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Development of proficiency test items for *Salmonella* spp. research in chocolate matrix

Desenvolvimento de itens de ensaio de proficiência para pesquisa de *Salmonella* spp. em matriz chocolate

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ABSTRACT

The aim of this study was to develop lyophilized test items (TI) containing *Salmonella* spp., in chocolate matrix to be used in proficiency testing programs (PTP). Microbial analysis was conducted on samples of granulated chocolate to verify that the sample was free of the target microorganisms. Homogeneity and stability studies in long and short term were carried out to monitor TI quality; the presence of vacuum in the samples was also verified, to ensure the efficiency of the lyophilization process. The results of the microbial testing indicated no contamination by *Salmonella* spp.; thus, the sample was appropriate to be used as matrix. The lyophilization technique, using trehalose as cryoprotectant, has proven to be effective for desiccation of TI produced. The *Salmonella* batch proved to be sufficiently homogeneous, because the microorganism was present in all analyzed flasks. The batch was held stable at -20°C (five weeks) and -70°C (26 weeks). As for the transportation stability, the batch was considered stable at 4°C (in four days). The TI produced batch in this study showed a quality level that makes it suitable to be used in PTP, to contribute to the increasing reliability of the test results from laboratories and to provide subsidies for identification of problems and troubleshooting.

KEYWORDS: Test Item; Proficiency Testing Programs; Chocolate; *Salmonella* spp; Sanitary Surveillance

RESUMO

O objetivo desse estudo foi desenvolver itens de ensaio (IE) liofilizados contendo *Salmonella* spp., em matriz chocolate, para utilização em ensaio de proficiência (EP). Foi realizada a análise microbiológica de uma amostra de chocolate granulado para verificar se estava livre do micro-organismo alvo. Para monitoramento da qualidade dos IE, realizou-se estudos de homogeneidade e estabilidade em longo e curto prazo, bem como verificou-se a presença de vácuo nas amostras garantindo a eficiência do processo de liofilização. A análise microbiológica do chocolate indicou ausência de contaminação por *Salmonella* spp., estando apto para ser utilizado como matriz. A técnica de liofilização, com uso de trealose como crioprotetor, se mostrou eficaz para dessecação dos IE produzidos. O lote produzido se apresentou suficientemente homogêneo, pois o micro-organismo estava presente em todos os frascos analisados. O lote se apresentou estável à temperatura de -20ºC (em cinco semanas) e -70ºC (em 26 semanas); na estabilidade de transporte, foi considerado estável a 4ºC (em quatro dias). O lote de IE produzido nesse estudo apresentou qualidade que o torna apto para uso em EP, o que visou contribuir para o aumento da confiabilidade dos resultados das análises dos laboratórios e propiciar subsídios para a identificação e solução de problemas.

PALAVRAS-CHAVE: Item de Ensaio; Ensaio de Proficiência; Chocolate; *Salmonella* spp; Vigilância Sanitária

INTRODUCTION

Chocolate is the predominant form of cocoa consumption, accounting for about 90% of the cocoa market[1]. Chocolate is consumed by 75% of Brazil's population, making the country the world's fourth largest consumer and the third largest producer of chocolate. In 2013, the chocolate industry produced 800,000 tons of the product[2]. The average *per capita* consumption of chocolate in Brazil is 2.8 kg per person yearly[2].

RDC n. 12, of January 2, 2001, sets the microbiological standards for food products[3]. For the assessment of the microbiological quality of chocolate, the legislation includes the absence of *Salmonella* spp. in 25 grams.

Salmonella is a type of bacteria that is widely distributed in nature. The intestinal tract of humans and animals is its main reservoir. This microorganism is eliminated in the feces and can be transmitted via fecal-oral route. Salmonella infections in humans are characterized by intestinal infections that may progress to systemic infections. Many foodstuffs have been identified as vehicles for the transmission of this pathogenic agent to humans such as eggs, pork and poultry meat, milk, chocolate, fruit and vegetables[4].

Foodborne diseases and food poisoning are serious public health problems. According to the Center for Disease Control and Prevention (CDC), every year, one of every six Americans (or 48 million people) have foodborne illnesses (FBD)[5,6]. In Brazil, from 2000 to 2014, there were 9,719 outbreaks of FBD. *Salmonella* spp. was the most prevalent agent, with 1,564 outbreaks[7].

