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Effect of time and storage condition on the performance of an electrochemical immunosensor for *Salmonella*

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Abstract

Storage of immunosensors has been rarely studied despite its importance for technology transfer to diagnostic companies. In this study was proposed a novel stabilizer storage solution of an electrochemical immunosensor for the detection of *Salmonella*. A commercial stabilizer solution for immunoglobulin was also tested for the devices. The immunosensors were kept in solution for 15, 30, 60, 90 and 120 days at refrigerated (4 ºC) and room temperature (25 ºC). There was a statistical difference ($p < 0.05$) between the two stabilizer solutions and the temperatures. The best condition found for the storage of the immunosensors was the formulated solution under refrigeration (4 ºC).

Keywords: electrochemical biosensor; storage; immunosensor; stabilizer solution; *Salmonella*.

Practical Application: A storage condition for an electrochemical immunosensor was proposed in this work.

1 Introduction

Bacteria of the genus *Salmonella* are considered one of the main causes of outbreaks involving several kinds of food. The pathogen is responsible for causing foodborne disease: salmonellosis, typhoid and paratyphoid fevers, thus being an important public health (Hammack et al., 2012; Silva et al., 2018).

The standard method (bacterial culture) for detecting the pathogen is laborious, has a high consumption of material and reagents besides lasting about 5 days. This long time is incompatible with the dynamism of the industry that needs to release quickly lots of their products. The use of reliable, simple and fast alternative methods is an attractive option to reduce the analysis time since it eliminates steps of a conventional method such as pre-enrichment/ pre-treatment and selective enrichment. In the specific case of some biosensors, these steps can be eliminated or reduced in time and consequently the analysis is faster (Andrews et al., 2018).

Biosensors are alternative tools that have shown promising results in the detection of *Salmonella* sp. (Brandão et al., 2015; Melo et al., 2016; Silva et al., 2018). Electrochemical biosensors are found in the market and are too known due to their specificity, ease of automation, high precision and rapid time of analysis (Felix & Angnes, 2018; Mahato et al., 2018). Although electrochemical immunosensors are found commercially in minor quantity than enzymatic sensors they are quite sensitive and specific. In the literature, it is common that immunosensors are assembled and the analysis for analyte identification occurs soon without determinate storage condition. Many studies (some few) have evaluated brief storage in buffer solution (Sun et al., 2011, Zhou et al., 2015, Pilas et al., 2018, Jayanthi et al., 2019). However, maintaining these devices in a buffered medium with other preservatives could improve their stability over time. It is known that the presence of proteases, metals and changes in pH can cause the leakage or denaturation of biomolecules (Mahato et al., 2018; Mendonça et al., 2011; Wang et al., 2007) and because of that, it is important to include inhibitors and preservatives in the formulation. Devices stored under inadequate conditions will have a short shelf-life, which could make them commercially unviable.

In this study, a stabilizer solution was formulated and an electrochemical immunosensor for the detection of *Salmonella* sp. was stored in this solution at room temperature and refrigerated for 120 days. A commercial stabilizer solution for immunoglobulin was evaluated and compared to the formulated stabilizer solution.

2 Materials and methods

2.1 Reagents

Horseradish peroxidase (HRP) (250 U mg−1), glutaraldehyde (25%), N-hydroxysuccinimide (NHS), N-ethyl-N′-(3-

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dimethylamino propyl) carbodiimide hydrochloride (EDC), cysteamine, protein A from *Staphylococcus aureus*, bovine serum albumin (BSA), hydroquinone, hydrogen peroxide and BioStab Antibody Stabilizer were purchased from Sigma- Aldrich (St. Louis, MO, USA). The culture media, brain heart infusion agar (BHI agar), brain heart infusion broth (BHI broth), nutrient agar and nutrient broth were acquired from Difco™ (Maryland, USA). *Salmonella* Typhimurium (*Salmonella enterica* subsp. *enterica* serovar Typhimurium, ATCC® 51812) was purchased from Microbiologics (Saint Cloud, MN, USA), and *Salmonella* Antiserum Poly A-I & Vi from Difco™ (Maryland, USA). Ethylenediaminetetraacetic acid (EDTA) and sodium azide were acquired from Synth® (São Paulo, Brazil).

