Production of certified reference material *Escherichia coli* in milk-based product

Le Thi Phuong Thao[1*], Đang Thi Oanh[1], Đang Huu Cuong[1], Nguyen Thi Hang[1], Nguyen Thi Minh Hoa[1], Vu Thi Hai Ha[2], Le Thi Hong Hao[3]

[1]*Quality Assurance laboratory, National Institute for Food Control, Hanoi, Vietnam*

[2]*Microbiology and GMO laboratory, National Institute for Food Control, Hanoi, Vietnam*

[3]*National Institute for Food Control, Hanoi, Vietnam*

(Received: 04/09/2022; Accepted: 29/09/2022)

Abstract

Reference materials are an important tool in ensuring the validity of the test results efficiently and economically. National Institute for Food Control has implemented several studies, characterization of microbiology chemistry for food and feeding stuffs certified reference materials (CRM) in order to support laboratories in control and maintaining test results quality. CRM for *Escherichia coli* (*E. coli*) in food is our study's first interest, because *E. coli* indicates unfavorable hygienic conditions and fecal contamination in foods. Milk and dairy products are used for any stage of human life from infancy to elder. CRM for *E. coli* in milk-based products were characterized by using a network of competency laboratories leading to certified values in accordance with ISO 17034:2016 requirements. These certified reference materials produced are accredited conforming to ISO 17034:2016 by the America Association of Laboratories Association.

Keywords: *Reference material, NIFC, food testing, ensuring validity of the test, E. coli*

1. INTRODUCTION

Certified reference material (CRM) is defined as "Material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability" [1]. Reference material (RM) and CRM play a critical role in ensuring the validity of the test results. CRM is especially useful for method validation, internal and external quality control of test methods, performance

[*] *Corresponding author: Tel: +84 989293425 Email: thaoltp@nifc.gov.vn*

testing of culture media, calibration of equipment, training of employees, and proficiency testing. CRM in food matrix could be for microbiology or chemistry analysis.

Microbiology may be contaminated at any stage in the food chain production and it is the main cause of food-borne diseases. *Escherichia coli* (*E. coli*) are major bacteria species, that can normally live in the intestines of people and animals. Most *E. coli* strains do not cause disease, some can cause serious food poisoning if people eat contaminated food. The presence of *E. coli* in food indicates unfavorable hygienic conditions and fecal contamination. Therefore, CRM for *E. coli* in food is our study's first interest.

Among food product used for any stage of human life from infancy to adulthood, milk and dairy products shall be the first to be mentioned, as those products are rich sources of nutrients and it is a good culture medium for microorganism growth, including that of *E. coli*. Therefore, milk-based products are one of the good matrices for CRM production.

In Vietnam, the maximum contaminant level of *E. coli* in food must comply with the National technical regulation of Microbiological contaminants in food, QCVN 8-3:2012/BYT. Requirements of *E. coli* contamination are not detected in natural mineral waters, drinking waters, edible ice, crustacea and heat treatment Mollusca, raw eating vegetables and instant fruit; for other food, *E. Coli* permission limit depends on the food categories. Generally, the maximum permission limit of *E. Coli* in food is 5×10^3 CFU/g (for meat and its products required heat treatment before use) [2]. Therefore, the assigned value of the CRM production target is less than 5×10^3 CFU/g.

Production technic is one of the important factors for product quality and long-lasting. Freeze drying is one of the technics used in microbiology sample production. "Freeze-drying has been widely used in microbiology for many decades to stabilize and store cultures" [3]. Freeze drying of microorganisms is a multistep process that involves culturing the microbes, suspending them in a lyophilization medium/buffer, subjecting them to the freeze-drying process, and then subsequently storing them properly. Solid reference materials are prepared by freeze drying, one sample can contain many microorganisms, and is used for control of multiple microorganisms at the same time.

