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Guidance document on Measurement Uncertainty for GMO Testing Laboratories – 3rd Edition

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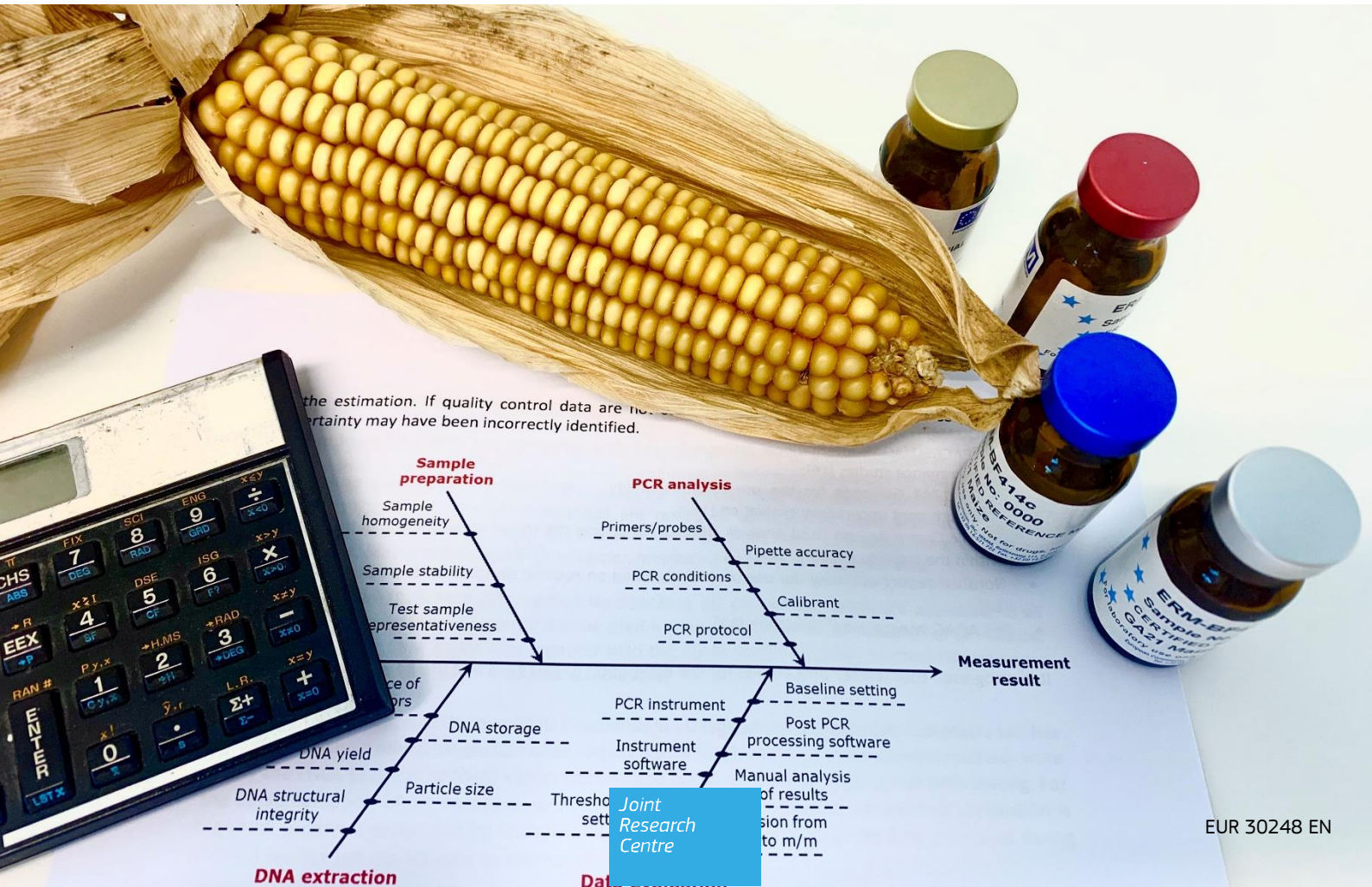

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Glossary

This glossary lists the abbreviations used in this guidance document. Parameters and symbols used for the various calculations are listed in Annex I.

ANOVA	Analysis of variance
CRM	certified reference material
DNA	deoxyribonucleic acid
dPCR	digital PCR
ENGL	European Network of GMO Laboratories
ERM	European Reference Material (code used by the JRC for its CRMs)
EC	European Commission
EU	European Union
EU-RL	European Union Reference Laboratory
EU-RL GMFF	EU-RL for GM Food and Feed
EURACHEM	Network of analytical chemistry organisations in Europe
GM	genetically modified
GMO	genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurement (ISO guide)
ISO	International Organization for Standardization
IEC	International Electrotechnical Commission
IUPAC	International Union of Pure and Applied Chemistry
JRC	Joint Research Centre (of the EC)
LOD	limit of detection
LOQ	limit of quantification
m/m	mass fraction
MU	measurement uncertainty
NMKL	Nordic Committee on Food Analysis
PCR	polymerase chain reaction
qPCR	quantitative (real-time) PCR
QC	quality control
QUAM	Quantifying Uncertainty in Analytical Measurement (EURACHEM guide)
RSDr	repeatability standard deviation

1 Introduction

This document provides guidance on how to estimate measurement uncertainty (MU) and supports the enforcement of EU food and feed labelling legislation in the GMO sector. Measurement uncertainty is a parameter which is always associated with the result of a measurement, and characterises the dispersion of values attributed to that result. This measurement uncertainty needs to be estimated when compliance is investigated.

The first version of this guidance document was written on request of the European Network of GMO Laboratories (ENGL) as a follow-up to a workshop on MU in the GMO sector organised by the European Commission, Joint Research Centre and was published in 2007 [1]. It was updated in 2009 [2]. The current version takes into account current EU legislation, availability of certified reference materials (CRMs) and validated quantification methods and the need for control laboratories which carry out measurements for the enforcement of EU legislation to be accredited according to ISO/IEC 17025 [3].