2.2 Preparation of primary and secondary antibody

The antibodies were purified by precipitation of the serum with 45% $(NH_4)_2SO_4$, and the concentration was determined by spsctrophotometer (NanoDrop® ND- 1000 UV-VIS). The antibody solutions were prepared by dilution in 0.1 mol.L^{-1} phosphate buffer saline- PBS (pH 7.4). The secondary antibody was conjugated to HRP (Avrameas, 1969).

2.3 Biosensor assembly

Gold screen-printed electrodes (Dropsense-C220AT®) were modified by immersing in 10 mM cysteamine for 3 h (Pimenta-Martins et al., 2012). The electrodes were immersed in a solution containing 2 mM EDC/5 mM NHS and 7.5 mg.mL^{-1} protein A from *Staphylococcus aureus* for 1 h. The electrodes were incubated overnight in a solution of primary antibody against *Salmonella* sp. (2 mg.mL^{-1}). After each incubation procedure, the modified electrodes were washed with 0.1 mol.L^{-1} PBS (pH 7.4). Finally, non-specified binding sites of the modified electrode were blocked with 1% BSA for 1 h.

2.4 Biosensor storage

A stabilizer solution was formulated with 1 mM PBS, 1 mM EDTA, 0.2% (w / v) Sodium azide and 1 mM BSA. The immunosensors were dipped into a formulated stabilizer solution (FSS) and an antibody commercial stabilizer solution (CSS) (Sigma-Aldrich®). The electrical contact of the electrodes with the equipment was protected using an adhesive and without dipping this part in the solution. Analysis of the electrodes was performed every 30 days in the presence of the pathogen. The shelf-life of the devices stored in stabilizer solution was based on the time that the biosensor showed positive responses in the presence of milk samples spiked with *Salmonella*.

Initially, the devices were kept without and with immersion into stabilizer solution at refrigerated temperature (4 °C) with 14% relative humidity and at room temperature (25 °C) with 69% relative humidity for 24 h to verify the best storage condition. The performance of the biosensor was evaluated in samples of Ultra High Temperature (UHT) milk contaminated with *S.* Typhimurium (10^6 CFU.mL^{-1}).

2.5 Analytical response

The immunosensor performance was evaluated in Ultra High Temperature (UHT) milk purchased in local stores. The milk samples were spiked with *S.* Typhimurium at 10^6 CFU.mL^{-1}. The contaminated milk samples were centrifugated (5000 rpm at 4 °C for 30 min) to remove the fat. The control samples were with no bacteria. Thereafter, the immunosensors were incubated with labeled secondary antibodies with horseradish peroxidase (HRP) (0.7 mg.mL^{-1}) for 1 h. After each incubation step, the electrode was rinsed with 0.1 mol.L^{-1} PBS (pH 7.4). The electrochemical responses were carried out using an electrochemical cell (10 mL) containing 0.1 mol.L^{-1} PBS (pH 7.4), 300 mM H_2O_2 and 3 mM hydroquinone. Electrochemical measurements were performed using potentiostat/galvanostat Autolab/PGSTAT12 and NOVA software (v.4.9) and applying a constant potential of 75 mV for 120 seconds.

A *cut-off* point was defined considering: W + 3 x SD, where W represents the average of the electric currents of the immunosensor freshly prepared in the absence of the pathogen and DP is the sample standard deviation. Responses were considered positive when the electric current values were above the *cut-off* and negative responses when they showed the same value or below the *cut-off*. The *cut-off* point was the same found for the detection limit.

2.6 Performance parameters of the immunosensor

The performance parameters of the immunosensor: sensitivity, false negative, false positive and specificity of the immunosensor were calculated according to the recommendation of AOAC for qualitative methods of microbiological analysis (Association of Official Analytical Chemists, 2012; Feldsine et al., 2002). Milk samples were contaminated with *Salmonella* in the concentration of 10^1 CFU.mL^{-1}. A negative was used with milk no contaminated. The reference method used was the bacterial culture according to Bacteriological Analytical Manual (BAM) (Andrews et al., 2018).

2.7 Statistical analysis

A split-plot design with the repeated measure in time was used in this work with two treatments: stabilizer solution (formulated and commercial stabilizer solution) and temperature (refrigerated and room). The data were transformed into $\sqrt{x}$ to approximate the data of the normal distribution. The analysis of variance was performed using the Statistic software version 13 (Dell). Confidence intervals were established at the 5% probability level.