The availability of microorganisms RM and CRM are important to laboratories in quality control since they demonstrate that methods used are fit for purpose. RM and CRM from oversea suppliers requires excessive time and cost, hence, decreasing the efficiency and lowering working progress. To deal with those difficulties, the National Institute for Food Control (NIFC) carried out many studies and produced RM and CRM for microbiology and chemicals in food and feeding stuff. Those RM and CRM products are accredited conforming to ISO 17034:2016 requirements [4-5]. The accredited microbiology CRM including those for milk-based, cereal based products, vegetable and animal feeding stuff.

Milk-based products are diversified with many kinds of food including dairy, infant formular, food supplements, health supplements, etc. Therefore, milk-based matrix is useful

for certified reference materials use. In this article, we report results of *E. coli* in milk-based certified reference materials study.

2. MATERIALS AND METHOD

2.1. Materials

All prepared materials are provided by suppliers following/under the Purchasing and supplies services procedure of NIFC, including:

Strain: *Escherichia coli* ATCC 25922.

Culture medium prepared with: Tryptic Soy Agar (TSA, Merck - Germany, Code: 1054580500, Lot: VM916758012); Brain Heart Infusion broth (BHI, Merck - Germany, Code: 1104930500, Lot: VM854193845); Tryptone Bile X-glucuronide agar (TBX, Oxoid - Bristish, Code: CM0945B, Lot: 3194947) and Buffered Peptone Water (BD - American, Code: 218105, Lot: 0174039).

Stabilizer solution was prepared with Myo-Inositol (2g), Glucose (2g); Lactose (5g) and water (100mL).

Matrix: Milk powder prepared for the matrix are tested before use to ensure it is not contaminated with microbiology (*E. coli*, total bacteria, total yeasts and molds), which may affect on spiked targets and background flora.

Packaging materials including: Glass vial 10 mL with rubber cap and aluminum foil cover; PVC bag size 16×25 cm for vacuum packaging 4 vials/bag; Shockproof nylon cover sheet and Dry ice.

Main facilities used including: Freeze drying machine Model: MODULYOD-230; Vial crimper; Vacuum sealing machine, Ultra-Low Temperature Freezer Model: MDF-U700VX-PB, Sanyo Incubator Model: MIR10201567, Cabinet Mode: SafeFAST Elite 215D and Other laboratory facilities.

2.2. Methods

The stock strains were cultured on non-selective agar dishes.

Enrich BHI culture from one colony and check for biochemical properties. The culture of organisms is prepared using method McFarland 0.5 and checked by plate count method if necessary.

Let dilute suspension of bacterial culture reach desired concentration and subsequently, transfer it into stabilized solution. Mix well by using magnetic mixer. The stabilized solution contains bacteria called solution A.

Mix matrix solution: Mix milk-based product in PBS buffer with ratio of 1:2 (by mass), this is called solution B.

Production planning designed with 100 glass vials produced in production protocol: Transfer 2 mL of solution B into a glass vial and add 0.5 mL of solution A into the vial that was filled with solution B. Loose the rubber stoppers, put the sample into liquid nitrogen, stored at - 80°C refrigerator in 12 hours, after that freeze drying at - 45°C in 24 hours. Test

the target microbiological concentration before, after stored at - 80°C, and after freeze-drying.

Test method used: Microbiological concentration test by Microbiology laboratory of NIFC with accredited test method - enumeration by colony count technique [6-7].

2.2.1. Assessment of homogeneity

Ten vials random sampling to be tested in duplicate [8, 9]. The one-way analysis of variance is used to test for a significant between-unit difference [9], using R software for homogeneity evaluation. The between-unit difference was not statistically significant at the 95% level of confidence, using the F test. Between-unit deviation (s_{bb}^2) shall be less than $c = F_1 (0.3\ \sigma_{pt})^2 + F_2 s_w^2$. Where F_1 and F_2 is drawn from ISO 13528 [10], σ_{pt} is proficiency testing standard deviation, s_{an}^2 is the within-unit deviation.

