UNCERTAINTY FROM SAMPLING IN FOOD MICROBIOLOGY

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Abstract:
The aim of this study was to estimate uncertainty arising from the sampling of food for microbiological analysis. A balanced experimental design using the duplicate method was a method of choice for empirical estimation of uncertainty. Duplicate test portions were drawn from both test samples and analyzed in duplicate. This approach allowed the estimation of overall measurement uncertainty and its components; analytical uncertainty and sampling uncertainty. Classical ANOVA and Robust ANOVA were used for the calculation. Based on 12 sampling targets of minced meat, uncertainty from sampling was estimated as 0.10-0.11 log10 cfu/g for microbiological parameter aerobic colony count, while analytical uncertainty was 0.04 log10 cfu/g. Estimated uncertainties proved to be fit-for-purpose. Results showed that uncertainty from sampling is the major contributor to overall measurement uncertainty.

Keywords: measurement uncertainty; sampling; sampling uncertainty, food microbiology

1. INTRODUCTION

Due to International Standard ISO 17025 [1] measurement uncertainty had become a “hot” topic among testing and calibrating laboratories. The new 2017 revision of the Standard introduced an even “hotter” topic –the uncertainty from sampling. In the last decade, the number of scientific and/or professional papers on this subject had been very scarce so there is not much data for comparison.

Sources of uncertainty in food microbiology include errors and variations in sampling, weighing, volumes of diluent, sample preparation, delays during analysis, microbiological culture media, incubation conditions, competitive microflora, and intrinsic distribution of colonies [2]. It is generally accepted that in conditions where these sources of variability are minimized, repeatability of enumerated microbiological data may only be precise to about 0.5 $\log_{10}$ units [3].

Measurement uncertainty is a result of combined analytical and sampling uncertainty. In food microbiology, three major components of analytical uncertainty had been recognized: technical uncertainty, matrix uncertainty, and distributional uncertainty [4]. Technical uncertainty arises from operational variability and is a characteristic of an analytical method. Matrix uncertainty however refers only to the effects of microbial distribution within a certain matrix and is a characteristic of that specific matrix. Distributional uncertainty arises from intrinsic variability associated with the distribution of microorganisms in the sample and its dilutions. Even without the contribution from sampling, uncertainty in food microbiology is expected to be high [5].

Sampling as an initial step in any analysis inevitably introduces some level of uncertainty that contributes to overall measurement uncertainty. In food microbiology, uncertainty from sampling is expected to be (very) high as it describes variability between and within the samples due to the heterogeneous distribution of analyte [6]. The heterogeneous distribution of microorganisms in foods has long been recognized, as well as the fact that their distribution in food matrices does not conform to the normal distribution [7]. In general, the distribution of microorganisms in most suspensions follows the Poisson distribution. However, in solid and multi-component food matrices the distribution of microorganisms is complex due to the presence of clumps and chains of microorganisms. The log-normal distribution most adequately describes the heterogeneity of microorganisms in food matrices [6].

According to Ramsey et al. [8], estimates of sampling uncertainty for chemical contaminants in foodstuffs are often larger than estimates of analytical uncertainty. Jarvis et al. [9] previously reported that estimated sampling uncertainty contributed two-thirds to the total measurement uncertainty of aerobic mesophilic count on prawns. The authors concluded that sampling uncertainty is likely to exceed analytical uncertainty in other foods as well. They also emphasized that ignoring sampling uncertainty leads to underestimates of total measurement uncertainty and may adversely affect the assessment for compliance of food with legislative and commercial microbiological criteria [9].

Main intention of this paper is to share experience on the estimation of uncertainty from sampling from the perspective of a small-to-moderate sample throughput laboratory that performs its sampling.

2. MATERIALS AND METHODS

The same sampling procedure according to ISO/TS 17728:2015 [10] was performed on 12 sampling targets of minced meat (retail lots). Sampling targets were chosen within the main criteria of fitness-for-purpose and to represent usual routine samples of our laboratory.

The samples were transported chilled to the laboratory where they were analyzed for aerobic colony count, according to the experimental design described below.

2.1. Experimental design

Uncertainty from sampling was estimated by a balanced design using the duplicate method as described in Eurachem Guide [11]. The duplicate method is based upon a single sampler duplicating a small proportion of the primary samples, selected at random to represent the typical composition of such targets. The duplicated samples were taken by repeating the same sampling protocol by a single person (sampler), with permitted variations that reflect the ambiguity in the sampling protocol and the effect of small-scale heterogeneity of the analyte of interest on the implementation of that protocol. Both of the duplicated samples were subjected to physical preparation resulting in two separate test samples. Duplicate test portions were drawn from both of the test samples and analyzed in duplicate. This system of duplicated sampling and analysis on both samples is known as a 'balanced design' (see Figure 1).

