EURL CPS
European Union Reference Laboratory for Coagulase Positive Staphylococci
http://eurl-staphylococci.anses.fr

EURL Campylobacter

EURL Lm
European Union Reference Laboratory for *Listeria monocytogenes*
http://eurl-listeria.anses.fr

Guide on the implementation of the Standard EN ISO 19036:2019 for the estimation of measurement uncertainty associated with the enumeration of *Campylobacter*, coagulase positive staphylococci and *Listeria monocytogenes* in the food chain

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Working group of the EURLs:

- *Campylobacter* (SVA, Uppsala, Sweden): Helena Höök, Elina Lahti and Hanna Skarin – eurl-campylobacter@sva.se
- Coagulase Positive Staphylococci (ANSES, Laboratory for Food Safety, Maisons-Alfort, France): Abakabir-Mahamat Abdelrahim - eurl-staphylococci@anses.fr
- *Listeria monocytogenes* (ANSES, Laboratory for Food Safety, Maisons-Alfort, France): Adrien Asséré, Léna Barre and Ludivine Bonanno - eurl-listeria@anses.fr
- Expert: Bertrand Lombard (ANSES, Strategy & Programmes Department, Maisons-Alfort, France) bertrand.lombard@anses.fr

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Introduction & scope

One of the main purposes of the European Union Reference Laboratories (EURLs) for *Campylobacter*, Coagulase Positive Staphylococci (CPS) and *Listeria monocytogenes (Lm)*, and the associated networks of National Reference Laboratories (NRLs) is to ensure the **reliability of official analyses** in their respective scope, in the frame of official controls.

The measurement uncertainty (MU) associated with the analyses performed by EURLs, NRLs and official laboratories is recognised to be an important criterion to assess the reliability of these analyses.

The above-mentioned laboratories should:

(i) estimate MU associated with their analyses for *Campylobacter*/CPS/*Lm* enumeration, according to the Standard EN ISO 19036:2019 (see clause 2);

(ii) obtain values which can be considered as acceptable (see clause 3).

This document intends to provide the above-mentioned laboratories with guidance on how to implement the Standard EN ISO 19036:2019[1] for MU estimation associated with *Campylobacter*/CPS/*Lm* enumeration in samples from the food chain. This guide does not cover MU associated to the microbiological analysis of water used in the food chain, since another standard, ISO 29201[2], deals with MU for the quantitative analysis of the different types of water (drinkable and natural waters). This document also provides guidance on how to assess the MU values obtained.

The present guide is specifically targeted to the enumeration of *Campylobacter*, CPS, *Lm* and the comparison of individual enumeration results to the quantitative limits of microbiological criteria related to these bacteria in the EC Regulation 2073/2005, Annex I: criteria 1.2, 1.3, 2.1.9, 2.2.3, 2.2.4, 2.2.5, 2.2.7, 2.4.1. However, it may also apply to the enumeration of any target bacteria, and to the comparison of an enumeration result to a quantitative limit *m*/*M* of any microbiological criterion.

The 2019 version of EN ISO 19036 has been prepared by the WG 2 "Statistics" of ISO/TC 34/SC 9 "Food products – Microbiology". This Standard replaced the first version, which was a Technical Specification (ISO/TS 19036, 2006, amended in 2009). As any European Standard, EN ISO 19036 has been taken over as a National Standard by each of the CEN Members, including all EU Member States. The harmonised and comprehensive approach described in this standard enables to enlarge MU estimation in the community of food microbiology laboratories across Europe.

This document avoids rephrasing the standard but brings clarifications and details on certain aspects of the standard for a harmonised implementation by the three EURLs, the corresponding NRLs networks and corresponding networks of official laboratories in EU Member States.

This document has been prepared on the basis of the existing EURL *Lm* guide on this topic ("Guide on measurement uncertainty for the enumeration of *Listeria monocytogenes*", V1 – 26/03/2009) and replaces it.

[1] EN ISO 19036: 2019 *Microbiology of the food chain — Estimation of measurement uncertainty for quantitative determinations*

[2] ISO 29201:2012 *Water quality — The variability of test results and the uncertainty of measurement of microbiological enumeration methods*

The first version of this guide was prepared by EURL *Campylobacter*, CPS and *Lm*, reviewed by the respective networks of NRLs, and approved by the Standing Committee on Plants, Animals, Food and Feed on 3 June 2022.