2.2.2. Assessment and monitoring of stability

Assessment and monitoring of stability by Long-term stability study is done by 3 vials random sampling each 1; 2 or 3 months until 12 months. Samples are stored at 2 - 8°C. Use Student t-test to test for statistically significant change to evaluate stability.

2.2.3. Characterization

Characterization measurand of CRM is performed by a network of competent laboratories [9], use results of 07 accredited laboratories participated in proficiency testing schemes organized by NIFC with the same test method and proficiency test results of |z-score| ≤ 1.

Assigned values are the mean of the p data set while mean y_i is applied as the assigned value y_{char} [9] in formula (1):

$$x_{CRM} = y_{char} = \frac{\sum y_i}{p} \quad (1)$$

Assigned uncertainty of the CRM is expressed as:

$$u_{CRM} = \sqrt{u_{char}^2 + u_{hom}^2 + u_{lts}^2} \quad (2)$$

In which:

+ u_{char} is uncertainty associated with reference materials characteristics calculated as the standard deviation of the mean of the p data set mean y_i:

$$u_{char} = s(y)/\sqrt{p} = \frac{1}{\sqrt{p}} \sqrt{\frac{\sum (y_i - y_{char})^2}{p - 1}} \quad (3)$$

+ u_{hom} is uncertainty associated with homogeneity is omitted due to heterogeneity is not permitted for production batch.

+ u_{lts} is uncertainty associated with predicted change given by standard error for the estimated slope, $s(b_1)$; the time interval between value assignment and the initial stability monitoring point (t_{m1}); and the period of validity of a certificate issued during that time (t_{cert}):

$$t_{lts} = s(b1)(t_{m1} + t_{cert}) \quad (4)$$

Certified value is the sum of assigned value (x_{CRM}) and expanded uncertainty ($U_{CRM} = ku_{CRM}$): $x_{CRM} + 2u_{CRM}$.

Where k is a coverage factor, k = 2, we assumed the distribution is approximately normal and the coverage probability is 95 %.

2.2.4. Data statistical analysis

In this study, we used R software for homogeneity evaluation and stability assessment; Excell software used for confirmation of R software statistic analysis, and shelf life calculation.

3. RESULTS AND DISCUSSION

3.1. Homogeneity assessment

Ten random vials were tested for *E. coli* colony forming unit with 2 replicates. The initial test (T_0) results are presented as in Table 1.

Table 1. *E. coli test results for homogeneity evaluation*

No.	Vials ordering number	Results							
		CFU/mL		Log_{10}					
		Replicate 1	Replicate 2	Replicate 1	Replicate 2	Mean	Bias (Wt)	Sum (Z)	Wt^2
1	23	3.0×10^3	2.8×10^3	3.477	3.447	3.46	0.030	6.924	0.0009
2	52	2.6×10^3	3.0×10^3	3.415	3.477	3.45	-0.062	6.892	0.0039
3	39	3.0×10^3	2.7×10^3	3.477	3.431	3.45	0.046	6.908	0.0021
4	22	2.8×10^3	2.6×10^3	3.447	3.415	3.43	0.032	6.862	0.0010
5	9	2.5×10^3	3.0×10^3	3.398	3.477	3.44	-0.079	6.875	0.0063
6	2	2.6×10^3	2.7×10^3	3.415	3.431	3.42	-0.016	6.846	0.0003
7	25	2.4×10^3	2.9×10^3	3.380	3.462	3.42	-0.082	6.843	0.0068
8	47	3.1×10^3	2.8×10^3	3.491	3.447	3.47	0.044	6.939	0.0020
9	44	2.7×10^3	3.0×10^3	3.431	3.477	3.45	-0.046	6.908	0.0021
10	15	2.7×10^3	2.6×10^3	3.431	3.415	3.42	0.016	6.846	0.0003
Total =				34.36	34.48	34.42	-0.117	68.844	0.0255
Mean =				3.44	3.45	3.44	-0.012	6.8844	0.0025
Variance (Var) =						0.001218			
Within-unit deviation (s_w^2) =						0.001275			
Deviation of sample averages (s_x^2) =						0.000609			
Between-unit deviation (s_{bb}^2) =						0.000000			

From the results of Table 1, use R software for one-way ANOVA analysis. As the results, P-value = 0.859 > 0.05, hence, the different between unit was not significant at 95% confident level.