This guidance document contributes towards a harmonised approach for how EU Member States check compliance of food and feed samples with EU legislation. Other documents, e.g. the flexible scope accreditation document [4] refer to this document concerning aspects related to MU.

[1] S Trapmann, M Burns, H Broll, R Macarthur, R Wood, J Zel (2007) Guidance Document on Measurement Uncertainty for GMO Testing Laboratories, EUR report EUR 22756 EN, ISBN: 978-92-79-05566-9

[2] S Trapmann, M Burns, H Broll, R Macarthur, R Wood, J Zel (2009) Guidance Document on Measurement Uncertainty for GMO Testing Laboratories, EUR report EUR 22756 EN/2, ISBN: 978-92-79-11228-7

[3] ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories

[4] S Trapmann, C Charles Delobel, P Corbisier, H Emons, L Hougs, P Philipp, M Sandberg, M Schulze (2014, 2nd version) European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs, Publications Office of the European Union, LU, EUR 26547 EN, ISBN 978-92-79-35936-1; https://europa.eu/!tT76ft

1.1 Scope

The guidance given in this document is addressed to testing laboratories entrusted with the enforcement of Regulation (EU) 2017/625 on the official control of the application of feed and food law [5]. More specifically it concerns Regulation (EC) No 1829/2003 on genetically modified food and feed [6], Regulation (EC) No 1830/2003 concerning the traceability and labelling of genetically modified organisms [7] and Regulation (EU) 619/2011 [8] on the official control of feed as regards the presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired.

Regulation (EC) No 1829/2003 [6] establishes a labelling threshold above 0.9 % per ingredient and taxon requiring that samples of food and feed products available on the EU market need to be checked for their compliance. Regulation (EU) No 619/2011 [8] considers the presence of GMOs in feed materials as non-compliant when the measurement result for one measured transformation event minus the expanded measurement uncertainty equals or exceeds the level of 0.1 (m/m) % of GM material. Figure 1 shows the decision tree for GMO compliance testing in the EU.

The scope of this document is limited to the estimation of MU for quantitative measurement results, as required for the labelling of GM food and feed products for the EU market (Regulation (EC) No 1829/2003 [6]). It deals with GMO events authorised for the EU market or falling under the specific rules for feed products (pending GMO authorisation or expired GMO authorisation (Regulation (EU) No 619/2011 [8]).
This guidance document addresses the MU arising from the measurement method but not the MU arising from sampling. Likewise it does not cover qualitative testing for presence/absence.

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[5] Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, amending Regulations (EC) No 999/2001, (EC) No 396/2005, (EC) No 1069/2009, (EC) No 1107/2009, (EU) No 1151/2012, (EU) No 652/2014, (EU) 2016/429 and (EU) 2016/2031 of the European Parliament and of the Council, Council Regulations (EC) No 1/2005 and (EC) No 1099/2009 and Council Directives 98/58/EC, 1999/74/EC, 2007/43/EC, 2008/119/EC and 2008/120/EC, and repealing Regulations (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council, Council Directives 89/608/EEC, 89/662/EEC, 90/425/EEC, 91/496/EEC, 96/23/EC, 96/93/EC and 97/78/EC and Council Decision 92/438/EEC, Official Journal of the European Union, L 95; https://europa.eu/!pR99nf

[6] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed, Official Journal of the European Union, L 268/1; https://europa.eu/!VF48Hq

[7] Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC, Official Journal of the European Union, L 268/24; https://europa.eu/!RT37vb

[8] Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired, Official Journal of the European Union, L 166/9; https://europa.eu/!Ff79fc

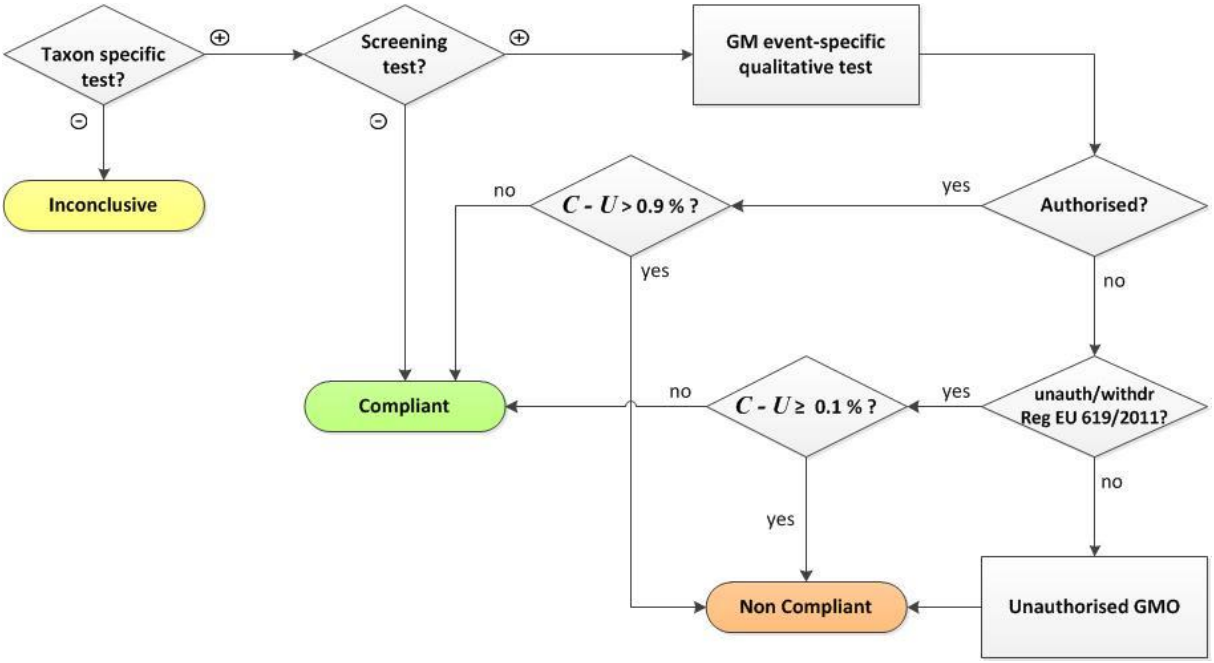

Figure 1: Decision tree compliance testing for food/feed products (with *C* being the measured GMO content and *U* the expanded uncertainty) not labelled for the presence of GMOs and their legal EU enforcement limits. Inconclusive denotes the situation in which the measurement request to detect and identify possible GM events for this taxon cannot be satisfied.

