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METHOD VALIDATION OF COMPACT DRY 'NISSUI' TOTAL COUNT TECHNIQUE IN COCOA AND COCOA PRODUCTS

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ABSTRACT - *In this study, method of Compact Dry Nissui for enumeration colony forming units of microorganisms was validating. The purpose of this method validation is to make sure the results obtained reliable throughout the study. This method is a rapid method kit for determining aerobic colony counts, which has been developed by Nissui Pharmaceutical Company for food application. The Compact Dry Nissui is a ready-to-use dry media sheet. The total aerobic colony count can be determined in the sample within 48 h of incubation. This method claim as the easiest and simple analysis tool for indicating colony forming units (cfu) for variety of cocoa and cocoa products. Therefore, the method was used to verify 4 different cocoa products sample; Chocolate Praline, Chocolate Block, Cocoa Powder and Cocoa Liquor. The verification parameters included in this study were Accuracy, Precision, Repeatability, Sensitivity/Specificity, and Limit of Detection (LOD). The results are as follows; Accuracy 98.8%, Precision 0.0918, Repeatability 9.18%, Sensitivity/specificity 100%, while for Limit of detection was 7.2/25g. From the result shown that this method fulfils the need of EURACHEM Microbiology Guideline®. Therefore, the Compact Dry Nissui plate can be considered as a convenient alternative method for routine microbiological testing in cocoa and cocoa products for enumeration and detection of aerobic microorganisms. The Compact Dry Nissui offers the advantage of the shorter time results compare to the conventional method.*

Keyword**:** Aerobic colony, Alternative method, Colony forming units, Rapid method kit, Validation,

INTRODUCTION

Compact Dry Total Count (TC) is a ready-to-use test method recommended for the determination of total aerobic bacterial counts in raw materials, finished products, or on environmental surfaces related to food. In performing the microbial testing, this test method helps to reduce the labour hours. Compact dry combines the features and benefits of the conventional plate media with the modern features of dehydrated film media. This unique combination will shorten the test time and increase lab efficiency, thus reducing the costs. Therefore, it allows maximizing the production by increasing efficiency (Kodaka *et. al*., 2005). The plates consist of 50 mm diameter petri dish containing a detection specific nutrient pad.

In this study compact dry total count (TC) was used as a comparison with conventional method (AOAC method) to determine aerobic colony count in cocoa powder and cocoa products. The compact dry TC is designated in a small and compact plate, thus it only required minimal physical spaces for storing, testing and incubating. The compact dry TC is portable plate and ready to use. The system of a compact dry TC makes sample diffuse automatically and spread evenly into the plate.

The big advantages of the Compact Dry 'Nissui' TC method are the reduced hands-on time and economical usage, as confirmed by the comparison test with conventional method. In terms of plate preparation, inoculation and reading the result, the Compact Dry 'Nissui' TC method also easier and quicker compare to the conventional method. Particles of cocoa powder in conventional test method made reading plates and counting colonies relatively more difficult, but when using Compact Dry 'Nissui' TC method, reading the plates become more faster, with the TTC indicator speeding up the counting (Kodaka *et. al*., 2005). It was also observed that cocoa powder particles, when present, did not appear to absorb the indicator.

The compact dry TC is a very convenient compact plate because it is small and

can be kept in room temperature within a long time shelf life duration. Once a liquid sample is inoculated, the dry coated medium transforms to gel and the plate is ready to incubate. Compact Dry TC contains a standard nutrient medium for detection of total plate count. After the incubation, the colonies on compact dry TC are easy to read because of the clear colour development by redox indicator. Tetrazolium salt which is integrated into the medium and serves as redox indicator die, will make the colonies grown in red coloured and therefore easily to identify and differentiable from possible food residues on the plate. Isolated colonies on the compact dry TC can also be subcultured individually to the other media. Compact Dry TC method has been approved by MicroVal (certificate Nr. RQA2007LR01), NordVal (certificate Nr.033) and AOAC-RI (certificate Nr. 010404)(Kodaka, *el. Al*., 2005).

According to the Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens (2019), method validation means a process by which a laboratory confirms by the examination, and provides objective evidence, and that particular requirements for specific uses are also fulfilled. Validation also defined as a process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirement for the intended application. In ISO/IEC 17025 defines validation as "the confirmation by examination and the provision of objective evidence that the particular requirements for specific intended use are fulfilled". Validation is specifically intended to be used as an analytical requirement.

In this study, the validation studies were conducted for 20 samples of cocoa powder and cocoa products received from local grinders and local market. Reference method is ISO4833 (2003), was performed exactly as specified, with no deviations or alterations.