Use the F test to confirm the homogeneity. Factors F1 = 1.88 and F2 = 1.01 as shown in table B.1 in ISO 13528 [10]; $\sigma_{pt} = 0.25$, and c = 0.01186. The result that $s_{bb}^2 < c$ is evidence of the fact that the batch of the product was sufficiently homogeneous.

3.2. Stability assessment and monitoring

Homogeneity assessment (T_0) was initially tested results for stability assessment. Following test caried out at T_1, T_2, T_3, T_4, T_5 and T_6 after 79, 122, 158, 210, 268 and 355 days from the production date. The average log_{10} results are presented in Table 2.

Table 2. *The Log_{10} results of E. coli test in sample at different time*

Test time / *Sample No.*	T_1	T_2	T_3	T_4	T_5	T_6
1	3.414	3.388	3.406	3.299	3.259	3.159
2	3.415	3.406	3.415	3.239	3.230	3.239
3	3.406	3.406	3.404	3.296	3.311	3.241
4	3.438					
5	3.429					

The initial test (T0) results were compared with the 355 days (12^{th} month – T6) test results in Table 2, using t-test two group samples for variances. As the results, p-value = 0.21>0.05. The difference between the two group variances is not significant. Therefore, the samples are stable until studied time (355 days ~ 12 months from production date).

3.3. Characterization

3.3.1. Assigned value

Seven accredited laboratories participated in the proficiency testing schemes organized by NIFC with the same test method and proficiency test results of |z-score| ≤1. The test results are shown in Table 3.

Table 3. *E. coli proficiency testing results of competent laboratories*

Lab No.	*Result*		*Test method*	*z-score*
	CFU/mL	*Log_{10}*		
1	1.6×10^3	3.204	TCVN 7924-2:2008	- 0.645
2	4.7×10^3	3.672	TCVN 7924-2:2008	0.692
3	1.4×10^3	3.146	TCVN 7924-2:2008	- 0.811
4	0.17×10^4	3.230	TCVN 7924:2008	- 0.570
5	4.6×10^3	3.663	TCVN 7924-2:2008	0.665
6	1.5×10^3	3.176	TCVN 7924-2:2008	- 0.725

7	2.0×10^3	3.301	ISO 16649-2:2001; TCVN 7924-2:2008	- 0.368

The Assigned value is calculated in accordance with formula (1) by data in Table 3, x_{CRM} = 3.342, equivalent with 2.2×10^3 CFU/g. The assigned value of CRM is less than the maximum permission limit (5×10^3 CFU/g) in food. Therefore, this CRM reaches the designed production target.

3.3.2. Uncertainty

Uncertainty of certified value **Log_{10}** calculated as formular (2) is: u_{CRM} = 0.233.

Extended uncertainty (k = 2): U_{CRM} = 0.466.

The upper limit (L_{up}) is 3.807 equivalence 6.4×10^3 CFU/g.

The lower limit (L_{low}) is 2.876 equivalence 7.5×10^2 CFU/g.

3.3.3. Shelf life

Simple linear regression analysis by R software shows the relationship between *E. coli* count in the sample and storage time (days) as shown in Figure 1.