1.2 Procedures for the estimation of measurement uncertainty

MU is generally thought of as applying to quantitative measurements. It is a parameter which characterises a measurement and should take account of all sources of uncertainty in a measurement process. MU is linked to the individual measurement performed. Therefore each control laboratory has to evaluate the specific MU for a measurement result obtained under defined conditions.

Figure 2 illustrates at which stages of the measurement process contributions to the estimation of the MU can be typically expected and which data can be used to estimate them.

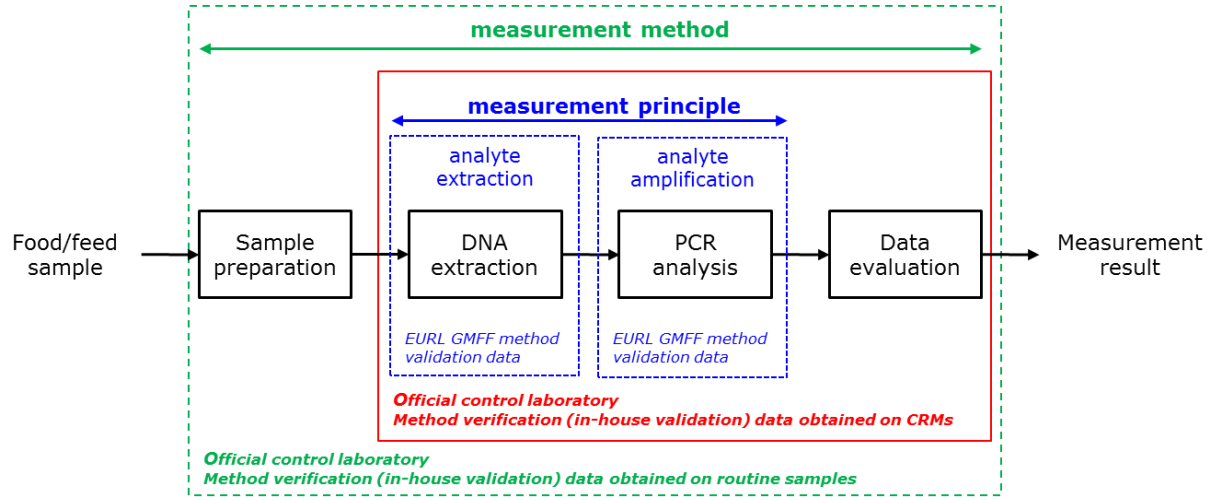

Figure 2: Measurement workflow for GMO quantification and representation of data available for the estimation of the related MU

Sampling (i.e. collection of the samples) often contributes significantly to the overall uncertainty. In most of the cases, it is difficult for laboratories to estimate the uncertainty derived from this step of the control since different protocols are used for the collection of representative portions (i.e. samples) depending by the type of food/feed [9]. Moreover, this step of the analysis is often carried out by other authorities than the laboratories performing the actual analysis. For this reason this guidance document addresses only the MU arising from the measurement method (see Figure 2). EURACHEM [10] and Codex Alimentarius [11] have published guidance on the uncertainty contribution from sampling.

MU arises from the preparation of the sample (reduction of the laboratory sample to test items), from pre-analytical steps (extraction, purification of the DNA), from the measurement itself (qPCR or dPCR) and from the data evaluation including calibration. Generally all sources of uncertainty need to be considered, unless it could be proven that specific uncertainty contributions are negligible.

There is always MU associated with a measurement result, whether it is reported or not. Official control laboratories testing for compliance with regulations (EU) 2017/625 [5], (EC) No 1829/2003 [6], (EC) No 1830/2003 [7] and (EU) 619/2011 [8] must report the measurement result together with the associated MU estimate. Furthermore, the ISO/IEC 17025 international standard also requires laboratories to use, where appropriate, procedures to estimate the related MU [3].

The first widely recognised approach to MU estimation was the 'Guide to Expression of Uncertainty in Measurement' (GUM) [12]. This guide introduced the concept of uncertainty, distinguishing it from errors and laying down general rules for the expression and estimation of MU. It describes the steps involved in the estimation of uncertainty. The GUM places emphasis on the component-by-component approach, in which the method is dissected and incremental calculations of uncertainty are made and eventually added up to provide a combined uncertainty. The correct evaluation of MU associated with a method requires the analyst to look closely at all of the possible sources of uncertainty.

The GUM implements cause and effect diagrams (also referred to as fishbone diagrams) as visualisation aids, and practical studies are carried out to help identify the major sources of uncertainty associated with the measurement. Figure 3 provides examples of possible sources of MU for qPCR measurements. For further details the reader is referred to other documents exploiting this approach [13, 14].

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[9] CEN/TS 15568 (2006) Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Sampling strategies

[10] EURACHEM / CITAC (2007): Measurement uncertainty arising from sampling: A guide to methods and approaches; https://bit.ly/2AVVzSQ

[11] Codex Alimentarius (2013) Codex Principles for the Use of Sampling and Testing in International Food Trade, CAC/GL 83-2013; https://stanford.io/30uMQlk

[12] ISO/IEC Guide 98-3:2008, Uncertainty of measurement -- Part 3: Guide to the expression of uncertainty in measurement (GUM). The HTML version of JCGM 100, on which ISO/IEC Guide 98-3:2008 is based, can be found at https://bit.ly/2AWtBX9.