3 Results and discussion

3.1 Performance parameters of the immunosensor

This immunosensor was previously studied by group for performance in milk (Brito et al., 2020; Alexandre et al., 2018). This immunosensor had a qualitative performance and showed 100% of sensitivity and specificity for *Salmonella* Typhimurium detection. Additionally, the rate of false negative and false positive

results was zero. This result is quite interesting, as the biosensor performed similarly to the standard method (bacterial culture). Most *Salmonella* detection methods use pre-enrichment step, however, the immunosensor presented in this work has the advantage of eliminating this step, which reduces the analysis time.

The stability of biomolecules depends on many abiotic factors such as temperature, pressure, pH and biotic factors such as enzymes that may cause proteolysis (Wang et al., 2007). It has been long recognized, for example, that the kind of buffering substance and concentration may affect the stability of proteins (Wang, 1999). Biodevices like immunosensors must observe these factors during the storage and analysis in order to guarantee their maximum performance. In this work, a stabilizer solution based on chelator, protease inhibitor, preservative and buffer was evaluated to store an amperometric immunosensor at 4 ºC and 25 ºC for *Salmonella* Typhimurium detection. The concentrations of preservative substances used in the formulation of the stabilizer solution were preliminarily tested to assess any interference of ions such as Na^+ in the electrochemical response. EDTA and sodium azide not caused variation of the current electrical of the biosensor in the presence and absence of the *Salmonella* (Bezerra et al., 2019).

3.2 Performance of the immunosensor during time

Devices stored dry for 24 h at room temperature not detected the pathogen in the milk samples. On the other hand, when the devices were stored under immersion into a stabilizer solution at temperatures of 4ºC and 25 ºC, the devices satisfactorily differentiated the contaminated and non-contaminated milk sample. Probably, the drying of the immunosensor that occurred naturally at room temperature caused a destabilization or leakage in the binding molecules with a loss in the electrical signal. Contrary, some studies reported storage of immunosensor at dry condition and found a good stability that is performed up to a maximum of 10 weeks (Aydın & Sezgintürk, 2017; Aydin, 2020, Cordeiro et al., 2020).

Biosensors depend on the reaction between the immobilized biomolecules and the analyte that produces or consumes electrons or ions, which affects the electrical properties of the solution, such as electric potential or current. Thus, variations in the stability of different types of immunosensors may happen due to changes in the surface microenvironment and degree of multipoint attachment of biomolecules related to the chemical immobilization method of antibody, assembly structure of the biosensor, and biocompatibility of the electrode (Mohamad et al., 2015).

In the literature, there are few studies about storage solutions of immunosensors and there is no commercial stabilizing solution available on the market suitable for this kind of analytical tool. The storage of the biosensor in a commercial stabilizer solution (CSS) for immunoglobulin was chosen for comparating with a formulated stabilizer solution (FSS) because the recognition biomolecule used in the sensor surface. Both stabilizer solutions were evaluated at 4ºC and 25 ºC for 120 days. The analysis of the treatments (temperature and stabilizer) over time was performed through analysis of variance (ANOVA) (Table 1). According to ANOVA, the stabilizer solutions and temperatures tested were statistically different ($p < 0.05$).

Table 1.

A statistic study of the time x solution x temperature interaction can be seen in Figure 1. At 15 days, the electrochemical responses of the biosensors stored in stabilizer solutions at two temperatures had the same behavior ($p > 0.05$). This means that the immunosensor dipped into stabilizer solution may be maintained at room temperature for 15 days. It is known that antibodies compared with other proteins, they seem to be more resistant to thermal stress and can withstand temperatures up to 70 ºC (Wang et al., 2007). Stabilizer solution for immunosensors is important to avoid leakage or denaturation, maintaining biological activity for a long time (Ron et al., 1995).

From Figure 2 is possible to evaluate the loss in the response of the immunosensors during the storage. In this study, the reduction in the current was monitored and compared to cut off in order to find out if the response (binary) was positive or negative. The immunosensors at room temperature (25 ºC) exhibited the highest percentage of decrease in cathodic electrical current, in other words, greater loss of performance over time. In CSS, the devices were able to detect the pathogen for 30 days, when they exhibited a 72.9% reduction in the amperometric response. In FSS, the devices also remained fit for 30 days, when the loss observed was 58.5%. Notably, maintenance at room temperature decreased the performance of devices. Temperature is one of the main factors to be considered for studies involving biomolecules (Wang et al., 2007).

