SAMPLE PREPARATION FOR MICROBIOLOGICAL ANALYSES: CHALLENGES AND IMPROVEMENTS IN THE FOOD INDUSTRY

David Tomás Fornés (david.tomasfornes@rdls.nestle.com)

Microbial and Molecular Analytics. Food Safety and Quality Department.

Nestlé Research Center. Lausanne (Switzerland)

Introduction

Sample preparation protocols must be applied in routine laboratories to assure the effectiveness of food microbiological analysis and pathogen monitoring for food safety and consumer protection.

Microbiological methods are usually considered as horizontal methods applicable to "all foods" including raw materials, finished food and feed products and environmental samples. However, different sample preparation instructions must be applied to different foods. Specific sample preparation protocols should be also adapted for some finish products (depending on the food recipe), raw materials and ingredients (e.g for ingredients with inhibitory properties, probiotics, etc...).

Sample preparation is also an horizontal step for different analytical methods regardless of the detection technology used in subsequent steps (cultural, molecular, immunological...). Advances and improvements have been focused in detection technologies but the "upstream" sample preparation step has not been always adequately developed (Brehm-Stecher et al., 2009).

Sampling plans can be also improved in some cases without increasing the analytical workload through the sample preparation protocols, for example, through the analysis of multiple test portions at the same time, provided validation/verification studies demonstrate equivalent results.

Collaboration between food industries, diagnostic companies and standardization bodies is crucial to apply the right sample preparation protocols and to assure the good performance of the methods and the quality of the whole food chain.

Sample preparation protocols

According ISO 17025, "*The laboratory shall use appropriate procedures for all tests (...) including preparation of items to be tested*".

Standard sample preparation instructions can be found in the series of standards ISO 6887: Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination:

- Part 1: General rules for the preparation of the initial suspension and decimal dilutions.
- Part 2: Specific rules for the preparation of meat and meat products.
- Part 3: Specific rules for the preparation of fish and fishery products.
- Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products.
- Part 5: Specific rules for the preparation of milk and milk products.
- Part 6: Specific rules for the preparation of samples taken at the primary production stage.

These standards include:

- Diluents for general uses (peptone salt solution, buffered peptone water) and for specific uses.
- Preparation of samples for frozen products, hard and dry products, liquid and heterogeneous products.
- General procedures for acidic products and high fat foods.
- Specific protocols for some matrices.

In some cases, standard reference protocols are not available or are not "fit-for-purpose" for specific matrices. In this case, new sample preparation protocols must be developed, evaluated and validated (if needed) by the laboratory. Alternative methods also contain in some cases specific sample preparation protocols. These protocols must be validated by the supplier and followed when using the by the laboratory.

Evaluation of sample preparation protocols.

The development of reference and alternative methods include the evaluation of a limited number of different food matrices (e.g. from 5 different food categories according ISO 16410). Both reference and alternative methods mention that "*because of the large variety of food and feed products, these horizontal methods may not be appropriate in every detail for certain products*".

It is important to highlight that, even with the most sensible and sophisticated analytical technology, an incorrect sample preparation can produce false negative results and samples contaminated with pathogens can be released with a high impact in public health. Food industries and laboratories have the final responsibility to confirm that method is appropriate for the food matrices analysed.

For this reason, evaluation of the sample preparation protocol for specific matrices should be considered:

- During the development phase. Studies should be designed to "challenge" the method, and should include samples inoculated with stressed strains usually isolated from food products or environment. The advantage to include challenging samples in this early stage is to detect limitations in the scope of the method and to develop specific sample preparation protocols if needed.
- In the validation phase. Specific sample preparation instructions should be tested and validated. Validation should be done following international recognized protocols (e.g. ISO 16140).
- During the use in routine analysis. Due to the limited number of matrices tested during the development and validation phase, performance of the sample preparation protocol must be evaluated for matrices routinely analysed. The protocol to evaluate matrices should be simple to facilitate the implementation in the routine laboratories.

For example, during routine analysis, matrices with inhibitory effects (matrices of concern) should be identified and if needed, alternative sample preparation protocols must be evaluated and implemented for these matrices.

Sample preparation assessment studies can be applied when sample preparation needs to be adapted for food items having an impact on the method performance. For example, this can result from inhibition of microorganisms growth or from interference with the method principle. Figure 1 shows some simple protocols to determine the right dilution factor for samples with inhibitory effects.

