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Validation of test portion pooling for *Salmonella* spp. detection in foods

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ABSTRACT

Pathogen monitoring programs play a crucial role in the verification of the effectiveness of implemented hygiene control measures. Sampling and testing procedures included in pathogen monitoring involve the analysis of multiple test portions where all samples must be negative for the presence of pathogens for a certain test portion size. Many food safety programs require increased testing due to the risks that a pathogen may be present. Analyzing more than one test portion could prove to be expensive and labor intensive. When more than one test portion for a specified food item is to be tested, the test portions could be combined to form a pooled test portion to reduce laboratory workload, costs of reagents and further confirmatory steps, but only when evidence is available that pooling does not affect on the number of false negative results for different matrices. This study has been performed to demonstrate the equivalence of test portion pooling for *Salmonella* detection with five different methods using cultural, ELISA and Real Time PCR technologies. Twenty-three (23) different food items including confectionary products, meal components, infant formula, pet food and powdered beverages were validated. Other complementary parameters like impact of minimum and maximum incubation time for pre-enrichment, temperature profile, pH and *Salmonella* concentration after the pre-enrichment and background flora have also been considered in the study. The results showed that pooling test portions up to 375 g for *Salmonella* detection is valid for the methods that were tested. Relative level of detection ($RLOD_{50}$) values for 22 of the food items tested were acceptable (i.e. lower than 2.5) when comparing the reference sample size (25 g) against the alternative pooled sample size (375 g), provided the enrichment broth was pre-warmed and maximum incubation time is respected.

1. Introduction

Pathogen monitoring is a mandatory element for food business operators (EC, 2004) and is part of Good Manufacturing Practices, playing a crucial role in verifying the effectiveness of implemented hygiene control measures as defined in operational prerequisite programs as well as in HACCP plans.

Regulation for microbiological criteria for foodstuffs and product-specific instructions developed by food companies define pathogen monitoring sampling plans, including sampling procedures and analytical methods. These sampling and testing procedures, in some cases, involve the analysis of multiple test portions from the same food item, of which none must be positive for a certain test portion size for pathogens like *Salmonella* (EC, 2005).

When multiple test portions for a specified food item must be analysed, the single test portions can be combined to form a pooled test portion (ISO 6579: Anonymous, 2002, Andrews and Hammack, 1998), when evidence is available that pooling does not affect the result for that particular food (ISO/FDIS 6887-1, Anonymous, 2016a). The validation of pooling for representative food items allows reduction on laboratory workload, cost of reagents and further confirmatory steps (Eijkelkamp et al., 2009).

Pooling validation studies (AFNOR Validation, n.d.) are limited to a single method and a limited number of food items. Publications evaluating *Salmonella* pooling (Mooijman et al., 2013; Pasquali et al., 2014) show limited information on the foods tested (raw poultry meat, eggs) and do not meet the ISO criteria to be applied across different food categories. Levels of different *Salmonella* serovars after pre-enrichment in BPW was also published (Margot et al., 2015) but only for milk powder food items. Publications for the validation of pooling for microorganisms like *Cronobacter* (Bennour Miled et al., 2010; Bennour Miled et al., 2011) or *Listeria monocytogenes* (Vitas et al., 2014) evaluate pre-enriched test portions and also in a limited number of matrices like powdered infant formula or smoked salmon.

This study has been conducted to demonstrate whether equivalence exists between test portion pooling for *Salmonella* detection up to 375 g and the reference 25 g test portion for 23 different food items. Comparing the limit of detection for which the probability of detection is 50% (LOD_{50}) for both sample sizes and several microbiological methods

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(cultural, ELISA and Real-Time PCR based methods), according the approach included the international validation standards ISO 16140-2 (Anonymous, 2016b).

Specifically, the aim of the study is to check the effect of pooling with regards to the level of detection. If a 25 g test portion contains a single viable *Salmonella* cell and that test sample is pooled with 14 further test samples that are free from *Salmonella*; the overall incidence of contamination is reduced. So the critical issue is whether, in the larger pre-enrichment volume, *Salmonella* will grow to the detectable level that is associated with the method.

Linked to this, is the incubation time of the pre-enrichment culture. Minimum incubation times may not provide sufficient time for an adequate recovery and growth of sub lethally damaged target microorganisms (Jarvis, 2007). For this reason, this study was conducted using both the minimum and maximum incubation time. In addition, other factors that may affect pre-enrichment step like pH changes, background flora were investigated, to assess their effect on the level of detection.

2. Materials and methods

2.1. Food types

Twenty three different food items were selected from six different food categories (according ISO 16140-2:2016 Annex A) to cover a broad range of food categories that are available worldwide from the Nestlé portfolio (see Table 1). Matrices with potential inhibitory effects due to the presence of spices, coffee, salted products were included in some categories to simulate a "worst case scenario".

2.2. Bacterial strains and inoculum preparation

Serovars of *Salmonella enterica* subsp. *enterica* characterised following the Kauffman-White Scheme were used to inoculate the food items. Serovars were selected based on the food item from which they were isolated and matched to the food items in this study (see Table 2).