Laboratories are essential for the sanitary control of health products. Through fiscal analysis and quality control, they intervene in scientific and technological production, product conformity assessment and risk analysis and management[8]. Laboratory quality is obtained through technical and administrative activities with careful organization and planning, from the sampling phase to the release of the results. The objective is to ensure these results are precise, accurate, traceable and, consequently, reliable[9]. To manage quality, the main standard is ISO/IEC 17.025[10]. One of the items required for the accreditation of tests by this standard is periodic participation in proficiency tests (PT) and/or interlaboratory comparisons. PTs are interlaboratory studies used as tools for external quality assessment and demonstration of the reliability of analytical results for clients, accreditation bodies and regulators. They also serve to identify failures and enable corrective or preventive action to be taken[11,12].

In the area of food microbiology, the number of PT providers is low and the costs of participation in these trials are usually very high. Therefore, the development of national test items for PT in the area of food microbiology enables greater participation of Brazilian laboratories in these tests[13].

According to ABNT ISO/IEC 17.043[11], a proficiency test item (PTI) is a sample, product, artifact, reference material, piece of equipment, standard, set of data or other piece of information used in the proficiency test.

To ensure a high level of reliability in the generated data, the composition of the PTI should be similar to that of the sample and there should be an adequate concentration of the analyte of interest[10]. Furthermore, the PTI should be sufficiently homogeneous and stable[14].

In the case of microbiological PTI, one of the most effective preservation methods for most microorganisms is freeze-drying, which consists in the removal of water vapor directly from frozen samples and vacuum drying until stable material is produced[15].

The use of cryoprotectants in this technique enables an increase in the survival of the bacteria over a long period in the matrix[16]. There are several substances that can be used as cryoprotectants, such as carbohydrates, proteins and polymers[17]. Carbohydrates, especially disaccharides like sucrose and trehalose, are the most commonly used materials[16]. These sugar molecules can replace water molecules that hydrate proteins and membranes, preventing the denaturation of proteins[18]. Trehalose has been indicated as an essential component for maintaining the viability of yeast, fungi, bacteria, insect and plant cells under stress conditions[19]. Some bacteria like *Escherichia coli* and *Salmonella* can produce endogenous trehalose to protect the cell in response to adverse growth conditions like osmotic stress and nutrient reduction, stationary phase and media with low nutrient concentration[20].

The objective of the present study was to develop PTI containing *Salmonella* spp. in a chocolate matrix, using the freeze-drying method, to be used in PT in food microbiology.

METHOD

Microbiological Analysis of Chocolate Sprinkles

A microbiological survey of *Salmonella* spp. was done according to the methodology recommended by Andrews et al.[21].

Preparation of Bacterial Suspension and Production of Test Items

We prepared a batch containing *Salmonella* with approximately 210 vials, with 1 g of chocolate sprinkles.

To prepare the suspension of *Salmonella*, a strain of *Salmonella* Enteritidis PT4 was deposited in the research collection of the Laboratory of Reference Microorganisms of INCQS/FIOCRUZ, identified as P3440, and isolated from food. A volume of 100 µL of a cryopreserved suspension was transferred to 10 mL of brain heart infusion culture medium (BHI) (Merck, Germany). After a 24-hour incubation period at 35° C ± 2° C, 500 µL of the obtained growth were seeded in 10 mL of the Luria Bertani (LB) culture medium (BD, France) with 10% NaCl and incubated at 35° C ± 2° C for 28 hours. After incubation, the culture was centrifuged and the pellet was resuspended in 0.1% peptone saline (0.1% SSP) with 100 mM trehalose, the cell concentration reading was performed on photocolorimeter (Libra S2, Biochrom, England) at a wavelength of 520 nm to reach approximately 2.0 x 10^9 CFU/mL. Dilutions in

0.1% SSP were done until we obtained a concentration of about 2.0 x 10^6 CFU/mL. Upon reaching this concentration a 1:100 dilution was performed by adding three milliliters of the bacterial suspension in 297 mL of 0.1% SSP with 100 mM trehalose, resulting in a concentration of approximately 2.0 x 10^4 CFU/mL. The final suspension was homogenized on an unheated stirring plate (Corning, USA) using a magnet for 20 minutes. With the aid of a peristaltic pump (Watson-Marlow, England), 0.5 ml of the bacterial suspension was dispensed into the 210 glass vials containing 1 g of chocolate sprinkles. The vials were then stored in an ultra-freezer at about -70° C (Thermo, USA) for 24 hours. After this period, they were removed from the freezer and subjected to a 24-hour freeze-drying cycle (Liotop, Brazil).