2.2. Method

Aerobic colony count (ACC) was enumerated according to ISO 4833-1:2012 [12], by pour plate technique using Plate Count Agar (Oxoid, UK). The plates were incubated at 30±1 °C for 72±3 hours. Analysis was conducted in repeatability conditions. Colonies were counted and calculated according to ISO 4833-1:2012. Results were expressed as colony-forming units per gram (cfu/g), which were then normalized by $\log_{10}$ transformation. Logarithm values were used in further statistical analysis.

2.3. Statistical procedures

Before further calculations results were examined for outliers using Grubb's test and tested for normality with Shapiro-Wilk and Kolmogorov-Smirnov tests using *Analyse-it*® for Excel (Analyse-it Software Ltd, Leeds, UK, v6.01.1.)

Standard uncertainty (u) for a single target was estimated using equation (1).

$$u = s_{meas} = \sqrt{s^2_{\text{sampling}} + s^2_{\text{analytical}}} \quad (1)$$

Standard uncertainty ($u = s_{total}$) for multiple targets was estimated using equation (2).

$$u = \sqrt{s^2_{\text{btw-targets}} + s^2_{\text{sampling}} + s^2_{\text{analytical}}} \quad (2)$$

Analysis of variance was performed by both classical ANOVA and robust ANOVA, using RANOVA3 software of the Analytical Methods Committee (AMC) of the Royal Society of Chemistry was used, available on their website (https://www.rsc.org). RANOVA3 software calculated total standard uncertainty for multiple targets, the variance between targets, sampling uncertainty, and analytical uncertainty.

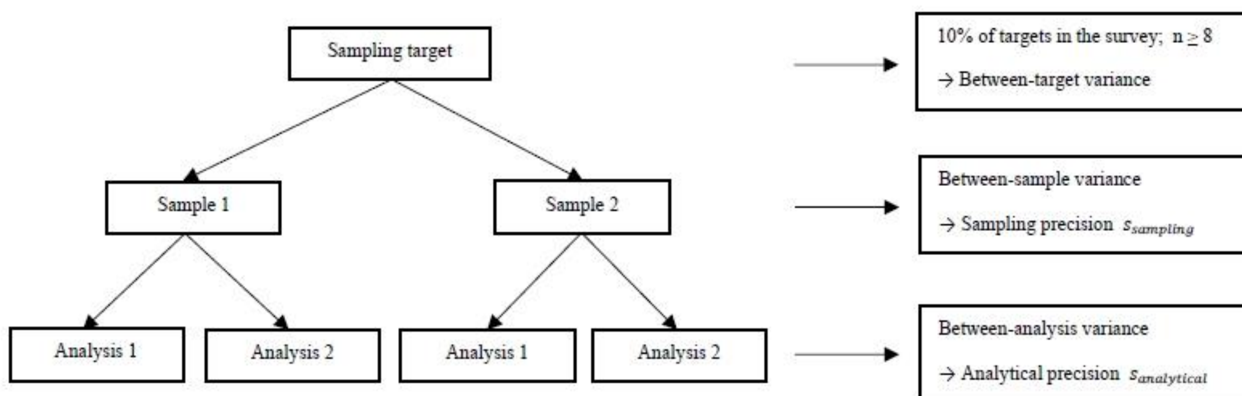

Figure 1: Balanced experimental design for estimation of uncertainty from sampling using the duplicate method [11]

3. RESULTS

Aerobic colony counts of all samples taken in duplicate during sampling (sample 1 and sample 2) and analyzed in duplicate (A1 and A2) are shown in Table 1.

Table 1: Results of aerobic colony counts, as $\log_{10}$ cfu/g, for 12 sampling targets

Sampling target	Sample 1		Sample 2	
	A1	A2	A1	A2
1	4.28	4.29	4.32	4.30
2	4.33	4.35	4.32	4.29
3	4.89	4.81	4.74	4.72
4	5.20	5.10	5.19	5.18
5	5.13	5.18	5.13	5.17
6	5.07	5.06	5.08	5.10
7	4.86	4.86	4.46	4.61
8	4.72	4.65	4.78	4.85
9	5.02	5.08	5.40	5.38
10	5.25	5.24	5.09	5.15
11	5.89	5.80	5.91	5.97
12	5.92	5.93	5.73	5.73

Normality tests showed that data conform to the normal distribution (normality was not rejected by statistical tests). Grubb's test recognized one value as further than the rest but not a significant outlier, so no outliers were detected.

