
Method Validation Report for *Clostridium perfringens* as per ISO 7937: 2004

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ABSTRACT

Clostridium perfringens food poisoning ranks among the most common gastrointestinal diseases in developed countries. The method for the enumeration of *Clostridium perfringens* in food samples as per ISO 7937:2004, was validated for a project involving the use of the standard test method in commercial laboratories across different regions. The study was performed to attend precision in terms of linearity, repeatability, reproducibility, accuracy, and limit of detection. Samples were artificially contaminated (spiked) to achieve desired concentration of bacterial cells for validating the method. The precision depended on the type of the samples being analysed.



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INTRODUCTION

One serious threat to public health in both developed and developing countries is the microbial contamination of food. This problem poses a great challenge and consequently has economic implications. Causes of microbial contamination are diverse and these may be natural, environmental, or technological. The microbiological quality of most ready to-eat foods is of great significance to human health because they require minimal or no processing when consumed. ^[1, 6]



Today, many methods exist that are used to assess the microbiological safety and quality of raw materials and finished food products and to monitor the microbiological status of manufacturing processes. The developers, end-users and public health authorities need a reliable common protocol for the validation of such alternative methods. [2, 3, 5]

A test method must be shown to be fit for purpose so that a facility's customers can have confidence in the results produced by its application. Method validation provides objective evidence that a method is fit for purpose, meaning that the requirements for a specific intended use are fulfilled. Validation is always a balance between costs, risks, and technical possibilities. The extent of validation required will depend on the status of the method under consideration and the needs relating to its intended application. [2, 3, *Clostridium perfringens* food poisoning ranks among the most common gastrointestinal diseases in developed countries. *Clostridium perfringens* is a Gram-positive, strict anaerobic, sporulating bacterium belonging to the anaerobic sulphite-reducing bacteria. This ubiquitous species is an important food pathogen, displaying gastro-enteritis and enteric necrosis by the production of a complex cocktail of exotoxins [1, 5, 6]

The ISO 7937:2004 International Standard describes a horizontal method for the enumeration of viable

Clostridium perfringens. This test method is applicable to products intended for human consumption and the feeding of animals, and environmental samples in food production and food handling. [5]

For the validation the method was challenged with two different food matrices. For this study raw chicken breast and Milk sample were used. In addition, a reference material was used to identify any serious errors in any of the participant's performance and to determine the maximum precision possible as the ISO 7937:2004 is quantitative method. The food samples were artificially inoculated to achieve desired inoculum levels and homogeneity. These materials were tested extensively by four different trained analyst's and the data from the testing was used to calculate the following in relation with the food type and inoculum level –

1. Linearity- which is the property of a mathematical relationship or function that can be graphically represented as a straight line. Therefore, linearity uncertainty would be the uncertainty associated with non-linear behaviour observed across the range of an assumed linear function.
2. Repeatability is defined as the ability of the analytical procedure to obtain test results within a given range of accuracy and precision when the same sample is repeated in accordance with the same method protocol. Chicken breast and Milk samples were spiked with known concentration of culture suspension and the same sample was repeated 10 times, to calculate the Mean, SD and %RSD.



3. Reproducibility is defined as the ability of the analytical procedure to obtain test results within a given range of accuracy and precision when the same sample is performed by different analyst in accordance with the same method protocol. Chicken Breast and Milk samples were spiked with known concentration of bacterial cell suspension and the same sample was repeated by four different analysts', to calculate the Mean, SD and %RSD.

Accuracy is the closeness of agreement between a test result and accepted reference value. Accuracy of the tested sample was determined by using the following formulae –
(Test Result/Expected Value) *100

EXPERIMENTAL METHODS

Preparation of Stock Culture.

1 lyophilized pellet of *Clostridium perfringens* ATCC 13124 cultures provided by Microbiologic's is added to sterile phosphate buffer saline which was pre-incubated at 35°C for 30 minutes. This suspension is incubated at 35°C for 24 hrs under anaerobic conditions. Prepare a serial dilution of the culture to verify the known concentration.

Spiking of samples.

Sterile chicken breast and sterile milk samples were spiked with the cell suspension which was prepared in 2.1.

The samples were spiked with different concentration of cell suspension ranging from 10^{-5} to 10^{-7} CFU/ml.

Method Validation

To perform the method validation different parameters were conducted which were-Linearity, Repeatability, Reproducibility, Accuracy and Limit of Detection.

RESULTS

Stock Culture.

Table 1- Stock Culture- *Clostridium perfringens* ATCC 13124

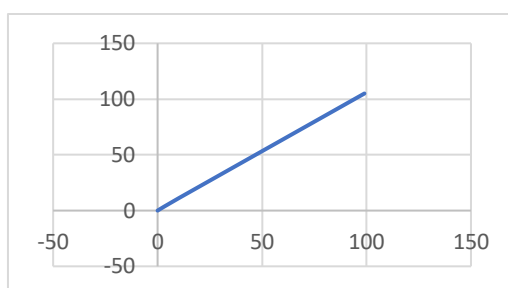
Dilution	Sterile Distilled Water										Mean	SD	% RSD
10^{-5}	126	12	10	11	12	10	13	11	11	99	115.9	10.35428	8.9338049
10^{-6}	10	9	9	10	11	9	12	10	9	8	97.0	1.16	1.1953627
10^{-7}	1	1	0	2	0	1	1	2	0	1	9.0	0.738	8.1984976
10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0	0

Linearity

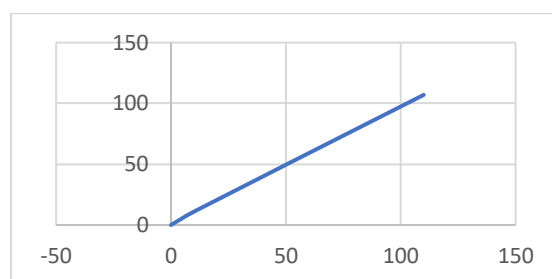
Table 2 – Linearity

Sample s Dilution	Milk					Chicken Breast				
	Values		Mean	SD	% RSD	Values		Mean	SD	% RSD
10 ⁻⁵	73	71	72	1.41	2.32	110	107	108.5	2.12	1.95
10 ⁻⁶	8	7	7.5	0.70	6.73	11	12	11.5	0.70	6.15
10 ⁻⁷	0	0	0	0	0	0	0	0	0	0

Graph 1a – Linearity – *Clostridium perfringens* Chicken Breast Sample



Graph 1b - Linearity – *Clostridium perfringens* Milk Sample



Repeatability

The results for the analysis are demonstrated in the tables which are given below.