Vacuum Check

After the vials were removed from the freeze-drier (Liotop, Brazil), we did the vacuum check of all vials of the batch using the electric spark emitting apparatus (Tesla Coil, Brazil) to evaluate the efficiency of the freeze-drying process. The vacuum vials were sealed, identified and stored at ≤ -70° C.

Homogeneity Study

For the evaluation of homogeneity, 21 vials were randomly selected. The analysis of each vial was done in duplicate. After the vials were removed from the freezer, they were kept at room temperature for 10 minutes. The lyophils were reconstituted with 1 mL of 0.1% SSP and kept at rest for 15 minutes. A 1:10 dilution was performed and then a 1 mL volume was transferred to the surface of two sterile plates. A 10 mL volume of red neutral crystal violet bile glucose agar (VRBG) (Difco, USA) was added to each plate. After solidification, a new layer of VRBG agar was added, in order to create an anaerobic environment, conducive to the growth of *Salmonella*. The plates were incubated at 35° C ± 2° C for 24 hours. After incubation, the colonies were counted on the seeded plates. Since this is a qualitative test, the criterion used for the assessment of homogeneity was the presence or absence of *Salmonella* spp. in the PTI evaluated. The items were considered homogeneous when all the results were positive for *Salmonella* spp., that is, the plates that had growth of colonies were considered positive.

Stability Studies

Stability studies were performed in short and long duration. The short-term study was performed at temperatures of 4ºC ± 4ºC and 35ºC ± 2ºC, aiming to simulate the transportation to the laboratories participating in a PT.

The short-term study was conducted during the four-day period and evaluated according to the criteria of ABNT ISO Guide 35[22]. Eighteen vials were randomly selected from the batch stored at ≤ -70° C (reference temperature). Every day, four vials were taken from the freezer and packaged in two containers for the transportation of biological material (Concepta, Brazil), with two vials per package. During the four days of the study, the boxes were kept at different temperatures; one of the boxes at 4º C ± 4º C and the other at 35º C ± 2º C. On the fourth day (day zero), all 18 vials were analyzed at the same time, under the same conditions of analysis (day zero counting was used for both study temperatures, since the vials were not incubated). The long-term study was performed at the reference temperature (≤ -70º C) and the storage temperature (-20º C ± 4º C) and evaluated according to the ABNT ISO Guide 35[22] criteria. Every day of analysis, two vials of the batch stored at ≤ -70º C were randomly selected and analyzed according to the methodology described in the homogeneity test, in a total period of 26 weeks (at times: zero, 2, 4, 6, 8, 10, 14, 18, 22 and 26 weeks), totaling 18 vials. The analysis at the temperature of -20º C ± 4º C was performed to simulate PTI storage in laboratories. For this purpose, in a period of five weeks, two vials stored at -20º C ± 4º C were randomly selected, every seven days, and analyzed according to the methodology described in the homogeneity test, totaling 12 vials.

Statistical evaluation was performed according to the ABNT ISO Guide 35[22]. It was based on linear regression analysis of the analyte concentration value. The counts in CFU/g of each vial were converted to $\log_{10}$.

RESULTS AND DISCUSSION

Chocolate is a type of food consumed by people of all ages because, in addition to its pleasant sensory properties, it has potential benefits to human health[2]. Outbreaks of salmonellosis were related to the consumption of chocolate, making the microbiological control of this product very important[23]. Microbiological analysis of chocolate sprinkles for *Salmonella* spp. indicated that the sample did not contain the target microorganism of the study and was considered satisfactory for use as a matrix for the production of PTI.

Brant et al.[24] have explained that the absence of *Salmonella* spp. in chocolate samples may be related to the lower competition capacity of this bacterium in relation to other microorganisms of higher occurrence, such as coliforms. Due to the importance of microbiological control, the laboratories conducting these analyses should ensure accurate and reliable results, since false-positive results can lead to unnecessary waste of food and financial impact, whereas false-negative results can cause serious problems of public health[25].