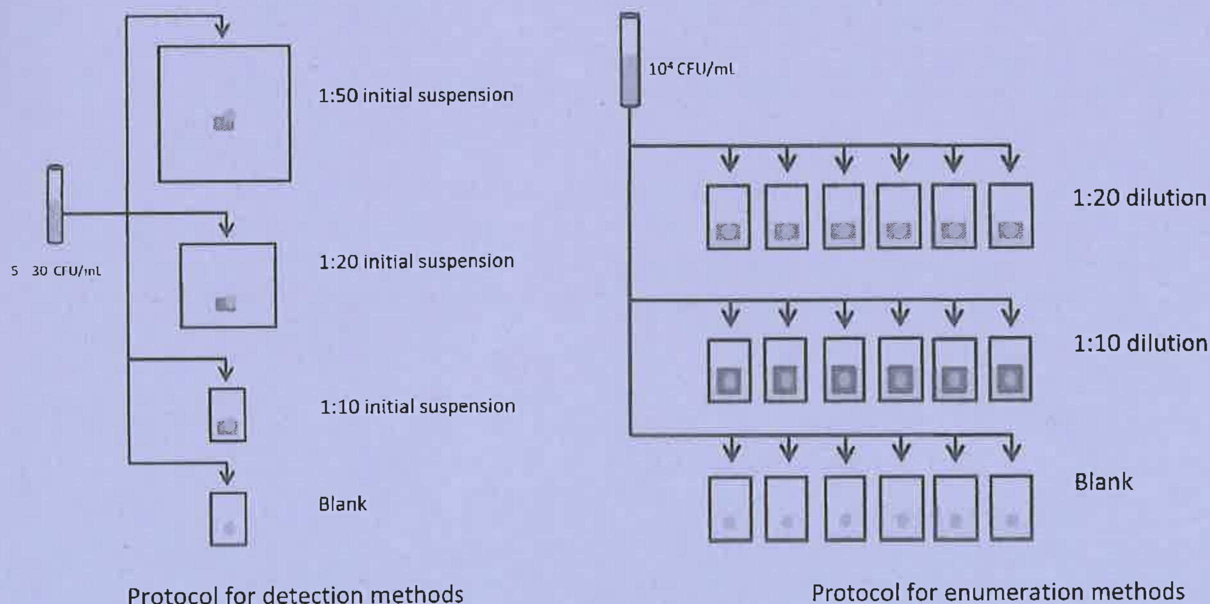


Fig. 1: Dilution assessment studies for inhibitory matrices

For detection methods, the minimum dilution factor for the matrix tested must be lower dilution with positive values.

For enumeration methods, SD_r and Bias are estimated for each series of dilution and compared with the blank series. Dilution can be accepted if tolerance interval is within acceptability limits corresponding to the accuracy profile limits according ISO 16140-2.

Dilution factor for inhibitory matrices

Protocols for sample preparation assessment can help to improve the sample preparation and to adapt the dilution factor for matrices with bacteriostatic or bactericidal properties detected in natural compounds.

Studies performed in Nestlé laboratories showed the need to include high dilution factors to neutralize the inhibitory compounds and obtain a reliable result for some matrices (see table 1).

	Dilution factor			Dilution factor	
Product	***Salmonella* detection**	**Enumeration**	**Product**	***Salmonella* detection**	**Enumeration**
Amaretto	1:100	1:200	Ground mustard flour	1:500	--
Black tea	1:100	--	Laurel (Bay leaf)	1:20	--
Café mocha	1:20	1:100	Lemon powder	--	1:1000
Chocol. mint powder	1:200	1:100	Mint powder	1:2000	--
Cinnamon	1:200 to 1:500	1:100 to 1:200	Ground rosemary	1:20	--
Ground clove	1:1500	--	Vanilla + chocolate	1:100	--
Coriander oil	1:1000	--	Vanilla + caramel	1:100 to 1:500	--
Crème brulee	1:100	--	Vanilla nut	1:100	1:10
Garlic powder	1:20 (BPW + K_2SO_3)	--	Vanilla powder	1:20 to 1:100	1:10
Green tea	1:20 to 1:100	--	Vanillin	1:2000 to 1:3000	1:100

Table 1: Examples of dilutions in different natural inhibitory products.