[13] EURACHEM / CITAC (2012): Quantifying Uncertainty in Analytical Measurement (QUAM), third edition; https://bit.ly/2MDK5X0

[14] M Burns, H Valdivia (2007): A procedural approach for the identification of sources of uncertainty associated with GM quantification and real-time quantitative PCR measurements, European Food Research and Technology (2007) 226: 7-18; https://doi.org/10.1007/s00217-006-0502-y

Once the MU has been estimated for a specific method on a particular sample in a particular laboratory, this estimate can be applied to subsequent results, provided that these results are obtained in the same laboratory under the same conditions and that quality control data confirm the correctness of the estimation. If quality control data are not covered by the MU estimate, major sources of uncertainty may have been incorrectly identified.

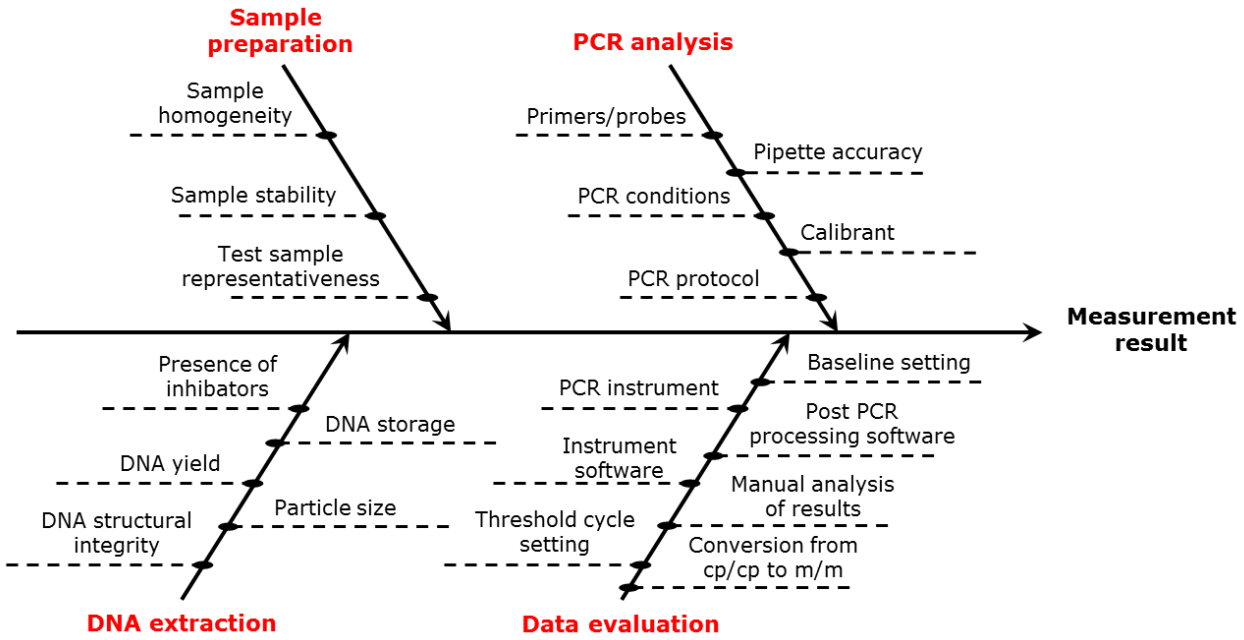

Figure 3: Cause and effect diagram ('fishbone diagram') illustrating a non-exhaustive list of possible sources of measurement uncertainty in the estimation of the GM content of a sample using qPCR (adapted and updated from [14]).

There has been some criticism on the practicability of the approach proposed by the GUM [12] and nowadays two general approaches are distinguished. While the 'bottom-up approach' described in the GUM requires a deep understanding of the measurement method, the 'top-down approach' makes use of existing measurement data.

In order to ensure that the MU covers all uncertainty sources, data used for the 'top-down approach' need to show all the variability which can arise from the preparation of a routine sample. Likewise data from collaborative trials can be used to estimate MU if the collaborative trails covered all steps of the measurement and if the laboratory can prove that it performs at the same level. For methods used for implementation of Regulation (EC) No 1829/2003 [6] or (EU) No 619/2011 [8] this means that the outcome of the collaborative trial has to meet the minimum performance criteria [15] and that the laboratory performance has to fulfil the method verification requirements established by the ENGL [16].
The repeatability standard deviation (RSDr) obtained during the collaborative trials organised for method validation by the EU-RL, can only be used by the laboratory, if their RSDr is smaller or equal to the one observed during method validation. Additionally, the RSDr needs to be amended with an uncertainty component covering the DNA extraction

[15] ENGL guidance (2015): Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing, JRC Technical report, JRC95544; https://europa.eu/!Wu89Ph

[16] ENGL guidance (2017): Verification of analytical methods for GMO testing when implementing interlaboratory validated methods, version 2, JRC Scientific and Technical report, EUR 29015 EN, ISBN 978-92-79-77310-5; https://europa.eu/!hH89Cg

step. Possibilities to estimate the effect and to combine it with the others uncertainty components are outlined in [17].

Interested readers can find more information about the estimation of MU in the following documents:

- ISO/IEC Guide 98-3 - Uncertainty of measurement - Guide to the expression of uncertainty in measurement (GUM) [12];
- EURACHEM/CITAC EURACHEM / CITAC - Quantifying Uncertainty in Analytical Measurement (QUAM) [13];
- IUPAC/ISO/AOAC International protocol for the design, conduct and interpretation of method performance studies [18];
- ISO 21748 Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty evaluation [17];
- Nordic Committee on Food Analysis (NMKL) suggesting the use of experimental data generated within the individual laboratory [19];
- Nordtest report outlining the use of data obtained on routine samples for the estimation of MU [20];
- The AOAC international approach [21].

It is recognised that further procedures for the estimation of MU exist and are being developed.

