

## Production of External Reference Materials in Food Microbiology

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**ABSTRACT**

*In order to help laboratories to face with their regulatory requirements, BIPEA (Bureau Interprofessionnel d'Études Analytiques) developed a production of external reference materials (ERM) for microbiology in food. These samples should allow laboratories to check the trueness of their results at any time, outside the regular proficiency-testing schemes (PTS).*

*For this purpose, stable and homogeneous samples of minced meat spiked with various bacterial strains are produced by BIPEA: Escherichia coli, Clostridium perfringens, Bacillus cereus, Staphylococcus aureus and Listeria monocytogenes, at levels between 10<sup>2</sup> to 10<sup>4</sup> microorganisms per gram. Controls were performed by analysing samples all along the production process, at the beginning of the study and regularly during a 6-month period.*

*The homogeneity was checked by calculating coefficients of variation, which were inferior to 25 % for all the analytical parameters. The stability was characterized by comparing means of three samples to the mean obtained at the beginning of the study. The samples produced were thus considered as being sufficiently homogeneous and stable to meet the ERM requirements: the results of enumeration of the different micro-organisms present in the minced meat after 6 months of storage at (-24 ± 6)°C showed a good stability, with a maximum deviation less than 0.5 log (CFU/g: colony forming unity per gram).*

**Keywords**

Stability, Homogeneity, External reference materials, Proficiency-testing schemes, Microbiology.

**Introduction**

The development of microbiological reference materials has a long history. In 1965 Kampelmacher was already using artificially inoculated minced meat samples in comparative studies [1] as a standard sample for the evaluation of the performance of the *Salmonella* detection procedure in various laboratories. Later *Salmonella* was dried onto milk powder which resulted in the production of a reference material (gelatin capsules containing spray dried milk artificially contaminated with *Salmonella*) for use in laboratories methods trials [2]. The development of other reference materials and their evaluation was initiated in 1986 when the first contract for water microbiology with the former European Communities Bureau of Reference (BCR) was agreed. A contract

for the development of reference materials for food microbiology followed in 1987. These reference materials are prepared from spray dried artificially contaminated milk. The initial spray dried powder (called highly contaminated milk powder or HCMP) is mixed with sterile milk powder to give the desired level of contamination and the mixed powder is subsequently packed into gelatin capsules.

Many regular proficiency-testing schemes offer real sample in microbiology but do not allow laboratories to verify the trueness of their results at any time. This is why BIPEA has developed new ERMs, particularly in the field of food microbiology.

The external reference materials, of BIPEA are a production surplus, consider as stable and homogenous enough and for which an assigned value has been estimated. The ERMs could have several uses: i) qualification of operators for laboratories accredited

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according to the requirements of ISO standard IEC 17025 [3], ii) validation of alternative methods according to ISO 16140 standard [4] or internal methods, iii) determination of reproducibility and repeatability of reference methods [5-7]. This article describes the different stages of the implementation of ERMs in minced meat matrix spiked with several bacterial strains, after storage at  $(-24 \pm 6)^{\circ}\text{C}$ .

### Experimental design

Sterile minced meat, divided into 25 grams samples, was spiked with several bacterial strains at given concentrations. Three of these samples were analyzed each month over a period of 6 months. To be used as ERMs, it is required to demonstrate the homogeneity between these samples and their stability over time, after storage at  $(-24 \pm 6)^{\circ}\text{C}$ .

## Materials and Methods

### Materials

Five bacterial strains were used for the contamination of minced meat matrix, *Escherichia coli*, *Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*. In the case of *Clostridium perfringens* and *Bacillus cereus*, these strains were used in their sporulated form in order to increase their stability.

### Methods

The minced meat was divided into 25 grams samples in sterile stomacher bags. These bags were then sealed and sterilized by ionization. The aim of this ionization is to sanitize microbiologically the matrix in order to avoid interactions between the target microorganisms and the endogenous flora.

Each bag was then inoculated individually from a pool of bacterial strains suspended in specific diluent. The target concentrations of the spiking suspensions were obtained by carrying out dilutions from the primary suspensions.

The samples were spiked at levels close to 103 CFU/g for all bacterial species tested except for *Clostridium perfringens* which was inoculated at a rate of about 2.102 CFU/g and *Bacillus cereus* at a rate of 5.102 CFU/g. The concentrations used reflect the rates generally observed in this type of matrix.

The samples were analyzed at different time intervals (at D0 and then each month during six months) according to the reference methods for each analytical parameter (Total viable count, *Escherichia coli*, Coliforms, Enterobacteria, *Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*).