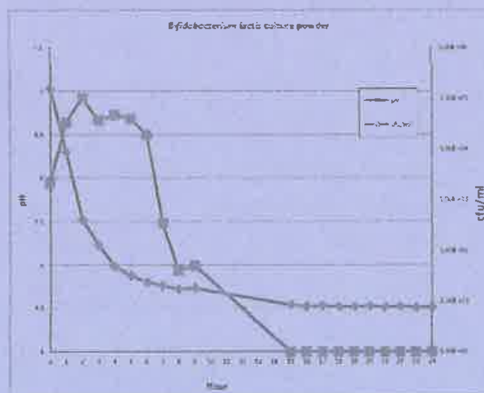
For these matrices, the use of high dilution factor to neutralize inhibitory effects is an easy solution, but is expensive and not user friendly. Improvements on sample preparation for these matrices are needed to reduce the dilution factor minimizing the risk of false negatives.

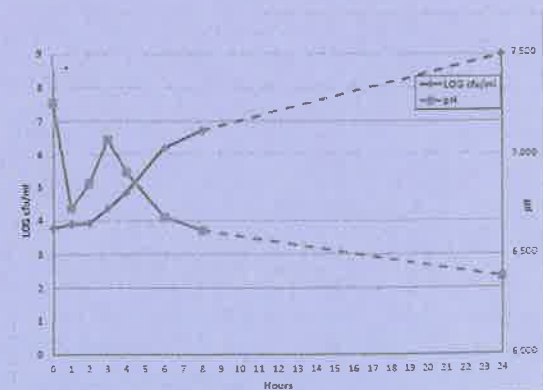
New diluents for pre-enrichment

In some cases, microbiological sample composition has a direct impact in the performance of the analytical methods because growth and detection of the target microorganisms can be affected, giving inaccurate results (like false negative results).

False negatives can also be obtained for pathogen detection in food samples with high levels of background flora when using conventional sample preparation protocols. One of the most challenging matrices for food industry are products containing probiotics.

The current procedures to detect pathogens like *Salmonella*, STEC or *Cronobacter* in products with high concentration of probiotics (>10^9 cfu/g) requires the application of a very high dilution factor to avoid false negatives (up to 1:800). Improvements in pre-enrichment broths to prevent acidification and increasing the buffer capacity of buffered peptone water in products containing high concentration of probiotics (>10^{11} cfu/g) enhance the growth of the target pathogens and allows a good limit of detection with lower dilution factors (see Fig. 2).





Pre-enrichment with conventional BPW　　　　Pre-enrichment with improved BPW

Fig. 2: *Salmonella* growth and pH during pre-enrichment of probiotic culture powder.

The use of improved pre-enrichment broths has been evaluated also to use only one-broth for the analysis of multiple pathogens (like *Salmonella*, STEC and *Cronobacter*) simultaneously. BPW supplemented with an available iron source and sodium pyruvate, along with low levels of 8-hydroxyquinoline and sodium deoxycholate (BPW-S) improved the recovery of stressed Gram-negative foodborne pathogens from dry products. However, a one-broth enrichment strategy based on BPW variants tested is not recommended for produce with a high level of Gram-negative background flora. (Margot et al., 2015).

Analysing multiple samples.

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. For example, according European Regulation 2073/2005 on microbiological criteria for foodstuffs, for milk powder 5 samples shall be tested with absence in 25g.

In this cases test portions could be combined to form a pooled test portion to reduce laboratory workload, costs of reagents and further confirmatory steps. However, pooling can be performed only when evidence is available than pooling does not affect the result for that particular food. This means that equivalent results shall be obtained between "normal analysis" and "pooled samples". (ISO 6579:2002; Bacteriological Analytical Manual FDA; ISO/WD 6887-1).

Test portions could be combined in the initial suspension (test portion pooling) or in the secondary enrichment broth ((pre-) enriched portion pooling). For test portion pooling (see fig. 3), samples from the same food item where the complete mixture is the pooled test portion are taken as a whole for examination in the laboratory.