[17] ISO 21748 (2017): Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty evaluation

[18] Horwitz W (1995): Protocol for the Design, Conduct and Interpretation of Method Performance Studies, Pure Appl. Chem., 67, 331-343; https://bit.ly/2YeJU9X

[19] NMKL (2003): Estimation and expression of measurement analysis in chemical analysis, procedure No5

[20] Magnusson B, Näykki T., Hovind H, Krysell M (2012): Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories, TR 537 Edition 3.1; https://bit.ly/3dNaiyj

[21] Horwitz W (2003): The Certainty of Uncertainty Journal of AOAC INTERNATIONAL, 86, 109-111; https://doi.org/10.1093/jaoac/86.1.109

1.3 Situation of EU official control laboratories

Since the implementation of (EC) No 1829/2003 [6] the availability of a quantification method for GMOs authorised for the EU market is assured. The European Reference Laboratory for GM Food and Feed (EU-RL GMFF) has systematically validated methods [22] for GMOs authorised under (EC) No 1829/2003 [6]. The qPCR methods are tested in collaborative trials with at least 12 participating laboratories per trial. The majority of these trials were conducted using extracted genomic DNA. Matrix effects and DNA extraction methods are tested in a separate step.

Likewise (EC) No 1829/2003 [6] and (EU) No 619/2011 [8] ensure that CRMs are accessible to all laboratories. These CRMs are intended to be used for the calibration of the validated qPCR method. Consequently, the CRM establishes together with the EU-RL GMFF validated method the reference system for the quantification of a specific GMO event.

For the implementation of the two GMO thresholds in EU legislation no maximum acceptable MU has been fixed. Instead minimum performance requirements for the applied measurement methods were set by the ENGL [15], above these values the method is not suitable for legal compliance testing. For the implementation of the measurement methods an in-house validation or method verification is required. Guidance on this can be found in a related ENGL document [16]. The data generated during method verification can be used to estimate MU.

This situation leads to the general recommendation for control laboratories to base their MU estimation on data obtained on routine samples, or if such samples are not yet available to base the MU estimation on measurements performed on CRMs.

The EU-RL GMFF method validation data derived from genomic DNA extracts can be used to estimate the additional uncertainty contribution related to the DNA extraction. This can be achieved using the approach outlined in ISO 21748 [17]. The laboratory has to verify that its performance is within the performance limits of the collaborative trial.

The methods validated by the EU-RL GMFF can be found on the corresponding webpage [22]. Further methods validated in collaborative trials can be found in ISO 21570 [23].

[22] Homepage of the European Union Reference Laboratory for GM food and feed; http://gmo-crl.jrc.ec.europa.eu/

[23] ISO 21570 (2005): Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Quantitative nucleic acid based methods

2 Estimating measurement uncertainty

This guidance document recommends estimating MU for the whole measurement method using results obtained from routine samples, or derived from the intermediate precision, reproducibility standard deviation and combined with the uncertainty contribution due to bias.

The following two approaches are presented:

1. Estimation of MU using data obtained on routine samples
   (see 2.1 and example in Annex III);
2. Estimation of MU using data obtained on one or more matrix CRM in the frame of method verification
   (see 2.2 and example in Annex IV).

It should be noted that these two approaches have a clear ranking. Whenever possible, a laboratory should use approach 1. Only when no routine samples are available should approach 2 be followed. Likewise, the estimation of MU should be carried out once more when approach 2 was followed and when routine samples become available.
In case the laboratory has no access to routine samples and no matrix CRMs are available, the control laboratory is unable to generate meaningful data which can be used to estimate MU. After having verified that the GMO quantification method validated by the EU-RL-GMFF is properly implemented (see [16]), the laboratory assumes a standard MU of 25 % for values measured above 2 g/kg (0.2 (m/m) %) and standard MU of 35 % for values above the LOQ, but below 2 g/kg. However, as this MU is most likely an overestimation of the real MU, laboratories are asked to move to approach 1 as soon as routine samples become available.

It is important that control laboratories demonstrate that their performances remain consistent over time as it is a requirement for laboratories accredited to ISO/IEC 17025 [3]. Data obtained on reference material or quality control (QC) materials can be used to verify that the estimated MU is realistic and covers the observed scatter of measurement results. If not, this is an indication that the MU might have been underestimated and needs to be re-evaluated.

The estimation of MU must include all steps of the measurement method. Hence, the intermediate precision standard deviation (s_{ip}) should derive from repeated independent analyses of samples that represent the measurement variation within the laboratory (e.g. different operators, stock solutions, new batches of critical reagents, recalibrations of equipment; different matrices, if applicable) at the content level of interest. In particular, samples with a GMO content close to legal thresholds of 9 g/kg (0.9 (m/m) % as stipulated in (EC) No 1829/2003 [6]) and 1 g/kg (0.1 (m/m) % as stipulated in (EC) No 619/2001 [8]) should be included.

The estimation of MU is independent from the unit of measurement, but it needs to be ensured that the unit of measurement is used consistently throughout the whole MU estimation. EU legislation requires expressing GMO measurement results in mass fractions (m/m, i.e. g/kg). Therefore, it is recommended, whenever possible, to use mass fractions and to avoid conversions.

MU estimates should be updated taking into account the additional results available. Once new results are generated, it is advised to review and remove older results from the estimation of the MU.

2.1 Estimation of MU using data obtained on routine samples

The first approach is recommended to laboratories having access to routine samples, since the uncertainty contribution related to the nature of the samples are covered by the MU estimation. This approach is in agreement with the NMKL and Nordtest procedures [19, 20]. In the absence of routine samples, MU has to be estimated using matrix CRMs. However, this second approach does not take into account the contribution due to DNA extraction from routine samples and is therefore prone to underestimate MU.