MATERIALS AND METHODS

Samples of cocoa products

Samples for monitoring purposes consisted of cocoa powder, chocolate block, chocolate praline and cocoa liquor. Cocoa powder samples and cocoa liquor were obtained from local cocoa manufacturers and traders, while chocolate samples were collected from local market. In total 5 chocolate praline samples, 5 chocolate block samples, 5 cocoa powder samples and 5 cocoa liquor samples were collected for this study. Samples were analysed in duplicate.

Selection and Preparation of Test

All 20 samples need to be sterilized before spiking process begins. To prepare a spiked sample containing approximately 10^2 cfu/g, 2 ml of the 10^{-6} culture dilution were added into 225 ml of suspension (225 ml of diluent containing 25 g of sample). At the same time, under the same condition, suspension of the reference culture was prepared by adding the same volume (1 ml) of culture suspension into 225 ml 0.1% peptone water.

Method Validation

The validation parameters included in this study were Accuracy, Precision, Repeatability, Sensitivity/Specificity, and Limit of Detection (LOD). The validation method followed the Guideline for The Validation of Microbiological Methods for the FDA Food program, 3rd Edition (FDA, 2019).

RESULTS AND DISCUSSION

Accuracy

The accuracy in analytical method means the degree of agreement of test results generated by the method to the true value. In the other hands, accuracy also means exactitude of an analytical method explain how close the mean test results obtained to the real value of analyte (Dilek *et al*., 2017). The replicate analysis of samples containing known amounts of the analyte determines the accuracy (Dilek *et al*., 2017, Riley, 2003). As the results expected are normally centered on the existing method, it is essential to test the accuracy by making a comparison between the old and new methods (PDA, 2000). Spiking trials are normally applied to get accuracy data. From this study on

accuracy, a mean result for spiking and reference samples were calculated, refer to **Table 1**. From the calculation, the accuracy of the test was counted. From this study, we found out the accuracy of this method is 98.9%, which is more than the acceptable value. The acceptable value for accuracy is more than 97% according to EURACHEM Microbiology Guideline. From the value, we can say that the test method was able to measure the true amount or concentration of a substance in a sample.

Table 1 : Mean result for spike and reference samples

No. of samples	Spike sample			Reference sample		
	Duplicate		Mean	Duplicate		Mean
	R1	R2		r1	r2	
1	100	82	91	99	100	99.5
2	70	64	67	97	97	97
3	101	97	99	79	79	79
4	100	95	97.5	99	100	99.5
5	82	97	89.5	100	98	99
6	102	68	85	96	96	96
7	92	84	88	95	87	91
8	105	101	103	100	98	99
9	103	94	98.5	102	89	95.5
10	101	95	98	97	100	98.5
11	90	90	90	103	97	100
12	103	99	101	99	93	96
13	98	95	96.5	101	95	98
14	102	101	101.5	95	89	92
15	97	79	88	100	94	97
16	102	102	102	90	92	91
17	92	87	89.5	99	92	95.5
18	102	99	100.5	98	77	87.5
19	86	100	93	101	95	98
20	96	85	90.5	98	66	82
	ΣR		**1869**	**Σr**		**1891**

Precision

Analytical precision method explains the closeness of individual measured of an analyte when the procedure is done again and again on various aliquots with a homogeneous volume of biological matrix (CDER, 2015). Precision is very much important as an identification method because trending isolates can be hard if the same organisms are given different identities at each time it is isolated (Eurachem, 2014). As demonstrated in **Table 2** is the calculation of Intermediate Precision RSD for Compact Dry 'Nissui' Total Count. The RSD value in this study was 0.029. This RSD value met the method performance criteria and indicates the good precision and accuracy of the method.

Repeatability

Repeatability is calculated when authenticating a method being measure of agreement of replicate tests carried out on the same material in the same laboratory by the analysts (Eurachem, 2014). The presicion assessments were done by determining relative standard deviation (RSD) of 20 blank samples spiked with selected microorganism. Result showed that the RSD observed were in acceptable value.