Based on obtained results in Table 1 total standard uncertainty, which includes variance between targets, sampling, and analytical variance was calculated using RANOVA3 software (Figure 2 and Figure 3). There was no significant difference between variances calculated by classical and robust ANOVA. Uncertainty from sampling was estimated as 0.11 $\log_{10}$ cfu/g by classical ANOVA and 0.10 $\log_{10}$ cfu/g by robust ANOVA, which corresponds to relative uncertainty of 25.3% and 23% respectively (conversion of the decimal logarithm to natural logarithm). Analytical uncertainty was estimated as 0.04 $\log_{10}$ cfu/g (relative uncertainty 9.2%).

Classical ANOVA

Mean	5,0315	No. Targets	12	
Total SD (std dev)	0,50819			
	Btn Target	Sampling	Analysis	Measure
SD (or u)	0,49415	0,11183	0,039555	0,11862
% of total variance	94,55	4,84	0,61	5,45
U' (Exp relative uncertainty) (95%)		4,45	1,57	4,72
^{F}U (Uncertainty Factor) (95%)		1,0453	1,0162	1,0483

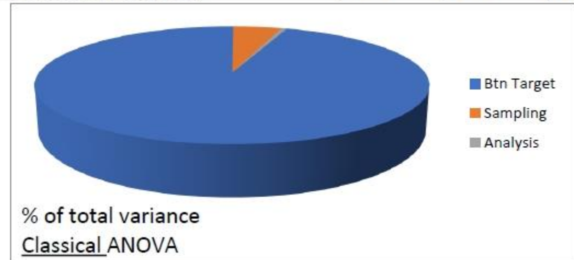

Figure 2: Contributions to total variance calculated by Classical ANOVA

Robust ANOVA

Mean	5,0249			
Total SD (std dev)	0,55927			
	Btn Target	Sampling	Analysis	Measure
SD (or u)	0,54952	0,096778	0,038019	0,10398
% of total variance	96,54	2,99	0,46	3,46
U' (Exp relative uncertainty) (95%)		3,85	1,51	4,14

u = standard uncertainty

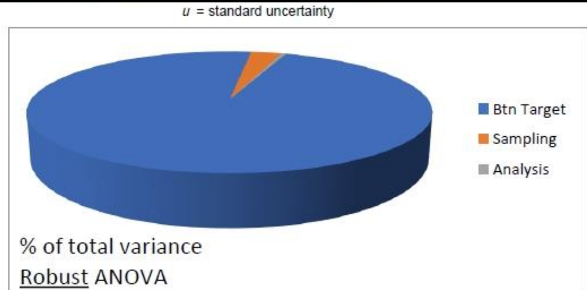

Figure 3: Contributions to total variance calculated by Robust ANOVA

Results also showed that the contribution from sampling to total measurement variance is 86-88% (Table 2). According to both classical and robust ANOVA, the variance between targets is the greatest contribution to the total variance, while sampling is the major contributor to the variance of measurement; see Table 2, Figure 2 and Figure 3.

Table 2. Sampling, analytical and total measurement standards deviations and variances, as $\log_{10}$ cfu/g

Classical ANOVA	Sampling	Analytical	Total measurement
Standard deviation (SD)	0.11	0.04	0.12
Variance	0.013	0.002	0.014
% of total measurement variance	88.9	11.1	-
Robust ANOVA	**Sampling**	**Analytical**	**Total measurement**
Standard deviation (SD)	0.10	0.04	0.10
Variance	0.009	0.001	0.011
% of total measurement variance	86.6	13.4	-

4. DISCUSSION

The aim of this study was to estimate the uncertainty arising from sampling. At first, we obtained a broad range of results (from 10^3 cfu/g to 10^9 cfu/g) since the microbiological quality of target samples varied a lot. Such data did not conform to the normal distribution and also included a certain number of outliers (unpublished data). A need to restrict the number of results and data values for aerobic colony counts around the median value was recognized. At that point, we decided to repeat the study on more sampling targets to collect samples of the approximately same microbiological quality. The biggest challenge of this study was to obtain results that are statistically valid and can be correctly evaluated so that valid conclusions are made. The same statistical problems with results in food microbiology were described by Hedges and Jarvis [13] as well as Jarvis et al.[3]. Hedges and Jarvis [13] stated that in most cases microbiological data are approximately lognormal due to over-dispersion and other theoretical distributions (such as the negative binomial) may give better fits but their use may introduce problems of calculation.