Thompson *et al.* [24] presented the general concept of the 'uncertainty function' (u) (Equation 1 and Figure 4) which depends on a parameter 'α' describing the constant contribution at GMO contents close to the limit of detection (LOD), and of a parameter 'β' representing the constant relative standard deviation at higher GM contents (C). This relation does not take into consideration the bias (see Section 2.3)

$$u = \sqrt{\alpha^2 + (\beta \cdot C)^2} \qquad \text{Equation 1}$$

Note: Equation 1 is similar to the "fitness" function described in Commission Regulation (EC) No 401/2006 [25] which specifies maximum levels of (standard) uncertainty regarded as fit-for-purpose: $u_f = \sqrt{(LOD/2)^2 + (\beta \cdot C)^2}$

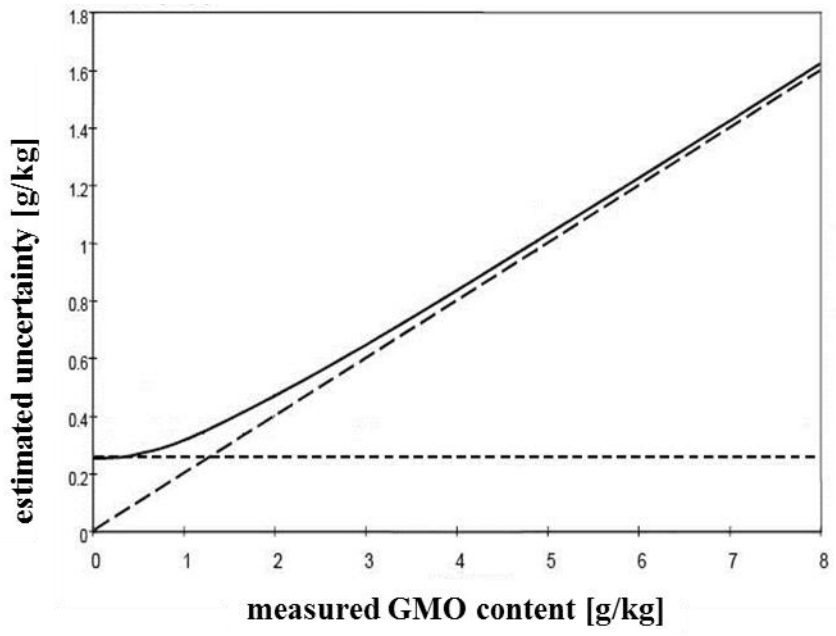

Figure 4: Model for the MU and its relationship to the measured GMO content (bold line). The uncertainty function is composed of a constant uncertainty contribution and a relative standard deviation (dashed lines).

[24] Thompson M, Mathieson K, Damant AP, Wood R (2008) A general model for interlaboratory precision accounts for statistics from proficiency testing in food analysis. Accred. Qual Assur, 13:223-230; https://doi.org/10.1007/s00769-008-0356-z

[25] Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs, , Official Journal of the European Union, L 70/12/; https://europa.eu/!nB66Hq

A minimum of 15 routine samples (N = 15) should be analysed in two independent replicates (n = 2). Independent analyses in this context means that two extractions are carried out for each sample. If a control laboratory performs two PCR measurements per extract, a total of 60 PCR analyses will then be required. An example is provided in Annex III.

Determination of α

Two independent extractions should be performed on at least 6 samples with the mean GMO contents close to the limit of detection (LOD) for the determination of the **constant standard deviation** (α, Equation 1) at low GM content. For each sample (i), the absolute difference ($|d_i|$) of the two replicate results ($C_{1,i}$ and $C_{2,i}$) is calculated as:

$$|d_i| = |C_{1,i} - C_{2,i}| \qquad \text{Equation 2}$$

The constant standard deviation α is calculated as the ratio of the average of the six absolute differences obtained $|\overline{d}|\ (= \sum_1^6 |d_i|/6)$ divided by the factor F_n (Table 1, [20]) which depends on the number of extraction replicates tested:

$$\alpha = \overline{|d|}/F_n \qquad \text{Equation 3}$$

Table 1: Values of the F_n factor as a function of the number of independent replicate measurement results (n) [20, Appendix 8 'Estimation of standard deviation from range' with d_2 being the symbol used for F_n]

n	2	3	4	5	6	7	8	9	10
F_n	1.128	1.693	2.059	2.326	2.534	2.704	2.847	2.970	3.078

Determination of β

Similarly, two independent extractions should be performed on at least nine samples with higher GMO contents for the determination of the **constant relative standard deviation** (β, Equation 1).

At first, the average content ($\overline{C_i}$) and the relative differences ($|d_i|_{rel} = |d_i|/\overline{C_i}$) of the two replicate measurement results are calculated for each sample. The average of the nine relative differences $\overline{|d|_{rel}}(= \sum_1^9 |d_i|_{rel}/9)$ is then divided by the factor F_n (Table 1, [20]) to derive β:

$$\beta = \overline{|d|_{rel}}/F_n \qquad \text{Equation 4}$$

Equation 1 is then applied at the content level of interest (C) using the α and β values determined above. In addition, a bias control check needs to be performed – measuring relevant CRMs to demonstrate the absence of significant bias, and to estimate the corresponding uncertainty contribution to be taken into account (see Section 2.3).

2.2 Estimation of MU using data obtained on CRMs in the frame of the method verification

The availability of routine samples is often the limiting factor of the approach described in Section 2.1. The laboratory may be forced to estimate the MU on fewer samples and/or different sample matrices or by using CRMs.

Since official GMO control laboratories must be accredited to ISO/IEC 17025 [3], the measurement methods applied have to be in-house verified [16] to demonstrate that (i) they were properly implemented and that (ii) they are fit for the intended purpose.

In this context, the control laboratory has to measure the GMO content in a CRM (with a certified mass fraction close to the relevant threshold of the GMO event concerned) on different runs individually calibrated and in several extraction replicates (e.g. p = 5 runs and n = 5 replicates). Depending upon the precision associated with the method (the repeatability and reproducibility), the number of technical replicates and runs can be reduced. However, in the majority of the cases, this can lead to larger uncertainty estimates and is therefore generally not recommended. One-way ANOVA can then be applied to further partition the variance, based on contributions from the between replicates variation (repeatability) and the variation between days. An example is provided in Annex IV.