## Results and Discussions

### Rate of recovery

At D0, which corresponds to the day of preparation of the spiked samples, the data show that the recovery rate reaches 100% for all strains species tested except for *Listeria monocytogenes* strains, which recovered at a 90% rate. These results show that

the recovery rate for all microorganisms is satisfactory to start the trials (Table 1).

### Coefficient of variation – Homogeneity

The coefficients of variation (CVs) [8] in the study show the reliability that can be attributed to the assigned values which are compared to assess the stability of the samples. From an analytical point of view, the coefficients of variation allow to sorting out which analysis is under control and which raises difficulties.

The coefficients of variation observed (table 1) range from 0.5% to 22.2%. The inter-samples variation is therefore sufficiently low (CVs less than 25%) to conclude that these samples are homogeneous regarding to the variability of the analytical methods and the matrix composition.

### Stability

One of the aims of this study is, in addition to the development of homogeneous samples, to determine over which period of time these samples can remain stable. Since microorganisms are living organisms, their concentration can be expected to evolve over time. It may increase, if the elements necessary for their multiplication are present in the matrix used with favorable conditions in terms of temperature and aero philia, or it may on the contrary decrease due to the death of these microorganisms [9]. However, some techniques such as freezing can preserve their concentration [10]. Indeed, freezing has the effect of slowing down, or even almost halting, the processes allowing them to live and develop without causing their death. Therefore, it was decided to store the samples at  $(-24 \pm 6)^{\circ}\text{C}$ , in order to stabilize as much as possible, the concentration of the bacterial strains within the minced meat.

The results of the different analyses show that the difference of enumeration of microorganisms between the analysis of these samples at D0 and their analysis after several months does not vary by more than 0.5 log. This data confirms that the enumeration of microorganisms in minced meat is stable after six months of freezing.

## Conclusions

The results of the counting of the various microorganisms present in the minced meat after 6 months of storage at  $(-24 \pm 6)^{\circ}\text{C}$  show satisfactory stability of the samples with a difference of extreme values less than 0.5 log for all the analytical parameters. The homogeneity of the samples is also satisfactory with coefficients of variation below 25% for all the analytical parameters.

The results of homogeneity and stability of the samples are therefore in accordance with those expected: satisfactory homogeneity and stability for 6 months at  $(-24 \pm 6)^{\circ}\text{C}$ .

This study enabled the implementation of a test production for trustworthy ERM's storage and shipping conditions.

Eight analytical parameters can be studied at any time by the laboratories to check their performance: total viable count,

