Pre-Collaborative Validation of an Amperometric Immunosensor for *Salmonella* †

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† Presented at the 1st International Electronic Conference on Biosensors, 2–17 November 2020; Available online: https://iecb2020.sciforum.net/.

Published: 2 November 2020

**Abstract:** The method of *Salmonella* detection recommended is cultural, but it is laborious, presents a high consumption of material, and requires about five days for presumptive results. Immunosensor is an alternative tool that has shown promising and rapid results, although many devices have their performance evaluated only under buffering conditions and few achieve the validation stage. The objective was to perform a pre-collaborative validation of an electrochemical immunosensor assembled on screen-printed electrodes for the detection of *Salmonella* sp. in milk. The antibodies were immobilized by cysteamine self-assembled monolayer. The sandwich-type amperometric immunosensor was evaluated for contaminated raw and whole UHT milk and compared to performance with a gold standard reference method (BAM) according to AOAC recommendations for a single laboratory. A binary response (positive/negative) of the immunosensor was used based on a cut off established from current electric obtained for the absence of the pathogen. There was no significant difference for the results of the biosensor and the reference method, in the absence and the levels from 10¹ to 10³ CFU mL⁻¹ of *Salmonella* Typhimurium for the two types of milk. This result indicates the efficiency of the biosensor in detecting the pathogen into a complex matrix.

**Keywords:** immunosensor; *Salmonella*; milk; validation; amperometric

1. Introduction

Salmonellosis is considered one of the most important diseases transmitted by food considering the number of people affected, its complications, and the number of contaminated food products [1]. The detection method of the pathogen usually employed is cultural, laborious, presents a high consumption of material, and lasts about five days, for presumptive results. The use of methods that are reliable, fast, and practical is required for *Salmonella* analysis to reduce the time of results and costs related to the laboratory structure.
Biosensors for *Salmonella* are alternative methods that have shown promising results [2–5]. However, it is common to evaluate the performance of biosensors under conditions considered optimized in detriment to real samples. Validation is the process that gives validity to an analytical method or instrument, whose performance is accepted, and the results are reliable in real samples. An alternative method of analysis should always be compared in terms of performance to a reference method, using statistical criteria determined in the validation protocols approved by recognized Technical Standards Organizations. This procedure is important to standardize and eliminate technical barriers between countries [6–8].

In this study, an amperometric immunosensor previously assembled and characterized [9] had its performance evaluated in milk samples contaminated with Salmonella, following the recommendations of a pre-collaborative study from Association of Official Analytical Chemists (AOAC) for qualitative methods using as reference the cultural method recommended by the Bacteriological Analytical Manual (BAM) [10].

### 2. Methodology

#### 2.1. Growth and Preparation of the Reference Strain

The reference strain was *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC® 51812 TM (Microbiologics®, Saint Cloud, MN, USA). The lyophilized strain was inoculated in brain–heart infusion broth (Becton, Dickinson and Company, Bergen, NJ, USA) at 35 °C for 24 h. Isolated colonies were obtained after being spiked in brain heart infusion agar at 35 °C for 24 h. The culture was kept on brain-inclined heart infusion agar at 4 °C and in brain–heart infusion broth with 25% glycerol (80% v/v), kept at −80 °C.

The cultures used for contamination of the samples were prepared according to [4], from the growth of five colonies of the reference strain in 10 mL of nutrient broth (Becton, Dickinson and Company, Bergen, NJ, USA) at 35 °C for 24 h. The culture medium was replaced with 10 mL of 10 mM PBS buffer (pH 7.4).

#### 2.2. Purification of Antibodies

Polyvalent anti-Salmonella sera Poly A-I and V (Difco™) were precipitated with (NH₄)₂SO₄ with 45% saturation. The solution was stirred for 30 min and kept at 8 °C for 24 h to then be centrifuged at 10,000 rpm at 4 °C for 15 min. The precipitated solution was collected and dialyzed for 24 h. After dialysis, the antibodies were lyophilized (Lioprop LP-510). A concentrated solution of antibody was prepared in 1 mL of 10 mM PBS buffer (pH 7.4). Furthermore the concentration was determined using a spectrophotometer (NanoDrop® ND-1000 UV-VIS). Antibody reactivity was evaluated by slide agglutination assay according to the manufacturer (Difco™). Antibody was conjugated to horseradish peroxidase enzyme (HRP) (Sigma-Aldrich Brasil®, Sao Paulo, Brazil) [11].

#### 2.3. Microbiological Analysis of Milk

Ultra-High Temperature (UHT) milk was purchased from the local market. Raw milk was purchased from a dairy located in Fortaleza, Brazil. The raw milk was placed in sterile glasses of 1 L. The samples were immediately transported in isothermal boxes at 8 °C to the laboratory.