According to ANOVA, the repeatability standard deviation (s_r) and the contribution to MU due to the between group variation ($s_{between}$) are calculated as

$$s_r = \sqrt{MS_w} \qquad \text{Equation 5}$$

$$s_{between} = \sqrt{\frac{MS_b - MS_w}{n}} \qquad \text{Equation 6}$$

where MS_w and MS_b are the within and between group mean squares respectively. The intermediate precision can then be estimated as

$$s_{ip} = \sqrt{s_r^2 + s_{between}^2} \qquad \text{Equation 7}$$

However, if a laboratory applies an in-house verified method to analyse several (n) replicates of an unknown sample under repeatability conditions, the uncertainty of the mean result would be:

$$u = \sqrt{\frac{s_r^2}{n} + s_{between}^2} \qquad \text{Equation 8}$$

A bias control check needs to be performed – measuring the certified GMO content in a CRM to demonstrate the absence of significant bias (b), and to estimate the additional uncertainty contribution to be taken into account (see Section 2.3). Ideally the certified and measured GMO content should be as close as possible to the threshold stipulated in legislation.

Note: Guidance on how to use GMO CRMs which are not available at the adequate GMO content can be found in [4].

Note: The MU estimation based on CRMs may underestimate the real MU, therefore the estimation should be compared to routine measurement results once they become available and the MU estimation shall be repeated, if needed.

Note: The laboratory may consider adding an uncertainty component for the parts which can (currently) not be investigated due to the lack of samples representative for routine analysis. Such an additional uncertainty component can for instance be estimated on the basis of observations made with other species and/or matrices.

2.3 Bias control and bias uncertainty

Replicate measurements of a CRM are required to estimate a bias. The bias (b) is calculated subtracting the certified value (C_{CRM}) from the average of the measured results ($\overline{C_i}$):

$$b = \overline{C_i} - C_{CRM} \qquad \text{Equation 9}$$

The standard uncertainty associated with the bias (u_{bias}) is obtained by combining the standard uncertainty associated with the average measurement result ($u(\overline{C_i})$) and the one associated with the CRM (u_{CRM}):

$$u_{bias} = \sqrt{u_{CRM}^2 + u(\overline{C_i})^2} \qquad \text{Equation 10}$$

where u_{CRM} is calculated dividing the expanded uncertainty provided in the CRM certificate (U_{CRM}) by the corresponding coverage factor (k), while $u(\overline{C_i})$ derives from the standard deviation (s_{Ci}) of the n replicate measurements:

$$u(\overline{C_i}) = s_{Ci}/\sqrt{n} \qquad \text{Equation 11}$$

Note: The average of measurement results has a much smaller uncertainty than a single measurement result since systematic errors are neglected in this approach.

No significant bias is detected when the absolute value of the bias is smaller than the expanded uncertainty of the bias (U_{bias}):

$$|b| < U_{bias} \ (= 2 * u_{bias}) \qquad \text{Equation 12}$$

In case a significant bias is detected the cause of such bias needs to be identified and corrected for. Ideally the experimental protocol is to be modified until no bias is found.
If the cause of the bias cannot be eliminated, it has to be investigated whether the bias is a constant factor for all GMO contents measured or whether the bias is a relative factor depending on the GMO content measured. In the first case the (positive or negative) correction factor needs to be added to the measurement result, in the second case the correction factor needs to be multiplied with the measurement result. The uncertainty related to the bias check needs to be added, even if the bias was corrected for.

Note: Guidance on how to use GMO CRMs for bias control, if they are not available in the adequate GMO content can be found in [4].

Note: Several approaches to calculate a bias are described in GUM [12]; they have to be considered carefully as a bias may be a constant or proportional factor towards the GMO content. However, investigations like this require access to a higher number of GMO samples.

Note: The factor 2.8 mentioned in the Nordtest report [20] is not a coverage factor k; it should be used to check whether the estimated MU is applicable for measurements on a new sample. In case of an inhomogeneous sample or the method being out of statistical control, MU may not be applicable. If the absolute

difference between two measurements is higher than "2.8 times the standard deviation", MU has to be reconsidered and /or the sample homogeneity questioned.

Note: The same CRM used for the bias control should not be used for calibration. In case this cannot be avoided the analysis of a CRM with a low GM content calibrated with the diluted extracts of a CRM with a higher GM content should be considered.

2.4 Combined uncertainty

The combined uncertainty (u_c), expressed in g/kg, is calculated by combining the uncertainty due to bias (u_{bias}) and the measurement uncertainty (u, derived from Equation 1 or 8):

$$u_c = \sqrt{u^2 + u_{bias}^2} \qquad \text{Equation 13}$$

Note: In the case of Equation 8, u_r and u_{bias} are correlated since the repeatability component will be included twice. However, this double contribution may be negligible compared to the between-day variation (cf. intermediate precision).

2.5 Expanded uncertainty for reporting

The expanded uncertainty (U) corresponding to a confidence level of 95 % is then calculated as:

$$U = k * u_c \qquad \text{Equation 14}$$

where k is the coverage factor. This depends on the degrees of freedom (df = n-1) and the chosen confidence level, and can be estimated from a two-tailed student t-distribution. However, a coverage factor k = 2 can be used, provided that the minimum number of samples and replicates recommended in this guidance document are measured. For the MU estimation using data obtained on routine samples this is N = 15 each measured in 2 replicates (n = 2, see Annex III), for the MU estimation using data obtained on in-house verification data this is N = 5 (equal to the number of days) measured in 5 replicates (n = 5, see Annex IV).

2.6 Reporting measurement uncertainty

For compliance control with labelling thresholds of 0.9 % for authorised GMOs [6] and the feed acceptance threshold of 0.1 % (referred as minimum required performance limit) for GMOs in the authorisation procedure or for which the authorisation has expired [8], measured results above the limit of quantification (LOQ) should be reported as

$$C \pm U \text{ g/kg } (k = 2) \qquad \text{Equation 15}$$

where C is the average of the measured GMO contents (in a given sample).

The laboratory should explain how the MU has been calculated. An explanatory note could be provided to ease communication.

2.7 The specific situation of stacked GMO events

Regulations (EC) No 1829/2003 and (EU) No 619/2011 set a threshold for the sum of authorised [6] accepted [8] GMO content on ingredient basis. As a consequence GMO events in a food/feed sample need to be added up per species (e.g. all soya GMO events).
Within the EU single GMO events and stacked GMO events (composed out of several single events) are authorised for the food/feed market.