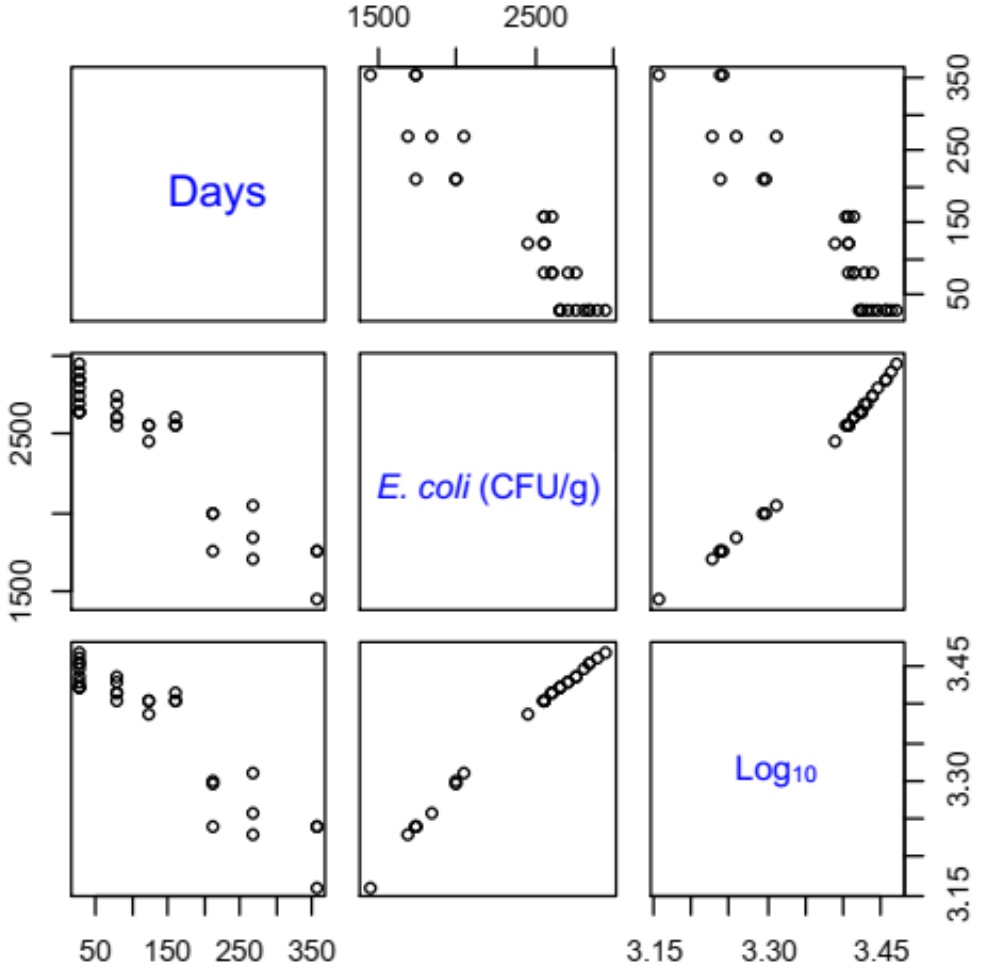

Figure 1. *The relationship of E. coli count and time*

The graph displays the change in *E. coli* numerical and its **Log$_{10}$** according to time (Day) as a few colonies are incapable of surviving or activated after a long time of storage.

The linear regression *E. coli* count and time are shown in Figure 2.

