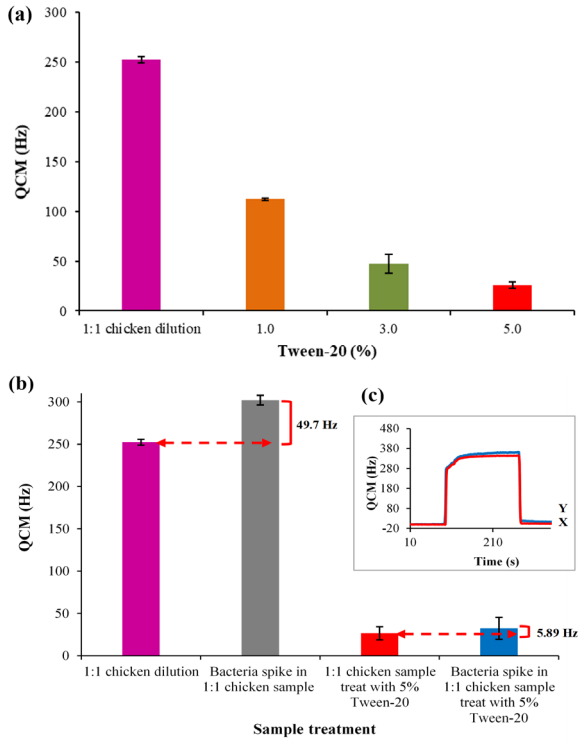

Fig. 4. Binding responses obtained from chicken samples (1:1 dilution) containing (a) several percentages of Tween-20 ranging from 0.5 to 5.0%, (b) the binding responses obtained with and without the addition of 5.0% Tween-20, and the responses obtained in the presence of 1×10^9 CFU mL^{-1} of *C. jejuni* in the samples with the addition of 5.0% Tween-20, and (c) the sensorgram in the form of an overlay plot demonstrating the responses obtained from the binding of chicken samples treated with 5.0% Tween-20 (insert top right, labelled X), and in the presence of 1×10^9 CFU mL^{-1} *C. jejuni* (insert top right, labelled Y) tested using the QCM sensor through a direct assay format. Error bars represent the average ± standard deviation of triplicates.

Effect of NaCl

NaCl is able to shield charges in the sample solution, and this shielding can prevent the charges on non-specific interfering proteins and other biological materials from interacting with charges on the ligand/bioreceptor and vice versa. However, it is crucial to make sure that the addition of salt does not affect ligand or analyte binding [27]. In this experiment, a 1:1 ratio of chicken samples containing 100, 300, and 500 mM of NaCl were tested separately. The results showed that NaCl at all concentrations removed 99% of the non-specific binding due to chicken samples (**Fig. 5a (i)**). When tested for *C. jejuni* binding at the concentration of 1×10^9 CFU mL^{-1}, NaCl concentrations of 100, 300 and 500 mM produced binding responses of 41.97, 32.77 and 27.61 Hz, respectively, after subtraction to control responses at each of the NaCl concentration, and this is a reduction of bacterial binding of 15.56, 34.06 and 44.44%, respectively, compared to control, which was 49.7 Hz (**Fig. 5b (i)**). This suggests that 100 mM NaCl was optimal and will be used for further assay development studies.