Table 3a – Repeatability for Chicken Breast Analyst 1

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	111	119	115	115	110	116	114	119	110	119	114.8	3.5839147	3.1218769
10 ⁻⁶	11	10	11	10	11	10	11	11	11	10	10.6	0.5163978	4.8716772

Table 3b – Repeatability for Chicken Breast Analyst 2

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	11 3	11 9	11 7	11 0	11 5	11 0	11 5	11 4	11 3	11 8	114.4	3.062315 8	2.676849 4
10 ⁻⁶	10	10	10	11	10	11	11	11	10	10	10.4	0.516397 8	4.965363 3

Table 3c – Repeatability for Chicken Breast Analyst 3

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	11 3	11 1	11 7	11 2	11 0	11 5	11 2	11 0	11 9	11 6	113.5	3.100179 2	2.731435 4
10 ⁻⁶	10	11	10	10	11	11	11	10	11	10	10.5	0.527046 3	5.019488 3

Table 3d – Repeatability for Chicken Breast Analyst 4

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	11 9	11 3	11 2	11 5	11 2	11 7	11 3	11 0	11 1	11 6	113.8	2.859681 4	2.512901 1
10 ⁻⁶	8	10	10	11	10	10	10	9	10	11	9.9	0.875595	8.844394 3

Table 3a – Repeatability for Milk Analyst 1

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	69	68	65	66	70	69	66	70	69	68	68	1.76	2.59
10 ⁻⁶	6	6	7	6	6	6	6	7	6	7	6.3	0.48	7.67

Table 3b – Repeatability for Milk Analyst 2

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	66	69	70	65	69	70	66	68	67	69	67.9	1.79	2.63
10 ⁻⁶	6	6	7	6	6	7	7	6	6	7	6.4	0.51	8.07

Table 3c – Repeatability for Milk Analyst 3

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	70	71	69	70	68	65	66	69	71	69	68.8	1.99	2.89
10 ⁻⁶	7	7	7	6	6	6	6	7	7	7	6.6	0.52	7.82

Table 3d – Repeatability for Milk Analyst 4

Dilution	Repeatability										Mean	SD	% RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	66	69	67	65	70	69	65	69	70	66	67.6	2.01	2.97
10 ⁻⁶	6	7	7	7	7	6	6	6	6	7	6.5	0.52	8.10

Reproducibility.

The results for the analysis are demonstrated in the table below-

Dilution	Reproducibility																			
	Analyst 1		Mean	SD	% RSD	Analyst 2		Mean	SD	% RSD	Analyst 3		Mean	SD	% RSD	Analyst 4		Mean	SD	% RSD
10-5	10	10	10	7.7	7.37	10	10	10	7.7	6.55	9	10	10	4.99	4.24	10	10	10	7.77	7.44
10-6	10	9	9	0.7	7.44	10	10	10	0.7	6.73	10	10	10	6.7	10	11	11	11	1.41	12.85

Table 4a- Reproducibility for Chicken Breast
Table 4a- Reproducibility for Milk

Dilution	Reproducibility																				
	Analyst 1		Mean	SD	% RSD	Analyst 2		Mean	SD	% RSD	Analyst 3		Mean	SD	% RSD	Analyst 4		Mean	SD	% RSD	
10-5	71	67	69	2.83	4.09	68	70	69	1.41	2.05	70	67	68	2.5	3.12	3.09	68	71	69	2.25	3.05
10-6	7	6	6.5	0.71	10.88	7	7	7	0	0	6	7	6.5	0.7	10.8	7	7	7	0	0	



Accuracy

Accuracy of the given test method was analysed from the data obtained after the samples spiked with known concentration of 10^{-5} and 10^{-6} of *Clostridium perfringens* was tested.

The known concentration of the culture suspension is obtained by referring to the mean values of the culture suspension in **Table 1**

Accuracy is calculated as 88.69%

Limit of Detection

Limit of Detection for enumeration of *Clostridium perfringens* was found to be 10 cfu/g

DISCUSSION

In the present study, the test parameter *Clostridium perfringens* was validated by using two different food matrices by the test method ISO 7937-2004. Two different matrix of food sample was tested by the same analysts under same laboratory conditions. Raw chicken and Milk sample were used for the entire study and the same spiked sample was used by the analysts to prevent more uncertainty in the testing. During the study the SD (standard deviation) and %RSD (Relative Standard Deviation) for the repeatability and reproducibility among the analysts was between the acceptable range. The analysis was performed by using an inoculum with low and high concentration of bacterial cells; and during the validation study it was observed that the test method ISO 7937:2004 was able to enumerate the *Clostridium perfringens* when spiked in the food samples.

CONCLUSION

In the present study Enumeration of *Clostridium perfringens* in food sample by test method ISO 7939:2004 was validated. The study shows that low and high concentration of *Clostridium perfringens* cells can be detected and enumerated, hence it is an efficient method which can be used for analysis of food samples.



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