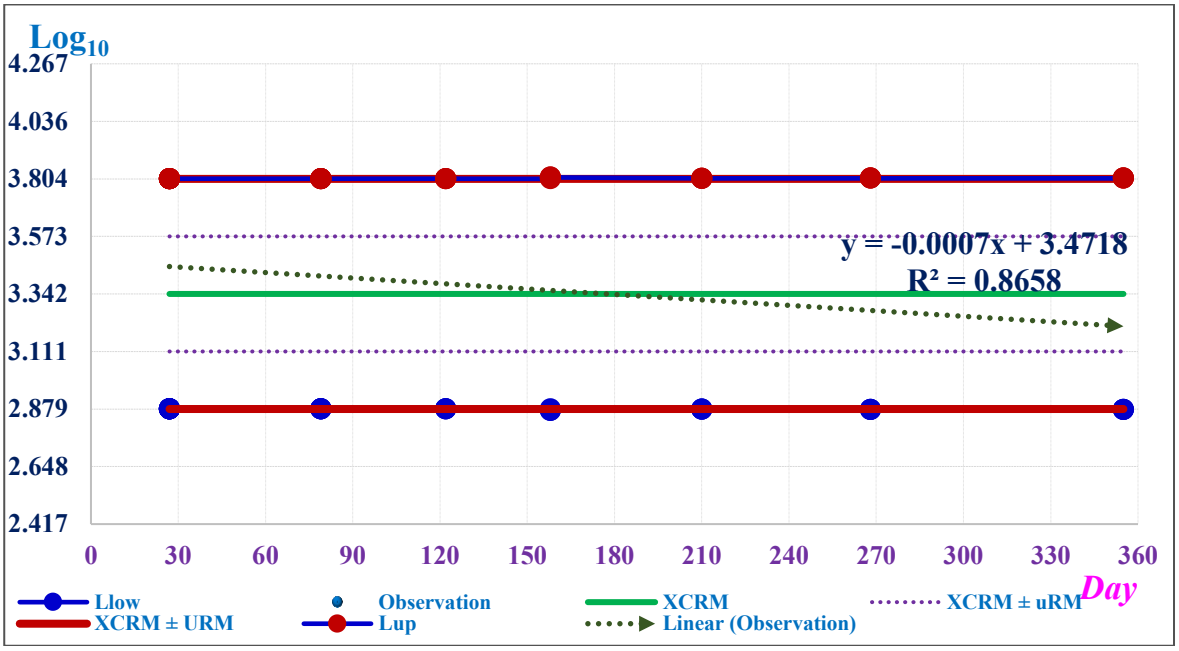

Figure 2. *The Log$_{10}$ of E. coli count following time (day)*

Figure 2 shows that the **Log$_{10}$** of *E. coli* count in the samples changes according to time ($p = 3.313e^{-14} < 0.05$) linearly with the regression coefficient $R^2 = 0.86$. The linear regression equation is:

$$Y = 0.0007 \times Days + 3.472 \qquad (5)$$

The predicted expiration date is a min of shelf life calculated by formula (5) according to L_{up} and L_{low}. As a results, the predicted shelf life is 454 days from the production date.

The certified reference material of *E. coli* produced by NIFC was accredited by the A2LA [4-5]. The CRM contributes source of resources not only for internal quality control, but also for external quality control, especially for second or third parties using in laboratory assessment.

4. CONCLUSION

The certified reference material of *E. coli* produced by NIFC was accredited by the American Association of Laboratory Accreditation. 100 vials are produced with characterization: Sample are evaluated homogenized; Assigned value: x_{CRM} = 3.342, equivalence with 2.2×10^3 CFU/g; Uncertainty: $u_{CRM} = 0.233$. Extended uncertainty (k = 2): U_{CRM} = 0.466; Sample stable until 12 months study from production date; Shelf life estimated as: 454 days from the production date. Domestically produced certified reference

materials are more convenient not only for internal quality control, but also for external quality control, especially for second or third parties using in laboratory assessment.

ACKNOWLEDGEMENT

First and foremost, study team would like to thank the National Institute for Food Control leadership in study orientation. Secondly, we would like to thank MSc. Nguyen Thanh Trung - head of Microbiology and GMO laboratory in technical advisory, and MSc. Tran Hong Ba with assistance and involvement in production process. Without their passionate participation, the study could not have been successfully conducted.

REFERENCE

[1]. National Technical committee TCVN/CASCO on conformity assessment, "General requirements for competence of reference material producers. International Standards Organization," *TCVN ISO 17034:2017*, Ha Noi: Directorate for Standards, Metrology and Quality, 2017.