Each strain was separately transferred from frozen stock culture and incubated. Following incubation, the strains used for inoculating the different matrices were submitted to a sub-lethal heat-stress by incubating at 50 °C for 10 min in a water bath to resemble the stress of the microorganisms found in the food item to be used for spiking. Degree of injuring was estimated by comparing number of colony forming units (cfu) on selective agar (XLD) versus number of cfu on non-selective agar (TSA). The degree of stress injury in the strains were between 43% and 68%, which is in line with the expected values (ISO 16140-2:2016).

Table 2
Strains used for inoculation.

Inoculating organism	Source	Food item inoculated
Salmonella Tennessee	Sesame seed	1, 2, 3
Salmonella Senftenberg	Milk powder	4, 5, 6, 7, 20
Salmonella Bareilly	Protein mix	8, 9, 10, 12, 13
Salmonella Typhimurium	Liquid egg	11
Salmonella Malmoe	Chocolate	14, 15, 16, 17
Salmonella Agona	Cereal	18, 19
Salmonella Enteritidis	ice cream	21
Salmonella Heidelberg	M&B meal	22, 23

In order to prepare the inoculum for the dry ingredients after heat stressing, the culture was centrifuged, the supernatant discarded and the bacterial pellet suspended in 10 ml of 10% non-fat dry milk (NFDM) for lyophilizing (Freeze Dryer Unit 4.5, LabConco, Kansas City, MO). After lyophilizing, the culture was ground to a fine powder with a mortar and pestle and mixed separately with 500 g each of the dry ingredients to make each seed inoculum.

Each seed inoculum was stored at 25 °C for 7 d to adapt the microorganism to the product. Samples were taken during the 7-day period for enumeration, and the inoculum level was determined by plating a serial dilution on TSA with a XLD agar overlay. The plates were incubated at 35 °C ± 1 °C for 24 h ± 2 h priori to enumeration.

2.3. Food item inoculation

An aerobic plate count determination (PCA, 30 °C ± 1 °C, 72 h ± 2 h) was performed from the uninoculated matrices to estimate the background microbial load. Additionally, a 25 g test portion of food item was screened for the presence of *Salmonella* using all methods as described below.

A total of 40 replicates were analysed for each food item. Twenty single test portions (25 g reference sample size) and twenty pooled test portions (375 g alternative sample size) were prepared. Pooled (375 g) test portions were prepared by adding 14 units of 25 g uninoculated test portions to one bulk inoculated 25 g test portion (see Fig. 1).

Table 1
Food items, types and categories included in the validation study.

Cod.	Food item	Food type	Food category
1	Noodles with Curry	Ready to (re)heat food: dry	Multicomponent foods or meal components
2	Cream of Mushroom Soup	Ready to (re)heat food: dry	Multicomponent foods or meal components
3	Fresh pasta	Ready to (re)heat food: refrigerated	Multicomponent foods or meal components
4	Soluble coffee mixtures	Dry powdered	Chocolate, bakery products and confectionary
5	Powdered malt beverages	Dry and sugared low moisture (aw <0.65)	Chocolate, bakery products and confectionary
6	Powdered chocolate beverages	Dry and sugared low moisture (aw <0.65)	Chocolate, bakery products and confectionary
7	Powdered coffee enhancers	Dry powdered	Chocolate, bakery products and confectionary
8	Growing up milk with cereals and fruits	Non-probiotic infant cereals	Infant formula and infant cereals
9	Breakfast Cereals for babies	Non-probiotic infant cereals	Infant formula and infant cereals
10	Powdered infant formula with soya	Non-probiotic infant formula	Infant formula and infant cereals
11	Liquid infant formula	Non-probiotic infant formula	Infant formula and infant cereals
12	Powder infant formula with probiotics	Probiotic infant formula	Infant formula and infant cereals
13	Growing up milk	Non-probiotic infant formula	Infant formula and infant cereals
14	Chocolate tablets	Dry and sugared low moisture (aw <0.85)	Chocolate, bakery products and confectionary
15	Dark chocolate drops	Dry powdered	Chocolate, bakery products and confectionary
16	Mint and chocolate tablets	Dry powdered	Chocolate, bakery products and confectionary
17	Sweet biscuits	Dry and sugared low moisture (aw <0.65)	Chocolate, bakery products and confectionary
18	Breakfast cereals	Dried cereals	Dried cereals, fruits, nuts, seeds and vegetables
19	Chocolate bar with peanut butter flavour	Dry and sugared low moisture (aw <0.65)	Chocolate, bakery products and confectionary
20	Clinical nutrition formula	Ready to (re)heat food: dry	Multicomponent foods or meal components
21	Vanilla ice cream	Pasteurized dairy products	Heat-processed milk and dairy products
22	Dry dog pet food	Dry food (aw ≤0.7)	Pet food and animal feed
23	Dry cat pet food	Dry food (aw ≤0.7)	Pet food and animal feed