In this study, we used the freeze-drying method to preserve the bacteria. This method stands out for most of the microorganisms because it preserves the original identity of the cell for long periods[26]. The vacuum inspection performed in the present study showed vacuum in 100% of the vials of the batch produced. Other studies using the freeze-drying method also showed their results of vacuum inspection, like those of Costa et al.[13], Brandão et al.[14] and Brandão et al.[16] in 96.5% of the vials produced in cheese matrix, 98.2% in cheese matrix and 94.2% in beef matrix, respectively.

The *Salmonella* spp. batch was considered sufficiently homogeneous, since the microorganism was present in all the vials that were analyzed (Table 1).

Other studies developed PTI for Food Microbiology through the freeze-drying method. They also produced homogeneous batches, like Costa et al., who produced batches for the research of *Salmonella* spp. in cheese matrix[13]. Brandão et al.[16] produced homogeneous batches for *Salmonella* spp. on raw beef and non-homogeneous batches of cooked and canned meat by freeze-drying. In milk matrix, Rosas et al.[27] obtained homogeneous batches for *Salmonella* spp. through freeze-drying and Schulten et al.[28], a homogeneous batch of *Salmonella* Typhimurium in milk powder capsules through spray-dryer.

Stability Assessment

The results regarding the study of short-term stability at temperatures of 4° C ± 4° C and 35° C ± 2° C are shown in Table 2 and Figure 1. The long-term stability study at the reference temperature (≤ -70° C) and storage temperature (-20° C ± 4° C) is shown in Table 3 and Figures 2 and 3.

After the trend analysis of the chart (Figure 1), we found that the batch was not stable enough at the temperature of 35 ± 2° C, since it was possible to clearly observe the decrease of the cellular concentration already on the second day, whereas on the third day there was absence of growth of the plated volume. This may have occurred due to the action of matrix components on the microorganisms, since other studies with other cheese and beef matrices were considered sufficiently stable at 35 ± 2° C[13,14,16,18]. At the temperature of 4 ± 4° C, since no trend was shown in the chart analysis (Figure 1), we did a linear regression test and the batch was considered sufficiently stable. This is because the 95% confidence interval covers the zero value (Table 2). In cheese matrix, Costa et al.[13] evaluated a batch of

Table 1. Result of counts in Log_{10} of the batch homogeneity test.

Test Item (Vial)	Log_{10}* x (colony forming units/gram)	
	Count 1	Count 2
2	3.17	3.03
14	3.26	3.12
21	3.21	3.12
31	3.07	2.96
43	3.03	2.96
50	3.08	2.9
60	3.13	2.72
72	3.14	2.94
79	3.11	2.98
89	2.91	2.91
101	3	2.83
108	3.2	3.14
118	3.03	3.02
130	2.86	2.72
137	2.82	2.78
147	2.87	2.84
159	2.73	2.72
166	2.87	2.81
176	2.98	2.88
188	2.76	2.75
195	2.79	2.72
Result: sufficiently homogeneous		

*log_{10} - base log 10

Table 2. Mean log score results in Log_{10} in the short-term stability study for four days at temperatures of 4° C and 35° C.

Days	Log_{10}* x (colony forming units/gram)	
	4ºC ± 4ºC	35ºC ± 2ºC
0	2.88	2.88
1	3.19	0.7
2	3.14	0
3	2.92	0
4	2.72	0
Angular coefficient	-0.0589	Not accomplished
Lower limit (95%)	-0.25654252	Not accomplished
Upper limit (95%)	0.13854252	Not accomplished

*log_{10} - base 10 log

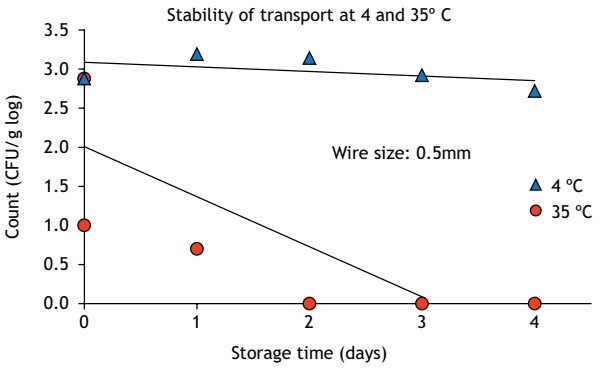

Figure 1. Variation of cell concentration from the short-term stability study for four days at temperatures of 4 ± 4° C and 35° C ± 2° C.