Raw and UHT milk were evaluated for the presence of *Listeria* sp., *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella* sp., and coliforms at 45 °C, according to the BAM cultural method methodology [10].

#### 2.4. Assembly of the Biosensor

Screen-printed electrodes Dropsense (C220AT®) were immersed in 10 mM cysteamine ethanolic solution (cys) for 3 h. Then, the electrodes were immersed in a solution of protein A (protA) 7.5 mg mL⁻¹ of *Staphylococcus aureus* (Sigma-Aldrich Brasil®, Sao Paulo, Brazil) containing N-hydroxysuccinimide/N-(3-dimethylamino propyl) -N’-ethylcarbodiimide (Sigma-Aldrich Brasil®,
Sao Paulo, Brazil) (EDC/NHS) (2 mM/5 mM) for 1 h. After each immersion procedure, the electrodes were washed with 10 mM phosphate buffer (PBS) (pH 7.4). The modified cis-protA electrode was then immersed overnight in a solution of anti-Salmonella (Ac) 2 mg mL$^{-1}$. The non-specific binding was blocked with 1% bovine serum albumin solution (w/v) (Sigma-Aldrich Brasil®, Sao Paulo, Brazil) for 1 h [9].

2.5. Analytical Response

Milk samples were contaminated with Salmonella strain (10$^1$ and 10$^3$ CFU mL$^{-1}$). Five replicates were used for each inoculation concentration. Detection probability (POD) and the difference in detection probability (dPOD) between the biosensor and the reference method were evaluated according to [6,8]. The lowest concentration of the POD was fractioned in 1:2 and 1:3 [6,8].

The samples after contamination were centrifuged to remove the fat, and the supernatant was discarded. The precipitate was suspended in 10 mL of 10 mM PBS buffer and vortexed for 1 min. The device was immersed in the solution for 1 h and then washed with PBS buffer (pH 7.4) for 1 h, and then in a conjugated antibody solution (0.7 mg mL$^{-1}$) with horseradish peroxidase enzyme (HRP) for 1 h.

The analytical response was obtained using a potentiostat/galvanostat (Autolab/PGSTAT12), NOVA software (v.4.9.007) in 10 mM PBS solution (pH 7.4) in the presence of 300 mM H$_2$O$_2$ and 3 mM hydroquinone. The chronoamperometry was performed at a constant potential of 75 mV for 120 s until the establishment of a baseline.

The interpretation of the analytical response (positive/negative) was based on the amperometric responses obtained in the presence of S. Typhimurium. The signal was compared with the instrumental response established for the negative control (absence of pathogen) plus a standard deviation and a statistical value, which was called cut-off point (Equation (1)) [12]. Amperometric responses above the cut-off point were considered positive.

$$\text{Cut-off point} = X + t(\alpha, \nu) \cdot \text{SD}$$  \hspace{1cm} (1)

where X is the average of the negative control, $t(\alpha, n-1)$ is the unilateral tabular t statistical value at a given level of significance ($\alpha$). Given that, $n$ is the sample size ($n = 5$), $\alpha$ (95%) and SD is the sample standard deviation of the negative control.

3. Result and Discussion

Microbiological Diagnosis of Milk

The raw and UHT milk were initially submitted to microbiological analysis to evaluate the presence of Salmonella and microorganisms that could interfere with the performance of the device. The results of the microbiological analyses are shown in Table 1. Raw milk, unlike the UHT sample, presented coliform bacteria at 45 °C in the concentration of 10$^3$ CFU mL$^{-1}$. In both kinds of milk, Salmonella sp was not found. Thus, the samples were contaminated with the reference strain of Salmonella.

| Sample    | Listeria sp. | Bacillus cereus (CFU mL$^{-1}$) | Staphylococcus aureus (CFU mL$^{-1}$) | Salmonella sp. | Coliforms 45 °C (CFU mL$^{-1}$) |
|-----------|--------------|---------------------------------|--------------------------------------|----------------|--------------------------------|
| Raw milk  | Absence      | <10                             | <10                                  | Absence        | 1.1 × 10$^3$                  |
| UHT milk  | Absence      | <10                             | <10                                  | Absence        | <3                             |

The amperometric immunosensor was evaluated according to [6] for pre-collaborative assays of qualitative analysis methods. The lowest concentration of Salmonella detected, and 10$^1$ CFU mL$^{-1}$ was fractionated (1:2 and 1:3) with five repetitions for each one. As can be seen in Table 2, the immunosensor presented POD similar to the reference method for the levels of inoculation: Negative control, 10$^1$ CFU mL$^{-1}$, and 10$^3$ CFU mL$^{-1}$ for both samples of raw milk and UHT.
According to [13], when the repetitions are evaluated for the highest concentration of the analyte, a POD = 1 is expected, since all results must be positive. In turn, for the replicates referring to the negative control, a POD = 0 is expected, indicating that there were no false-positive results. In Table 1 can be seen that the results obtained for the highest concentrations (10^3 CFU mL⁻¹) and negative control presented POD = 1 and POD = 0, respectively, demonstrating compliance with the requirements of AOAC [6].