At 60 days, the devices dipped into CSS under refrigeration showed a decrease in the reduction current. However, even with a significant decline in response, devices kept refrigerated were able to detect the pathogen at 60 days. Alternatively, biosensors kept in FSS under refrigeration showed an excellent performance in detecting the pathogen at 60 days. Antibodies are better stabilized at lower temperatures explaining the better performance of devices maintained in this condition (Gibson, 1999; Reverberi & Reverberi, 2007).

Table 1. Analysis of variance (ANOVA) of the transformed data for the biosensor response in UHT whole milk contaminated with *S.* Typhimurium (10^6 CFU. mL^{-1}).

Causes of Variation	DF	SS	MS	F
Solution	1	9.48	9.48	68.70*
Temperature	1	9.84	9.84	71.30*
Solution X Temperature	1	0.04	0.04	0.30
Residue	16			
Time	4	38.30	9.58	88.80*
Time x Solution	4	3.90	0.97	8.98*
Time x Temperature	4	8.70	2.17	20.11*
Time x Solution x Temperature	4	1.83	0.45	4.21*
Residue	64	6.90	0.11	

Transformed data √x. Some treatments were unbalanced data. ANOVA results: DF: degree of freedom; SS: sum of squares; MS: sum of mean squares; F: statistics. CFU: Colony Forming Unit. * Significant test at 5% probability level

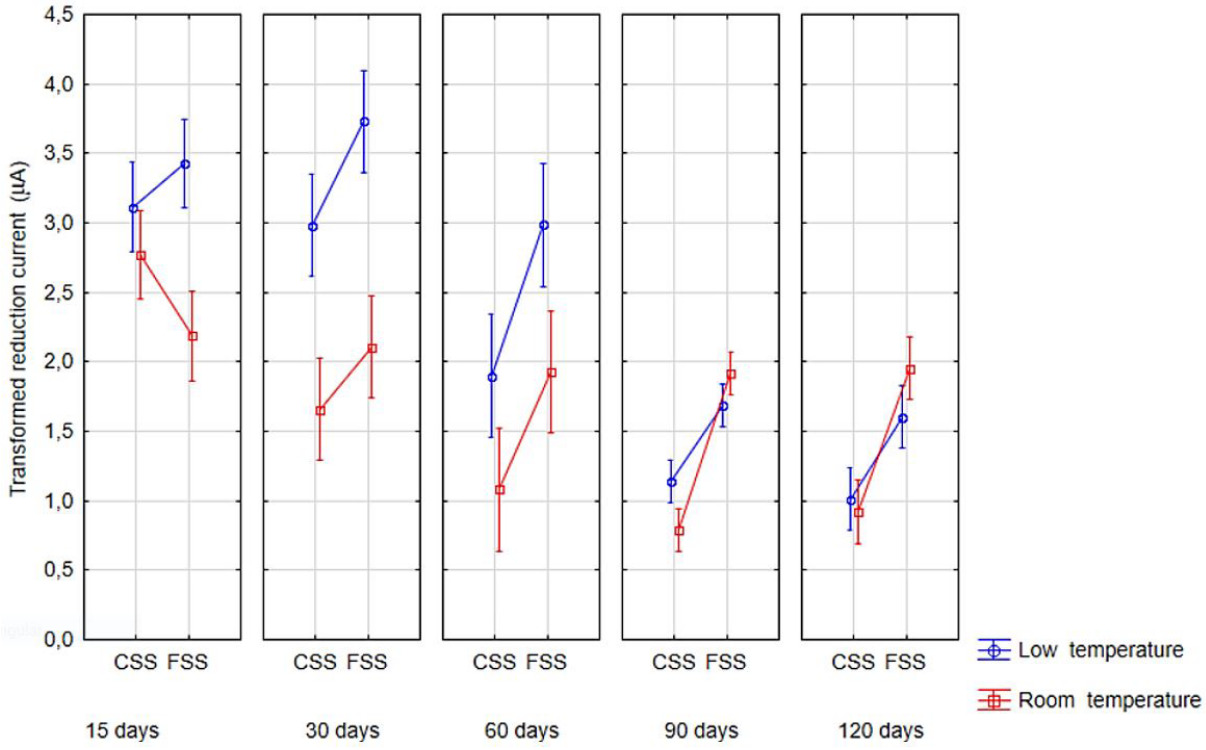

Figure 1. Confidence interval of the biosensor response in milk contaminated with *S.* Typhimurium (106 CFU.mL-1), transformed values, for interaction Time x Solution x Temperature after storage of the immunosensors in a commercial stabilizer solution (CSS) and a formulated stabilizer solution (FSS) in 15 ; 30; 60 and 90 and 120 days at room (25 ºC) and low (4 ºC) temperature. The standard deviation is shown for n = 10.