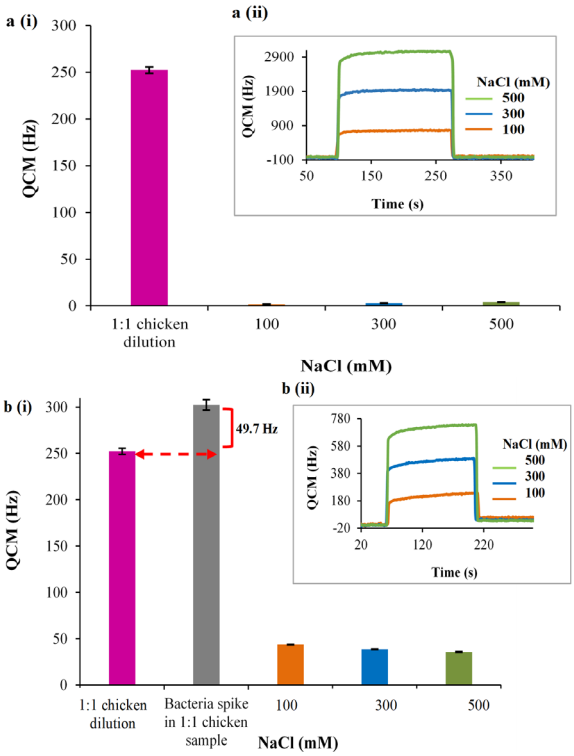

Fig. 5. Binding responses obtained from (a) (i) chicken samples (1:1 dilution) containing three different concentrations of NaCl, (a) (ii) the sensorgram responses obtained from three different concentrations of NaCl, (b) (i) effect of binding responses obtained for 1×10^9 CFU mL^{-1} of *C. jejuni* in chicken samples with and without the addition of NaCl, (b) (ii) the sensorgram demonstrating the binding responses for 1×10^9 CFU mL^{-1} *C. jejuni* in chicken samples at three different concentrations of NaCl.

Although some reduction of binding response was observed, the total elimination of the non-specific binding at 100 mM NaCl was much better than BSA and Tween-20, since NaCl significantly eliminated false positive results. The amount of binding response obtained was adequate and is within the binding response range reported in several pathogen detection works based on QCM of between 40 and 100 Hz [17–19]. NaCl is normally effective in reducing non-specific binding due to electrostatic interaction, indicating that electrostatic interactions significantly contribute to the non-specific binding caused by the chicken sample. This is the reason why the buffer commonly

used in immunosensors, PBS, already contains salts such as KCl (2.7 mM) and NaCl (137 mM) [6,7]. However, even though PBS contains about 140 mM salts, this is not enough to remove the non-specific binding, and further addition is needed when dealing with chicken samples, as observed in this study.

Development of the standard curve for the detection of *C. jejuni* in PBS and chicken samples

A calibration curve for *C. jejuni* was developed using QCMA-1 in PBS, along with chicken samples at a 1:1 dilution, which included 100 mM NaCl as an additive. The results, shown in **Figs. 6** and **7**, demonstrate a sigmoidal calibration curve for *C. jejuni*. This curve was modeled using a four-parameter logistic equation, yielding a LOD of 2×10^5 CFU mL^{-1} and 1.1×10^3 CFU mL^{-1}. The correlation coefficients for these values were 0.995 and 0.977, respectively, indicating a strong agreement between the experimental data and the regressed results.

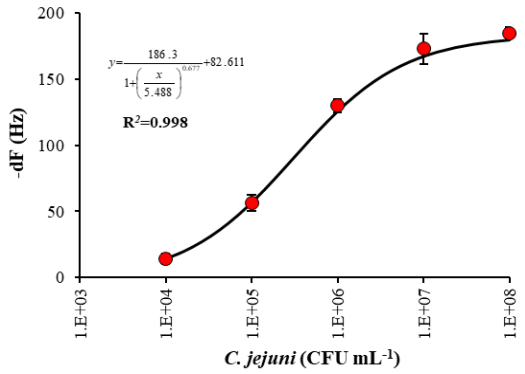

Fig. 6. The corresponding standard plot with -dF (Hz) versus *C. jejuni* concentration (CFUmL^{-1}) on the QCMA-1 sensor through a sandwich assay format in PBS. Error bars represent the average ± standard deviation of triplicates.