Coefficient of Variation, CV % = 100 x RSD = 2.9 %

Table 2: Calculation of Intermediate Precision RSD for Compact Dry 'Nissui' Total Count

Test No.	Standard Count Result (cfu/ml)					Difference, R_{LOG} ($loga_i$–$logb_i$)	Diff / mean ($loga_i$-$logb_i$)/x_i	Diff / Mn Sqrd [(($loga_i$-$logb_i$)/x_i]^2
	Plate a_i	Plate b_i	Log a_i	Log b_i	Mean x_i			
1	96	90	1.9823	1.9542	1.9683	0.0280	0.0142	0.0002
2	100	99	2.0000	1.9956	1.9978	0.0044	0.0022	0.0000
3	102	99	2.0086	1.9956	2.0021	0.0130	0.0065	0.0000
4	70	100	1.8451	2.0000	1.9225	0.1549	-0.0806	0.0065
5	92	85	1.9638	1.9294	1.9466	0.0344	0.0177	0.0003
6	101	87	2.0043	1.9395	1.9719	0.0648	0.0329	0.0011
7	102	101	2.0086	2.0043	2.0065	0.0043	0.0021	0.0000
8	100	102	2.0000	2.0086	2.0043	0.0086	-0.0043	0.0000
9	97	84	1.9868	1.9243	1.9555	0.0625	0.0320	0.0010
10	102	79	2.0086	1.8976	1.9531	0.1110	0.0568	0.0032
11	82	97	1.9138	1.9868	1.9503	0.0730	-0.0374	0.0014
12	102	101	2.0086	2.0043	2.0065	0.0043	0.0021	0.0000
13	98	68	1.9912	1.8325	1.9119	0.1587	0.0830	0.0069
14	92	99	1.9638	1.9956	1.9797	0.0318	-0.0161	0.0003
15	103	97	2.0128	1.9868	1.9998	0.0261	0.0130	0.0002
16	90	90	1.9542	1.9542	1.9542	0.0000	0.0000	0.0000
17	105	95	2.0212	1.9777	1.9995	0.0435	0.0217	0.0005
18	103	64	2.0128	1.8062	1.9095	0.2067	0.1082	0.0117
19	101	94	2.0043	1.9731	1.9887	0.0312	0.0157	0.0002
20	86	82	1.9345	1.9138	1.9242	0.0207	0.0107	0.0001

Sensitivity/Specificity

The following cumulative results were obtained from plate counts of all samples in sensitivity study:

a	Number of presumptive positives found positive (true positives)	380
b	Number of presumptive negatives found positive (false negatives)	0
c	Number of presumptive positives found negative (false positives)	0
d	Number of presumptive negatives found negative (true negatives)	18
	Total number of colony counts on plates, n	398

The observations of performance characteristics are shown as follows:

Sensitivity = a/(a+b) = (380/380)x100 = 100%

Specificity = d/(c+d) = (18/ 18)x100 = 100%

False positive rate = c/(a+c) = (0/380)x100 = 0%

False negative rate = b/(b+d) = (0/18)x100 = 0%

Sensitivity study of the test method was done. This is as to explain as the limit of accurate measurement. This parameter was used to show the limit of a method can be discriminated, with

a large measure of trust, between and above levels below several critical values close to zero. Sensitivity is the effectiveness of the gradient response curve or the change in instrument response to correspond with the change in analyte concentration (FOA, 2001, MAF,2002)

Limit of Detection (LOD)

Table 3: Limit of Detection (LOD) for microorganisms

Number of samples	Level of spike (ml)	Actual count (cfu/ml/25 g)
1	1	8
2	1	5
3	1	10
4	1	5
5	1	7
6	1	6
7	1	5
8	1	10
9	1	6
10	1	10
11	1	7
12	1	6
13	1	10
14	1	6
15	1	8
16	1	7
17	1	5
18	1	10
19	1	8
20	1	5

Reference material or spiked samples with 5 to 10cfu for each sample was used for accumulation of the limit of detection. Result reported based on taking the actual count of detectable microorganism for 20 samples, tabulated as shown in Table 5. LOD for this method was 7.2/25g. This is the ability of the method to measure only what it is intended to measure. The detection limit for qualitative tests is best described as the "LOD50", or number of organisms per gram of sample at which 50% of the tests are positive. "LOD50" not used in the analytical chemistry of LOD and LOQ. It is used in the microbiological because the methods are able to estimate around the level of a few particles (bacterium, virus, or genetic macromolecule) per analytical portion. This is possible because virtually unlimited amplifiability of such particles is possible due to their ability to multiply themselves in appropriate conditions.

CONCLUSION

In this study, the enumeration of colony forming units of microorganisms in cocoa and cocoa products using Compact Dry 'Nissui' TC Technique at 35°C were successfully developed and validated. This method has been applied in daily routine for enumeration of microorganisms in cocoa and cocoa products. This method showed good accuracy, precision, repeatability, sensitivity/Specificity, and Limit of Detection (LOD) which acceptable under the validation criteria of EURACHEM Microbiology

guidelines. Demonstration was achieved that the Compact Dry 'Nissui' TC method may constitute a useful alternative tool for rapid enumeration and could also be a convenient alternative method in routine microbiological testing for total aerobic count.

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