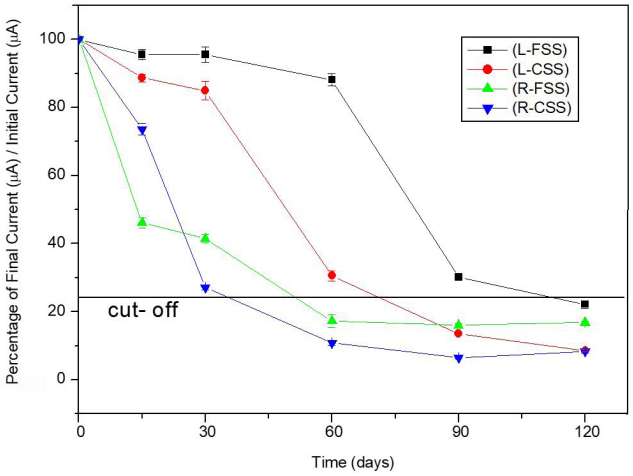

Figure 2. Percentage of decrease of the cathodic current of the devices after storage into a formulated (FSS) and commercial stabilizer solution (CSS) over time (15; 30; 60, 90 and 120 days) at refrigerated (4 ºC) and room (25 ºC) temperature.

At 90 days, devices kept in FSS showed an adequate response (presence/absence of *Salmonella*). It was noticed over time that there was a gradual decrease in the cathodic electric current and at 120 days the device response in the presence of *Salmonella* was lost with an amperometric response equal to the cut-off value, demonstrating a false-negative result. Stabilizer for immunosensors to the best of our knowledge, are not yet commercially available. In this study, an attempt was made to formulate a solution capable of stabilizing immunoglobulins in order to avoid the leakage of immobilized antibodies and not interfere with other molecules involved in the assembly of the immunosensor (thiol, protein A, BSA, peroxidase) without causing any variation in the amperometric response. The solution formulated and proposed in the present study was composed of a buffer, proteins that aid in the stability of antibodies, a preservative agent that prevents microbial contamination and a chelating agent.

Sodium azide is a preservative agent in low concentration and has a known inhibitory and bacteriostatic effect, not severely increasing the ionic effect of the solution (Hendrix et al., 2019). EDTA is one of the components used in formulations of commercial conjugated antibodies (Duerr & Friess, 2019). EDTA is an inhibitor of metalloproteases and proteases activated by divalent metals. The pH of a solution is another important factor to be considered to choose a buffer since it influences the stability of antibodies. The effect of pH can affect the physical stability of immunoglobulins, changing the number and distribution of charges on the surface of the protein, becoming difficult to bind the antigen. Besides, the aggregation seems to be lower at neutral pH (Dominguez & Holmes, 2011; Wang et al., 2007). In the formulated solution, the pH was adjusted to 7.4 in order to mimic the ideal biological condition for homeostasis of biological molecules, one of the factors that may have contributed directly to a better performance of the devices maintained in this solution.

4 Conclusions

The storage of the immunosensor in the formulated stabilizer solution maintained the performance at 90 days but lost stability by 120 days, indicating that the shelf-life must be established within this period. On the other hand, a commercial stabilizer solution for immunoglobulin did not maintain the performance of the device at 90 days. Other chemical components may be tested in order to extend more the shelf-life of the device, although with the use of this formulation has been found a shelf-life for the industry needs.

Conflict of interest

There are no conflicts to declare.

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References

Alexandre, D., Melo, A., Furtado, R., Borges, M., Figueiredo, E., Biswas, A., Cheng, H., & Alves, C. (2018). A rapid and specific biosensor for *Salmonella typhimurium* detection in milk. *Food and Bioprocess Technology*, 11(4), 748-756. http://dx.doi.org/10.1007/s11947-017-2051-8.