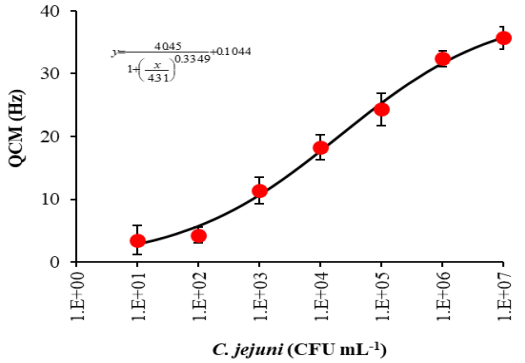

Fig. 7. The corresponding standard plot with -dF (Hz) versus *C. jejuni* concentration (CFUmL^{-1}) on the QCMA-1 sensor through a sandwich assay format in chicken samples. Error bars represent the average ± standard deviation of triplicates.

The LOD values of the same assay that was developed in the absence of chicken sample in PBS showed a more sensitive assay indicating that the presence of non-specific binding caused by the chicken samples affected assay sensitivity. This is despite the use of NaCl and the 1:1 dilution factor to reduce non-specific binding.

Sample additives are either added singly or in combination when dealing with chicken samples. For instance, in the detection of *S.* Typhimurium in chicken carcass samples, the PBS extraction buffer contains a combination of several sample additives consisting of 2 mg mL^{-1} BSA and 0.05% Tween-20 [28]. In the detection of *E. coli* O157:H7, PBS containing 1 mg/mL BSA and 0.05% Tween-20 was added to the sample and running buffer [29]. In another work on the detection of *E. coli, L. monocytogenes,* and *C. jejuni*, only 0.05% Tween-20 was added to the PBS in the running buffer [16]. The additives, such as BSA or Tween-20, are used to block immobilized antibody on sensor surfaces from further non-specific binding caused by chicken samples. These works include the detection of the pathogen *C. jejuni* [22,23], *Salmonella* spp. [5,19,20,30], *E. coli* O157:H7 [31], and *L. monocytogenes* [32].

The sample matrix is a known contributor of non-specific binding in many immunosensor works. Chicken samples contain proteinaceous materials that can include IgG, lipids, and nucleic acids. These can increase the non-specific binding, leading to a reduction in the sensitivity of the developed assay. As far as the QCM assay for pathogens is concerned, the effect of the sample matrix has shown mixed effects on assay development. Very few works on pathogen detection on QCM or piezoelectric devices using chicken samples exist in the literature. One of the earliest works was for the detection of *S.* Typhimurium on a QCM device in chicken samples. The authors reported that the LOD of the assay for the bacterium was not affected in the presence of chicken samples.

An immunomagnetic separation and pre-concentration are employed with a 10 times dilution of chicken samples utilized. In addition, the immobilized antibody was incubated with 1.0% BSA in PBS, blank chicken meat sample solution, and 10^8 CFU mL^{-1} of *E. coli* K12 to remove non-specific binding [33]. In another recent work on the same bacterium, no effect on the LOD is reported, and a similar blocking of the immobilized antibody with BSA and dilution of the chicken sample are carried out [34].

CONCLUSIONS

Non-specific binding poses a significant challenge in many pathogen detections assays, particularly due to the complex sample matrices found in food samples. Chicken samples are especially problematic, as they tend to elicit non-specific responses because of the presence of various interfering substances. These non-specific interactions can lead to false-positive signals in sensor systems. Research has shown that adding an additive during sample preparation can help mitigate this issue. Specifically, the incorporation of 100 mM NaCl in sample preparation has proven effective for assays developed in both PBS and chicken samples using the QCMA-1. Looking ahead, it is important to test other food products, such as meat, milk, and vegetables, for the presence of this bacterium. Cross-contamination with *C. jejuni* is frequently linked to these food items. Additionally, further investigation into the effects of various sample additives and strategies for reducing non-specific binding is necessary, depending on the type of food samples used.

ACKNOWLEDGEMENTS

The authors acknowledge and thank the Malaysian Agricultural Research and Development Institute (MARDI) and Cranfield University for funding and supporting this research project.

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