NOTE: the format of the decimal separator used in this guide is "," to be in line with ISO standards.

1. Definition of measurement uncertainty (MU)

For the definition of MU, refer to EN ISO 19036, coming from the definition of ISO/IEC Guide 98-3:2008.

Note that MU includes all sources of uncertainty at the laboratory linked to a given laboratory sample received by the laboratory. The laboratory sample may consist of a packaged food (food unit), an aliquot of bulk food, an environmental sample in the area of food production/food handling, or a sample at the stage of primary production. Thus, MU defined in such a way includes in particular the effects of sub-sampling (e.g. taking a test portion of 10 g or 25 g from the laboratory sample) but excludes any uncertainty linked to sampling (i.e. taking a sample from the production batch).

2. MU estimation

2.1 General aspects

Use EN ISO 19036 for MU estimation associated with *Campylobacter*, CPS and *Lm* enumeration in samples from the food chain.

Note that the EC *Guidance Document on official controls, under Regulation (EC) No 882/2004, concerning microbiological sampling and testing of foodstuffs*[3], in its existing version of 2006, recommends in its clause 8.2.2 the use of ISO/TS 19036 for MU estimation in the context of official controls in food microbiology. It is intended that a revised version of this guidance document, which is under preparation, will refer to EN ISO 19036:2019 instead of ISO/TS 19036.

EN ISO 19036 provides a procedure to estimate and express MU associated with results of quantitative analyses in food microbiology, conducted with both conventional (culture-based) and alternative methods, such as instrumental and molecular methods. According to this standard, MU comprises three main components detailed in clauses 2.2 to 2.4 of this guide:

- technical uncertainty;
- matrix uncertainty;
- distributional uncertainties.

We recommend using the complete approach of the standard as described in its clause 8.1.2 combining the three MU components mentioned in the former paragraph. Thus, we recommend not using the simplified option (MU restricted to technical uncertainty, see clause 8.1.3 of the standard), since values of matrix uncertainty are provided (see clause 2.3 of this guide).

We recommend using the main calculation approach described in the standard to simplify the calculations and their understanding; thus, **not** to correct the standard deviations estimating the technical uncertainty and the matrix uncertainty (i.e. not to implement the optional Annex D).

All calculations described in the standard are implemented in an Excel sheet, easy-to-use with a user's guide, developed and verified by ISO/TC 34/SC 9/WG 2. It is freely available[4].

[3] https://ec.europa.eu/food/safety/biosafety/food_hygiene/microbiological_criteria_en (bottom of page)

[4] https://committee.iso.org/sites/tc34sc9/home/general-standards/content-left-area/culture-media/iso-19036-estimation-of-measurem.html

2.2 Technical uncertainty

Technical uncertainty is linked to the implementation of a given method in a given laboratory. It is thus estimated per method (including the target analyte) by each laboratory estimating its MU. A global approach has been chosen in the standard to estimate the technical uncertainty, based on the reproducibility standard deviation of the final result of the measurement process.

EN ISO 19036 describes three options, in a decreasing order of preference, to estimate the reproducibility standard deviation (s_R):

1. Option 1: intra-laboratory s_R;
2. Option 2: inter-laboratory s_R derived from a method validation inter-laboratory study;
3. Option 3: inter-laboratory s_R derived from an inter-laboratory proficiency test.

We recommend using option 1, detailed in EN ISO 19036, and which is by far the preferred option since it provides a value of technical uncertainty linked to each laboratory. Another advantage of this option is that the same parameter, the intra-laboratory $s_{R,}$ with an identical experimental design, is used in EN ISO 16140-3[5] for the verification of quantitative methods.

The standard describes limitations to options 2 and 3. In particular for option 2, EN ISO 19036 refers to ISO 21748, which describes how to conduct an experimental study to verify that the repeatability and reproducibility estimates within the laboratory are not larger than the corresponding values obtained in the interlaboratory study. Such study is similar to the one to be conducted for option 1.

2.3 Matrix uncertainty

Matrix uncertainty corresponds to the uncertainty associated with taking the test portion from the laboratory sample; it is therefore different from the uncertainty linked to sampling (taking a sample from a batch of production). It is linked to the degree of the spatial heterogeneity of the microorganisms within the matrix. It is thus expected to be large for solid or multi-component matrices, and low for homogeneous matrices. It is assumed in the standard as being independent of the analytical method used (including target bacteria) and of the laboratory estimating it.