Andrews, W. H., Jacobson, A., & Hammack, T. S. (2018). Salmonella. In Food and Drug Administration (Org.), *Bacteriological Analytical Manual (BAM)*. Arlington: AOAC.

Association of Official Analytical Chemists – AOAC. (2012). *AOAC® Guidelines for Validation of Microbiological Methods* (Vol. 18, pp. 1-46). Arlington: AOAC.

Avrameas, S. (1969). Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. *Immunochemistry*, 6(1), 43-52. http://dx.doi.org/10.1016/0019-2791(69)90177-3. PMid:4975324.

Aydin, E. B. (2020). Highly sensitive impedimetric immunosensor for determination of interleukin 6 as a cancer biomarker by using conjugated polymer containing epoxy side groups modified disposable ITO electrode. *Talanta*, 215, 120909. http://dx.doi.org/10.1016/j.talanta.2020.120909. PMid:32312453.

Aydın, E. B., & Sezgintürk, M. K. (2017). A sensitive and disposable electrochemical immunosensor for detection of SOX2, a biomarker of cancer. *Talanta*, 172, 162-170. http://dx.doi.org/10.1016/j.talanta.2017.05.048. PMid:28602290.

Bezerra, L. C. R., Brito, F. A. E., Melo, A. M. A., Furtado, R. F., Borges, M. F., & Alves, C. R. (2019). Avaliação da estocagem de agentes antimicrobianos na estocagem de um biosensor eletroquímico. In Universidade Estadual do Ceará (Org.), *XXIV Semana Universitária da UECE*. Fortaleza: UECE.

Brandão, D., Liébana, S., & Pividori, I. (2015). Multiplexed detection of foodborne pathogens based on magnetic particles. *New Biotechnology*, 32(5), 511-520. http://dx.doi.org/10.1016/j.nbt.2015.03.011. PMid:25858812.

Brito, F., Bezerra, L., Furtado, R., Melo, A., Oliveira, M., Alves, C., Feitosa, T., & Figueiredo, E. (2020). Pre-Collaborative validation of an amperometric immunosensor for *Salmonella*. *Proceedings*, 60, 43. http://dx.doi.org/10.3390/IECB2020-07031.

Cordeiro, T. A. R., Martins, H. R., Franco, D. L., Santos, F. L. N., Celedon, P. A. F., Cantuária, V. L., Lana, M., Reis, A. B., & Ferreira, L. F. (2020). Impedimetric immunosensor for rapid and simultaneous detection of chagas and visceral leishmaniasis for point of care diagnosis. *Biosensors & Bioelectronics*, 169, 112573. http://dx.doi.org/10.1016/j.bios.2020.112573. PMid:32905944.

Dominguez, R., & Holmes, K. C. (2011). Antibody structure and function. *Focus*, 40, 58-78.

Duerr, C., & Friess, W. (2019). Antibody-drug conjugates-stability and formulation. *European Journal of Pharmaceutics and Biopharmaceutics*, 139, 168-176. http://dx.doi.org/10.1016/j.ejpb.2019.03.021. PMid:30940541.

Feldsine, P., Abeyta, C., & Andrews, W. H. (2002). International methods committee guidelines for validation of qualitative and quantitative food microbiological official methods of analysis. *Journal of AOAC International*, 85(5), 1187-1200. http://dx.doi.org/10.1093/jaoac/85.5.1187. PMid:12374420.

Felix, F. S., & Angnes, L. (2018). Electrochemical immunosensors—a powerful tool for analytical applications. *Biosensors & Bioelectronics*, 102, 470-478. http://dx.doi.org/10.1016/j.bios.2017.11.029. PMid:29182930.

Gibson, T. (1999). Biosensors: the stabilité problem. *Analusis*, 27(7), 630-638. http://dx.doi.org/10.1051/analusis:1999270630.

Hammack, T., Abraham, A., & Al-Khadi, S. (2012). *Bad bug book: handbook of foodborne pathogenic microorganisms and natural toxins*. Washington: U.S. Food & Drug Administration.

Hendrix, K., Bleyen, N., Mennecart, T., Bruggeman, C., & Valcke, E. (2019). Sodium azide used as microbial inhibitor caused unwanted by-products in anaerobic geochemical studies. *Applied Geochemistry*, 107, 120-130. http://dx.doi.org/10.1016/j.apgeochem.2019.05.014.