A great challenge was also the choice and interpretation of statistical methods for the analysis of data. Standard ANOVA is usually a statistical standard in the analysis of microbiological data. However, Hedges and Jarvis [13] showed that standard analysis of variance is often sub-optimal for analysis of microbial data and that robust methods that reduce the impact of outlier values are more appropriate when testing statistical significance. Jarvis et al. [3] stated that the choice of statistical methods by which the data are analyzed is also one of the major influences on the estimation of uncertainty. The standard ANOVA requires data that conform well to normal distribution. If that is not the case, the statistically generated results can be misleading. One should keep in mind that the removal of outliers can generate more reliable data, but at the same time can result in the loss of values that may be true estimates of the analyte [3].

In our study, the total standard deviation (uncertainty) for multiple targets was estimated at around 0.5 $\log_{10}$ cfu/g (see Figure 2 and Figure 3), with the greatest contribution from between-targets uncertainty. Uncertainty in food microbiology is regarded as very high, usually expected within the range ±0.5 $\log_{10}$ units. Jarvis et al. [3] reported reproducibility uncertainty as high as 0.77 $\log_{10}$ cfu/g. According to Corry et al. [14], estimates of repeatability and reproducibility uncertainty for ACC ranged from 0.11 to 0.60 $\log_{10}$ cfu/g.

Analytical uncertainty was the smallest contributor to overall uncertainty, estimated as 0.04 $\log_{10}$ cfu/g (relative uncertainty 9.2%) by both classical and robust ANOVA (see Figure 2 and Figure 3). This estimate is lower than other published data ([15]; [3]; [16]), due to repeatability conditions that were applied with the particular purpose of minimizing the analytical uncertainty. Previous work of Ljevaković-Musladin [16] showed that analytical uncertainty for ACC was at 0.08-0.09 $\log_{10}$ cfu/g (expressed as intralaboratory reproducibility standard deviation). In comparison, Augustin and Carlier reported that the standard deviation associated with the analytical method for ACC was on average 0.111 $\log_{10}$ cfu/g [15].

One of the components which can largely contribute to analytical uncertainty is matrix uncertainty. Although also caused by the heterogeneity of the sample, matrix uncertainty is regarded as different than the sampling uncertainty. In their ISO Trials on measurement uncertainty, Ah-Soon and Cornu [17] reported estimated matrix uncertainties for certain heterogeneous foods. Matrix uncertainty for homogenous foods and those foods which can be well homogenized is estimated at 0.1 $\log_{10}$ cfu/g. Minced meat is considered as a food that can be well homogenized in a laboratory. Our sampling uncertainty estimate at 0.10-0.11 $\log_{10}$ cfu/g resembled matrix uncertainty of minced meat. According to Augustin and Carlier matrix uncertainty needs to be included in the overall assessment of uncertainty [15].

In food microbiology uncertainty from sampling arises from the heterogeneity of the target sample, effects of sampling strategy, the physical state of the target sample, temperature effects, transportation, and storage of samples. Heterogeneity is the most important contributor to sampling variability [6].

To our knowledge, there is currently only one study on the contribution of sampling uncertainty to total measurement uncertainty in food microbiology, so there is not much data for comparison. According to a study by Jarvis et al. [9] sampling variances accounted for at least 50%, and in most cases for >85% of the overall reproducibility variance. The results of our study are in full agreement with the respective findings since our sampling uncertainty contributed to 86%-88% of the total measurement variance (see Table 2).

In their respective study, Jarvis et al. [9] also reported standard reproducibility deviation, which includes the contribution from sampling, ranged from 0.11 to 0.59 $\log_{10}$ cfu/g for ACC in different foods (0.5 $\log_{10}$ cfu/g in minced meat). Our estimate of sampling uncertainty at 0.10-0.11 $\log_{10}$ cfu/g was considered in agreement with the lower value of the range in those findings. According to Corry et al. [18], it is possible to determine microbial colony counts on diverse food matrices with a higher

analytical precision (lower uncertainty) by aseptic compositing of samples or by increasing the quantity of sample examined.

5. SUMMARY

Estimation of uncertainty from sampling is a demanding task, which requires substantial time, resources, and statistical knowledge. A major challenge in this task was obtaining results that comply with normal distribution so they can be statistically analyzed to make valid conclusions.

Our study showed that uncertainty from sampling is indeed the major contributor to the overall uncertainty of measurement but to a lesser degree than expected. For our routine sampling tasks, this uncertainty was deemed as “fit-for-purpose”. However, further monitoring is highly recommended to prove the level of uncertainty was well estimated.

6. ACKNOWLEDGMENT

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