The standard allows using values of matrix uncertainty already obtained for laboratory samples expected to have a similar matrix uncertainty (matrix homogeneity), see clause 6.4. We thus suggest using values of matrix uncertainty, per matrix type, obtained in a collaborative study organised (i) by the three EURLs *Campylobacter*, CPS, *Lm*, together with the associated NRLs networks, as well as (ii) at French level (AFNOR committee on food microbiology and NRLs CPS, *Lm*, *Salmonella*). For the EURLs study, see: https://sitesv2.anses.fr/en/minisite/listeria-monocytogenes/measurement-uncertainty

Based on an experimental study[6], EN ISO 19036 (clause 6.2) recommends using a fixed value of 0,1 $\log_{10}$ for liquids (thin, non-viscous fluids, e.g. milk and beverages) and powders as well as when the whole laboratory sample can be made homogeneous before taking the test portion. Details on homogenization techniques are provided in the ISO 6887 series, in particular in EN ISO 6887-part 1 (clause 9.1), which is referred to in clause 6.2 of ISO 19036. For homogeneous

[5] EN ISO 16140-3:2021 *Microbiology of the food chain — Method validation — Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory*

[6] Ah Soon C., Cornu M. Report of 2003/2004, ISO Trials about uncertainty measurement, June 2004, AFSSA, Maisons Alfort, France. Freely available for download at http:// standards .iso .org/ iso/ 19036

matrices that were not included in the EURLs collaborative study, the value of 0,1 $\log_{10}$ can be used as default value.

If there is no available matrix uncertainty value defined for the matrix to be analysed and the fixed 0,1 $\log_{10}$ value cannot be used, then an experimental study should be undertaken as described in clause 6.3 of the standard: analysis under repeatability conditions of multiple test portions from one/several laboratory sample(s). In particular, such study shall be conducted only with naturally contaminated samples.

2.4 Distributional uncertainties

The distributional uncertainties are linked to the nature of the technique used:

- For colony-count techniques (CCT):
    - Poisson uncertainty, linked to the distribution of the number of colonies in/on plates and significant at low numbers of colonies counted;
    - Confirmation uncertainty for methods giving presumptive counts which require confirmation of a certain number of colonies (in general 5);
- For Most Probable Number (MPN) techniques: intrinsic variability estimated by MPN uncertainty derived from the MPN design and detailed results.

The estimation of distributional uncertainties does not require any experimental study. Refer to EN ISO 19036, clause 7, for the calculations.

3. Acceptable MU values

3.1 Introduction

Once MU is estimated, we recommend examining that the obtained MU value can be considered as "acceptable".

As indicated in the introduction, the acceptability of the MU value appears to be an important criterion to assess the reliability of the analyses provided by a laboratory. This applies in particular to the laboratories involved in official controls.

3.2 Notion of acceptable MU values for colony-count techniques

The notion of "acceptable" MU value (or analytical tolerance) for CCT in food microbiology has been introduced in a scientific opinion from AFSSA (now ANSES)[7].

MU can be considered as "acceptable" if it is not too large, relatively to values usually obtained in other laboratories. Table 1 (below), extracted from the AFSSA opinion, gives guidance values of acceptable MU, which are based on (i) results obtained in the frame of proficiency tests organised by RAEMA[8], and (ii) results of ISO trials to estimate the MU component linked

[7]Opinion of the French Food Safety Agency (Afssa) on the references applicable to foodstuffs as process hygiene criteria, 2008. See p.8-10 and Table 1, https://www.anses.fr/en/search/site/2007sa0174

[8] Augustin, J.-C., Carlier, V. 2006. Lessons from the organization of a proficiency testing program in food microbiology by interlaboratory comparison: analytical methods in use, impact of methods on bacterial counts and measurement uncertainty of bacterial counts. *Food Microbiol*. 23, 1-38

to the sub-sampling of the test portion and to the preparation of initial suspension[9], referred to in clause 6.2 of EN ISO 19036. The values given in the table are derived from a large dataset on several bacteria, which included at the time of the publication (year 2006) CPS and *Lm* for instance, but not *Campylobacter*.