Jayanthi, V., Das, A. B., & Saxena, U. (2019). Fabrication of an immunosensor for quantitative detection of breast cancer biomarker UBE2C. *RSC Advances*, 9(29), 16738-16745. http://dx.doi.org/10.1039/C8RA10245G.

Mahato, K., Kumar, S., Srivastava, A., Maurya, P. K., Singh, R., & Chandra, P. (2018). Electrochemical immunosensors: fundamentals and applications in clinical diagnostics. In S. K. Vashist & J. H. T. Luon (Eds.), *Handbook of immunoassay technologies* (pp. 359-414). London: Academic Press.

Melo, A. M. A., Alexandre, D. L., Furtado, R. F., Borges, M. F., Figueiredo, E. A. T., Biswas, A., Cheng, H. N., & Alves, C. R. (2016). Electrochemical immunosensors for *Salmonella* detection in food. *Applied Microbiology and Biotechnology*, 100(12), 5301-5312. http://dx.doi.org/10.1007/s00253-016-7548-y. PMid:27138197.

Mendonça, E., Visôtto, L., Costa, N., Ribeiro, F. R., Oliveira, J. A., & Oliveira, M. G. A. (2011). Caracterização enzimática de isoformas de cisteíno protease de *Anticarsia Gemmatalis*. *Ciência e Agrotecnologia*, 35(3), 446-454. http://dx.doi.org/10.1590/S1413-70542011000300002.

Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., & Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnology, Biotechnological Equipment*, 29(2), 205-220. http://dx.doi.org/10.1080/13102818.2015.1008192. PMid:26019635.

Pilas, J., Yazici, Y., Selmer, T., Keusgen, M., & Schöning, M. (2018). Application of a portable multi-analyte biosensor for organic

acid determination in silage. *Sensors*, 18(5), 1470. http://dx.doi.org/10.3390/s18051470. PMid:29738487.

Pimenta-Martins, M. G. R., Furtado, R. F., Heneine, L. G. D., Dias, R. S., Borges, M. F., & Alves, C. R. (2012). Development of an amperometric immunosensor for detection of staphylococcal enterotoxin type A in cheese. *Journal of Microbiological Methods*, 91(1), 138-143. http://dx.doi.org/10.1016/j.mimet.2012.05.016. PMid:22710410.

Reverberi, R., & Reverberi, L. (2007). Factors affecting the antigen-antibody reaction. *Blood Transfusion*, 5(4), 227-240. PMid:19204779.

Ron, E., Freeman, A., & Solomon, B. (1995). Stabilization and surface modification of monoclonal antibodies by 'bi-layer encagement'. *Journal of Immunological Methods*, 180(2), 237-245. http://dx.doi.org/10.1016/0022-1759(94)00340-3. PMid:7714338.

Silva, N. F. D., Magalhães, J. M. C. S., Freire, C., & Delerue-Matos, C. (2018). Electrochemical Biosensors for *Salmonella*: state of the art and challenges in food safety assessment. *Biosensors & Bioelectronics*, 99, 667-682. http://dx.doi.org/10.1016/j.bios.2017.08.019. PMid:28858763.

Sun, X., Du, S., Wang, X., Zhao, W., & Li, Q. (2011). A label-free electrochemical immunosensor for carbofuran detection based on a sol-gel entrapped antibody. *Sensors*, 11(10), 9520-9531. http://dx.doi.org/10.3390/s111009520. PMid:22163709.

Wang, W. (1999). Instability, stabilization, and formulation of liquid protein pharmaceuticals. *International Journal of Pharmaceutics*, 185(2), 129-188. http://dx.doi.org/10.1016/S0378-5173(99)00152-0. PMid:10460913.

Wang, W., Singh, S., Zeng, D., King, K., & Nema, S. (2007). Antibody structure, instability, and formulation. *Journal of Pharmaceutical Sciences*, 96(1), 1-26. http://dx.doi.org/10.1002/jps.20727. PMid:16998873.

Zhou, C., Liu, D., Xu, L., Li, Q., Song, J., Xu, S., Xing, R., & Song, H. (2015). A sensitive label–free amperometric immunosensor for alpha-fetoprotein based on gold nanorods with different aspect ratio. *Scientific Reports*, 5(1), 9939. http://dx.doi.org/10.1038/srep09939. PMid:25909588.