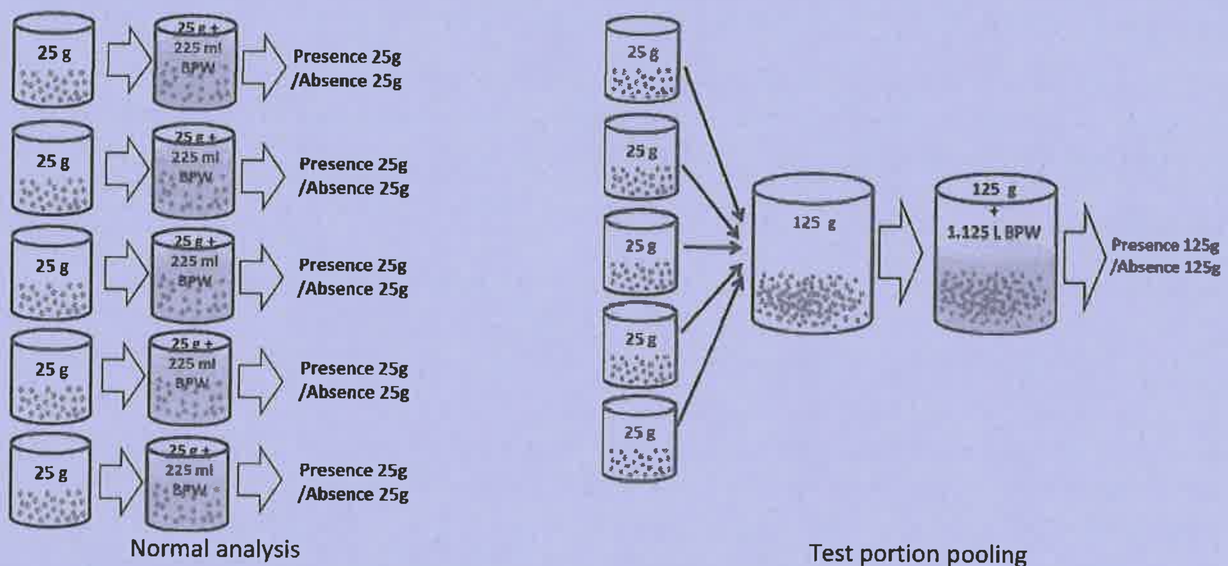


Fig. 3.- Example of pooling during sample preparation.

Protocols included in ISO 16140-2 can be performed to validate pooling for a selected method and a broad range of matrices. Using this approach, pooled samples can be considered the "alternative" method to be compared with the "reference" method with the normal already validated sample size.

Validation studies must include different stressed strains and food matrices inoculated at low levels to obtain fractional positive results. With this fractional positive results, level of detection for which 50% of the tests are positive (LOD_{50}) for pooled and simple size samples and must be estimated. Relative level of detection ($RLOD_{50}$) calculated from both LOD_{50} will give the comparison between pooled and "normal" sample size. Values of $RLOD_{50}$<2,5 are considered as equivalent (unpaired studies).

Different validation studies performed with alternative and reference *Salmonella* methods showed LOD_{50} values close to 1 CFU up to 375g samples (LOD_{50} = 0.3-1.1 CFU/250 g; LOD_{50} = 0.2-0.9 CFU / 375 g and LOD_{50} = 0.5-1.3 CFU / 375 g). Other studies reported that the sensitivity for Salmonella detection according ISO 6579 and a Real Time PCR method, was not affected by the dilution of one positive egg with 10 negative eggs (Pasquali et al., 2014). However, in other reference (Mooijman K. et al., 2013) a reduction in the number of Salmonella positives for pooled test portions versus single test portions in poultry meat was detected, but no references to LOD_{50} were provided.

In the case of *Listeria*, pooling of RTE meat and poultry products (frankfurters, deli turkey and deli ham) worked only up to 125 g sample size with culture method and PCR, but not with ELISA. In the same study, it has also been shown that pooling up to 375 g

did not work with any of the methods (Curiale, 2000). In another study by Becker et al., 2012, it has been showed that vegetable type (broccoli, tomato, onion and potato), sample size (125 g or 375 g) and methodology used (FDA BAM; ELFA or PCR) will affect the ability to detect *L. monocytogenes* from pooled test portions of frozen vegetables.

Due to these results and because of the large variety of food products, additional verification studies must be performed at quality control laboratories using test portions pooling to determine whether number of pooled test portions, food item, method selection, and specific laboratory conditions (incubators, culture media supplier…) are suitable.

These verification studies can be performed estimating and comparing LOD_{50} for both sample sizes or with a simple design doing replicates with a low level of inoculum (for example between 5-30 CFU/sample).

In addition, some practices like the use of pre-warmed enrichment broth or to respect maximum incubation time can reduce the risk of false negative results.

Conclusions

Sample preparation has a direct impact on the final results as well as in the analytical cost and workload and should be considered as a critical step for food microbiological analytical methods.

Horizontal methods used for microbiological food analysis should be applied with specific adequate sample preparation protocols to guarantee the results obtained. Specific protocols for sample preparation must be evaluated and adapted during the development, validation and application in routine laboratories.

Despite the high number of new rapid and alternative methods and technologies, sample preparation technologies and protocols have not been always adapted to the industrial needs and more research, development and improved protocols are needed to implement properly this critical step in routine laboratories.

Acknowledgments

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