From these data and in coherence with EN ISO 19036, it appeared that the guidance values of Table 1 mainly depend on:

- the matrix effect (homogeneous/heterogeneous);
- the number of colonies counted on plates (effect of the distribution of bacteria according to the Poisson distribution);
- the presence or not of a confirmation stage in the enumeration method;
- the value of intra-/interlaboratory reproducibility standard deviation.

Based on the ISO trials quoted above, certain food matrices are considered as homogeneous concerning their MU (see 2.3).

The MU values of other products than the above-mentioned ones are considered as heterogeneous.

Table 1: Guidance values of acceptable MU values for enumeration of bacteria with a colony-count technique (in log_{10} cfu/g).

	Homogeneous matrix		Heterogeneous matrix	
Total number of colonies counted on plate(s) retained for enumeration	Method without confirmation	Method with confirmation	Method without confirmation	Method with confirmation
≤5	0,7	0,7	0,7	0,8
6-10	0,5	0,6	0,6	0,7
11-15	0,4	0,5	0,5	0,6
16-150 or 16-300, depending on the method	0,3	0,5	0,5	0,6

*Source: AFSSA opinion, 2008[5]

[9] Ah Soon, C. et Cornu, M. Report of 2003/2004 ISO trials on measurement uncertainty, June 2004, AFSSA-LERQAP, Maisons-Alfort, France, available at: https://standards.iso.org/iso/19036/ed-1/en/

3.3 Acceptable MU values for *Campylobacter,* CPS and *Lm* enumeration

Based on the approach described above in 3.2, we recommend considering as acceptable the following MU values, taken as tentative values (to be followed up and verified through future proficiency tests organised by the 3 EURLs):

1. For enumeration with a CCT including a confirmation step, i.e. enumeration of *Campylobacter* according to EN ISO 10272-2, of *Lm* according to EN ISO 11290-2 and of CPS according to EN ISO 6888-1:
    - ca. 0,5 $\log_{10}$ when a sufficient number of colonies are counted on the plate(s) retained for enumeration (low numbers excluded, see case 3) and when the product analysed is homogeneously contaminated;
    - ca. 0,6 $\log_{10}$ when a sufficient number of colonies are counted on the plate(s) retained for enumeration (low numbers excluded, see case 3) and when the product analysed is not homogeneously contaminated.
2. For an enumeration method with a CCT without a confirmation step, i.e. CPS enumeration according to EN ISO 6888-2:
    - ca. 0,3 $\log_{10}$ when a sufficient number of colonies are counted on the plate(s) retained for enumeration (low numbers excluded, see case 3) and when the product analysed is homogeneously contaminated;
    - ca. 0,5 $\log_{10}$ when a sufficient number of colonies are counted on the plate(s) retained for enumeration (low numbers excluded) and when the product analysed is not homogeneously contaminated.
3. In the case of low numbers ($\leq$ 15 colonies), refer to Table 1, first three lines.

If an acceptable MU value is not obtained, we recommend carefully reviewing the analytical method and its implementation, by examining for example:

- if sub-sampling of the test portion and preparation of the initial suspension can be more representative of the whole laboratory sample;
- the competence of the operators and how reproducibly they conduct the different steps of the analysis;
- the metrology of the apparatus (e.g. stability of temperature of incubators);
- the quality assurance of culture media.

Corrective actions should be taken based on this review.

Finally, if different laboratories, in the same organisation, have obtained different MU values for the same determination (e.g. *L. monocytogenes* enumeration in smoked salmon), each laboratory has to use the MU value it has obtained. However, we encourage intensive exchanges between the laboratories to compare their methodologies, the analytical method itself, its implementation and the MU estimation technique.

4. Interpretation of MU

MU interpretation, i.e. how to take into account MU when interpreting an enumeration result in terms of conformity to a limit of a microbiological criterion, is dealt within clause 8.2 of the EC "Guidance Document on official controls, under Regulation (EC) No 882/2004, concerning microbiological sampling and testing of foodstuffs", 2006.

With the replacement of ISO/TS 19036 by the Standard EN ISO 19036:2019 and change in practices, it is expected that clause 8.2 will be modified in the frame of the on-going revision of this EC guidance document.

IMPORTANT NOTE: This aspect is not of the competence of the laboratories, thus in particular not the competence of the EURLs, NRLs and official laboratories, but of the risk managers (DG SANTE and national Competent Authorities).