According to the recommendation for AOAC qualitative pre-collaborative study [6], the data presented were analyzed statistically using the dPOD, which is calculated by the difference in the POD values of the methods evaluated. It was observed that there was no significant difference between the results obtained by the biosensor and the reference method for the inoculation concentrations: Negative control, 10^1 CFU mL⁻¹, and 10^3 CFU mL⁻¹, for the two kinds of milks. This result indicates the equivalence and efficiency of the biosensor in detecting the pathogen in these matrices and in the inoculation concentrations evaluated in comparison with the standard method. On the other hand, for fractional inoculation levels, 1:2 and 1:3, the methods differed statistically p > 0.05 [6].

The performance of the immunosensor equivalent to the traditional method for the detection of *Salmonella* sp. at concentrations 10^1 CFU mL⁻¹ and 10^3 CFU mL⁻¹ is remarkably interesting result. Besides, identifying the presence of the pathogen in low concentration within of a complex matrix, the device has the advantage of requiring less reagents and detection time (about 2 h and 30 min) than cultural methods (from 2 to 5 days). The reduction in the immunosensor analysis time occurs because the pre-enrichment step is not necessary. This step is required by some methods of microbiological analysis since they present a lower sensitivity of about 10^4 to 10^5 CFU mL⁻¹ colonies [3,5].

### Table 2. Comparison of the probability of detection (POD) obtained by the reference method (POD ref) and the biosensor (POD bio) at different levels of inoculation for analysis of *Salmonella* sp. in raw and UHT milk.

| Sample | Inoculum Level | Biosensor | Reference Method | Comparison between Methods |
|--------|---------------|-----------|------------------|---------------------------|
|        |               | N X POD  | 95% IC POD       | N X POD                   | dPOD bio/ref | 95% IC dPOD |
|        |               | bio      | 95% IC ref       | 95% IC bio                | 95% IC bio/ref |
|        |               | LI LS    | LI LS            |                           |
| Raw Milk | Negative Control | 5 0 0 | 0 0.43 | 0 0.43 | 0 | -0.43 | 0.43 |
|        | 10^1 | 5 5 1 | 0.57 1.00 | 5 1 | 0.57 1 | 0 | -0.43 | 0.43 |
|        | 1:2 | 5 2 0.4 | 0.12 0.77 | 5 1 | 0.57 1 | -0.60 | -0.88 | -0.03 |
|        | 1:3 | 5 0 0 | 0 0.43 | 5 1 | 0.57 1 | -1.00 | -1.00 | -0.39 |
|        | 10^3 | 5 5 1 | 0.57 1.00 | 5 1 | 0.57 1 | 0.00 | -0.43 | 0.43 |
| UHT Milk | Negative Control | 5 0 0 | 0 0.43 | 0 0.43 | 0 | -0.43 | 0.43 |
|        | 10^1 | 5 5 1 | 0.57 1.00 | 5 1 | 0.57 1 | 0.00 | -0.43 | 0.43 |
|        | 1:2 | 5 1 0.2 | 0.04 0.62 | 5 1 | 0.57 1 | -0.80 | -0.96 | -0.19 |
|        | 1:3 | 5 0 0 | 0 0.43 | 5 1 | 0.57 1 | -1.00 | -1.00 | -0.39 |
|        | 10^3 | 5 5 1 | 0.57 1.00 | 5 1 | 0.57 1 | 0 | -0.43 | 0.43 |

N, number of test portions; x, number of positive test portions; POD, positive results divided by N; PODref, reference method; PODbio, alternative method (biosensor), CI, confidence interval; LI, lower limit and LS, upper limit.

### 4. Conclusions

The biosensor had a good probability of detection and was able to detect the presence of *Salmonella* sp. in one of the lowest concentrations of the pathogen 10 CFU mL⁻¹ compared to other immunosensors. The biosensor was efficient in detecting the pathogen for the two types of milk evaluated without requiring a pre-enrichment step, which was decisive for the substantial reduction in analysis time compared to the reference method.
Author Contributions: The authors contributed of different complementary forms for the execution of experiments and written of this work. F.A.E.d.B., L.C.R.B., A.M.A.M., and M.d.A.O. worked directly in the formal analysis and original draft preparation. C.R.A., T.F.M., and E.A.T.d.F. were involved in conceptualization, supervision, and review and editing, R.F.F. realized activities of review and editing, conceptualization, supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: The authors thank Embrapa and CNPq (408599/2018-9 process) for their financial support, including the scholarship for Francisca Airlane Esteves de Brito (CNPq).

Conflicts of Interest: The authors declare no conflict of interest.

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