|                                |                                 | D0      | M1      | M2      | M3      | M4      | M5      | M6      |
|--------------------------------|---------------------------------|---------|---------|---------|---------|---------|---------|---------|
| <b>Total viable Count</b>      | mean concentration [CFU/g]      | 3.6E+03 | 3.2E+03 | 2.7E+03 | 2.5E+03 | 1.8E+03 | 1.7E+03 | 1.6E+03 |
|                                | mean concentration (log[CFU/g]) | 3.56    | 3.51    | 3.43    | 3.40    | 3.26    | 3.23    | 3.20    |
|                                | Coefficient of variation (%)    | 3.9     | 4.4     | 15.3    | 11.6    | 14.8    | 0.5     | 2.2     |
| <i>Escherichia coli</i>        | mean concentration [CFU/g]      | 1.1E+03 | 9.1E+02 | 7.5E+02 | 6.7E+02 | 5.7E+02 | 4.4E+02 | 4.4E+02 |
|                                | mean concentration (log[CFU/g]) | 3.04    | 2.96    | 2.88    | 2.83    | 2.76    | 2.64    | 2.64    |
|                                | Coefficient of variation (%)    | 7.5     | 5.7     | 5.6     | 7.5     | 9.7     | 5.2     | 12.5    |
| <b>Coliforms</b>               | mean concentration [CFU/g]      | 1.0E+03 | 8.8E+02 | 8.2E+02 | 6.9E+02 | 5.1E+02 | 4.3E+02 | 3.5E+02 |
|                                | mean concentration (log[CFU/g]) | 3.00    | 2.94    | 2.91    | 2.84    | 2.71    | 2.63    | 2.54    |
|                                | Coefficient of variation (%)    | 8.4     | 6.4     | 7.1     | 3.8     | 14.3    | 5.6     | 11.8    |
| <b>Enterobacteria</b>          | mean concentration [CFU/g]      | 1.0E+03 | 8.5E+02 | 8.1E+02 | 7.5E+02 | 4.8E+02 | 4.5E+02 | 3.6E+02 |
|                                | mean concentration (log[CFU/g]) | 3.00    | 2.93    | 2.91    | 2.88    | 2.68    | 2.65    | 2.56    |
|                                | Coefficient of variation (%)    | 5.6     | 3.2     | 8.5     | 9.7     | 14.7    | 5.4     | 7.8     |
| <i>Clostridium perfringens</i> | mean concentration [CFU/g]      | 2.0E+02 | 2.0E+02 | 2.2E+02 | 2.0E+02 | 2.2E+02 | 2.0E+02 | 1.8E+02 |
|                                | mean concentration (log[CFU/g]) | 2.30    | 2.30    | 2.34    | 2.30    | 2.34    | 2.30    | 2.26    |
|                                | Coefficient of variation (%)    | 5.3     | 9.1     | 16.6    | 10.3    | 8.3     | 11.3    | 7.9     |
| <i>Bacillus cereus</i>         | mean concentration [CFU/g]      | 5.1E+02 | 4.9E+02 | 4.0E+02 | 3.6E+02 | 3.1E+02 | 2.7E+02 | 2.4E+02 |
|                                | mean concentration (log[CFU/g]) | 2.71    | 2.69    | 2.60    | 2.56    | 2.49    | 2.43    | 2.38    |
|                                | Coefficient of variation (%)    | 4.1     | 9.4     | 2.3     | 3.9     | 1.6     | 3.0     | 7.7     |
| <i>Staphylococcus aureus</i>   | mean concentration [CFU/g]      | 1.1E+03 | 9.4E+02 | 7.4E+02 | 6.6E+02 | 5.2E+02 | 4.9E+02 | 5.0E+02 |
|                                | mean concentration (log[CFU/g]) | 3.04    | 2.97    | 2.87    | 2.82    | 2.72    | 2.69    | 2.70    |
|                                | Coefficient of variation (%)    | 4.8     | 7.7     | 7.8     | 3.5     | 21.5    | 6.7     | 9.3     |
| <i>Listeria monocytogenes</i>  | mean concentration [CFU/g]      | 9.3E+02 | 8.7E+02 | 6.9E+02 | 6.9E+02 | 5.0E+02 | 5.0E+02 | 5.0E+02 |
|                                | mean concentration (log[CFU/g]) | 2.97    | 2.94    | 2.84    | 2.84    | 2.70    | 2.70    | 2.70    |
|                                |                                 | 2.9     | 5.8     | 6.0     | 8.9     | 22.2    | 15.0    | 8.5     |

**Table 1:** Results obtained from the analysis of samples of minced meat contaminated with several bacterial strains over a period of 6 months after storage at  $(-24 \pm 6)^\circ\text{C}$ .

*Escherichia coli*, Coliforms, Enterobacteria, *Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*. The use of ERM is one of the important tool for the monitoring of quality assurance and should for example allow methods validation and operators' qualification using a real food matrix.

## References

- Kampelmacher EH. Comparative studies into the isolation of *Salmonella* from minced meat in five laboratories. Zbl. Bakt. I. Abt. Orig. 1967; 204: 100-111.
- Beckers HJ, van Leusden FM, Meijssen MJM, et al. Reference material for the evaluation of the standard method for the detection of *salmonellas* in foods and feeding stuffs. J. Appl. Bacteriol. 1985; 59: 507-512.
- International standard: ISO/IEC 17025:2005 - General requirements for the competence of testing and calibration laboratories.
- International standard: ISO 16140-2:2016 - Microbiology of the food chain -- Method validation -- Part 2: Protocol for the validation of alternative proprietary methods against a reference method.
- International standard: ISO 5725-1:1994 - Accuracy trueness and precision of measurement methods and results -- Part 1: General principles and definitions.
- International standard: ISO 5725-2:1994 - Accuracy trueness and precision of measurement methods and results -- Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method.
- International standard: ISO 5725-6:1994 - Accuracy trueness and precision of measurement methods and results -- Part 6: Use in practice of accuracy values
- Reed GF, Lynn F, Meade BD. Use of coefficient of variation in assessing variability of quantitative assays. Clin. Diagn. Lab. Immunol. 2002; 9: 1235-1239.
- Mihoub F, Mistou MY, Guillot A, et al. Cold adaptation of *Escherichia coli*: microbiological and proteomic approaches. Int. J. Food. Microbiol. 2003; 89: 171-184.
- El-Kest SE, Marth EH. Injury and death of frozen *Listeria monocytogenes* as affected by glycerol and milk components. J. Dairy Sci. 1991; 74: 1201-1208.