Table 3. Mean log score results in Log_{10} in the long-term stability study for five weeks at -20° C ± 4° C and for 26 weeks at ≤ -70° C.

Weeks	-20ºC	-70ºC
0	3.2	3.2
1	3.11	NA
2	2.92	2.89
3	3.11	NA
4	3	2.94
5	2.91	NA
6	NA	2.99
10	NA	3.21
14	NA	3.06
18	NA	3.09
22	NA	2.81
26	NA	3.13
Angular coefficient	-0.0272	-0.0004

NA: Not accomplished

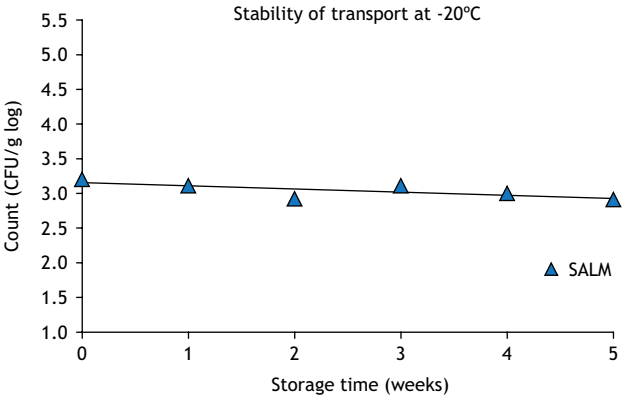

Figure 2. Variation of the cell concentration of the long-term stability study at -20° C ± 4° C for five weeks.

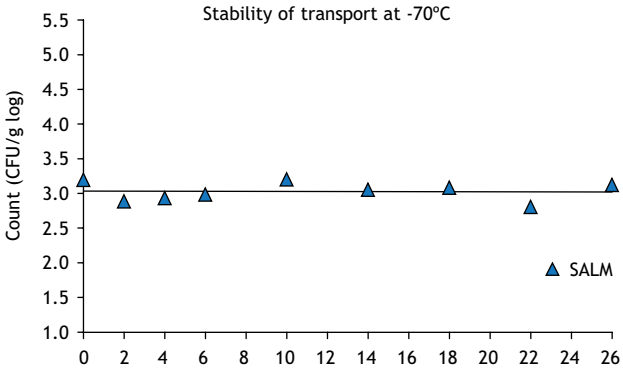

Figure 3. Variation of the cell concentration of the long-term stability study stored at ≤ -70° for 26 weeks.

PTI for *Salmonella* spp. for six days, indicating that it was stable at 4° C ± 4° C and 35° C ± 2° C. Also in cheese matrix, Brandão et al.[18] studied the stability of the PTI batches produced for coliforms over a period of six days, verifying that these were sufficiently stable at 4° C ± 4° C, 25° C ± 2° C and 35° C ± 2° C.

The PTI batch was considered sufficiently stable at -20° C ± 4° C and at ≤ -70° C according to the statistical calculations recommended by ABNT ISO Guide 35[22].

Costa et al.[13] evaluated the long-term stability of a batch of PTI for *Salmonella* spp. They identified that it was stable over the 168 days (24 weeks) of the study. Batches of PTI produced for *Salmonella* spp. and *S. aureus* also had their long-term stability studied by Rosas et al.[27]. They were found to be stable when stored for up to 3 months (approximately 13 weeks) at -20° C ± 4° C.

It is important to obtain sufficiently long-lasting PTI at temperatures above ≤ -70° C, since not all laboratories can store the PTI at that reference temperature[29]. The present study used trehalose as cryoprotectant in order to provide greater stability for the microorganisms in the matrix.

CONCLUSIONS

In view of the results we achieved, the freeze-drying technique proved efficient for desiccation of the PTI produced in a matrix of chocolate sprinkles. It also achieved satisfactory results in the vacuum control. The use of trehalose in the batch preparation proved suitable as a cryoprotectant of the PTI produced by freeze-drying.

The batch of *Salmonella* spp. was considered stable at 4° C ± 4° C for 4 days, at -20° C ± 4° C for five weeks and at ≤ -70° C for 26 weeks.

The batch of PTI in a matrix of chocolate sprinkles produced in this study presented quality levels that make it suitable for use in PT, contributing to quality management in the microbiological control of chocolate.

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.