[2]. National Technical regulation committee on limitation of chemical and biology, "National technical regulation of Microbiological contaminants in food," *QCVN 8-3:2012/BYT*, pp. 5-11, Ha Noi: Ministry of Health, 2012.

[3]. C. Morgan, G. Vesey, "Freeze-Drying of Microorganisms," *Encyclopedia of Microbiology (Third Edition)*, Elsevier Inc., pp. 162-173, 2009.

[4]. A2LA, 2021 [Online]. Available: https://customer.a2la.org/index.cfm?event=directory.detail&labPID=348F2B41-9AC3-4A5D-9AD3-836DEBCAEAAD [Accessed 20/9/2022].

[5]. National Institute for Food Control, 2022 [Online]. Available: https://nifc.gov.vn/a2la [Accessed 20/9/2022].

[6]. Technical committee, "Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia* coli - Part 2: Colony-count technique at 44°C using 5-bromo-4chloro-3-indolyl β-D-glucuronide," *ISO 16649-2:2001*, International Standardization Organization, 2001.

[7]. National Institute for Food Control, 2022 [Online]. Available: https://nifc.gov.vn/van-phong-cong-nhan-chat-luong [Accessed 20/9/2022].

[8]. Technical committee, "Microbiology of the food chain - Specific requirements and guidance for proficiency testing by interlaboratory comparison," *ISO 22117:2019*, International Standardization Organization, 2019.

[9]. Technical committee, "Reference materials - Guidance for characterization and assessment of homogeneity and stability," ISO Guide 35:2017, International Standardization Organization, 2017.

[10]. Technical committee, "Statistical methods for use in proficiency testing by interlaboratory comparison," *ISO 13528:2015*, corrected version 2016-10-15, International Standardization Organization, 2016.

Sản xuất mẫu chuẩn chứng nhận *Escherichia coli* trong sản phẩm từ sữa

Lê Thị Phương Thảo[1*], Đặng Thị Oanh[1], Đặng Hữu Cường[1], Nguyễn Thị Minh Hòa[1], Nguyễn Thị Hằng[1], Vũ Thị Hải Hà[2], Lê Thị Hồng Hảo[3]

[1]Khoa Đảm bảo chất lượng, Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia

[2]Khoa Vi sinh và GMO, Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia

[3]Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia, Hà Nội, Việt Nam

Tóm tắt

Mẫu chuẩn là công cụ quan trọng trong đảm bảo giá trị sử dụng của kết quả thử nghiệm một cách kinh tế và hiệu quả. Viện Kiểm nghiệm an toàn vệ sinh thực phẩm quốc gia đã thực hiện một số nghiên cứu, đưa ra các đặc tính của mẫu chuẩn chứng nhận cho thực phẩm và thức ăn chăn nuôi nhằm hỗ trợ các phòng thí nghiệm trong việc duy trì chất lượng của kết quả thử nghiệm. CRM *E. coli* trong thực phẩm được chúng tôi quan tâm nghiên cứu đầu tiên, vì *E. coli* chỉ thị điều kiện vệ sinh không phù hợp và sự nhiễm phân trong thực phẩm. Sữa và các sản phẩm từ sữa được sử dụng cho bất kỳ giai đoạn nào của cuộc đời con người từ trẻ sơ sinh đến người lớn tuổi. *Escherichia coli* trong các sản phẩm từ sữa được xác định các đặc tính đo lường bằng mạng lưới các phòng thí nghiệm có năng lực và đưa ra giá trị chứng nhận phù hợp theo yêu cầu của ISO 17034:2016. Các mẫu chuẩn chứng nhận này đã được công nhận phù hợp theo yêu cầu của ISO 17034:2016 bởi Hiệp hội công nhận phòng thí nghiệm của Hoa kỳ.

Từ khóa: *Reference material, NIFC, food testing, ensuring validity of the test, E. coli.*