Quantitative PCR and dPCR can discriminate between single GMO events and stacked GMO events if the measurements are carried out on individual seeds. However, statistical evaluation has to be applied to conclude how representative the outcome of several seeds tested is for the whole seed lot. No discrimination between single events and stacked events is currently possible in food/feed samples.

Laboratories should always report the measurement results such that no information is lost, i.e. for each single event per species. Competent Authorities may develop methodologies to extrapolate the results of detection of single events in the context of stacked events. Such methods should be based on science, taking into account available information about the product, evidence of adventitious and technical unavoidable presence as well as the underlying labelling and traceability obligations.
For single events and if there is more than one ingredient per taxon the contents need to be summed up per ingredient.

2.8 Compliance assessment using measurement uncertainty

EU legislation [5] and international standards [3] require that competent authorities estimate the MU associated with their measurements, in order to be able to decide/conclude whether a measurement result falls within the specification for food and feed control purposes. In practice, the analyst will determine the measurement result which includes the estimated MU applicable to the measured GMO content. The value obtained by subtracting the expanded uncertainty from the reported GMO mass fraction, is used to assess compliance. Only if that value is larger than the threshold stipulated in legislation, is it assumed 'beyond reasonable doubt' (at a confidence level of 95 %) that the content of the analyte in the sample is larger than the threshold stipulated in legislation (see Figure 5).

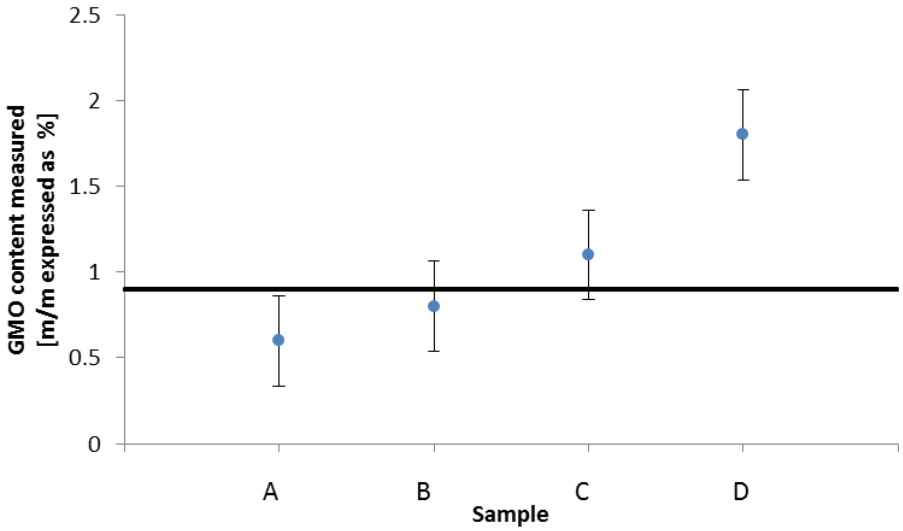

Figure 5: Measurement results and their expanded uncertainty obtained on samples A, B, C and D. The bold horizontal line indicates the labelling threshold. Only sample D needs to be labelled to contain GMO above the legal threshold of a mass fraction of 0.9 %.

The estimated MU must be reported as part of the measurement result. The uncertainty is of particular importance when the range of the expanded uncertainty encompasses the legal limit.

3 Estimation of MU for dPCR measurement results

Digital PCR (dPCR) does not require the use of a DNA calibrant and measures DNA copy number ratios. Hence a conversion (from copy number ratio into mass fraction) is necessary. The general principle is to relate a measurement result to a GMO quantity embedded in a specified CRM either directly or via one single conversion factor (CF_{CRM}) per event. This conversion factor and its related uncertainty need to be determined precisely for each CRM batch. The uncertainty associated with this CF_{CRM} must be integrated into the measurement uncertainty of the final results expressed in GM mass fraction [26, 27]. The CF_{CRM} are established and published by the EU-RL GMFF [22].

The recommended 'top-down approach' to estimate the MU related to qPCR measurement results based on data obtained on routine samples, facilitating the estimation of the intermediate precision of a laboratory, also works for dPCR. Additionally the following uncertainties need to be considered:

- $u_{CF(CRM)}$ - the uncertainty of the conversion factor CF_{CRM} (used to convert copy number ratio results produced by dPCR into mass fraction results);
- $u_{volume\ (dPCR)}$ - the uncertainty of the size of the partition or droplet size in which the dPRC reaction takes place. According to [28] the uncertainty related to the dPCR volume can be estimated as 1.7 %.

[26] P Corbisier, A Barbante, G Berben. W Broothaerts., M De Loose., H Emons, T Georgieva, A Lievens, M Mazzara., N Papazova, E Perri., S Sowa, D Stebih., V Terzi, S Trapmann (2017) Recommendation for the unit of measurement and the measuring system to report traceable and comparable results expressing GM content in accordance with EU legislation, Publications Office of the European Union, LU, EUR 28536 EN, ISBN 978-92-79-66971-2; https://europa.eu/!xh67dW

[27] P Corbisier, H. Emons (2019) Towards metrologically traceable and comparable results in GM quantification Anal. Bioanal. Chem. 411, 7-11; https://doi.org/10.1007/s00216-018-1457-0

[28] K R Emslie, J L H McLaughlin, K Griffiths, M Forbes-Smith, L B Pinheiro, D G Burke (2019) Droplet Volume Variability and Impact on Digital PCR Copy Number Concentration Measurements, Anal. Chem. 91, 4124−4131; https://bit.ly/30odPPN

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ANNEX I: Parameters and symbols

In this annex parameters and symbols used for the various calculations are explained. Abbreviations can be found in the glossary.

α	constant variation at contents close to the detection limit
β	constant relative standard deviation at high content
b	bias
C	GMO content (in a given sample)
CF_{CRM}	conversion factor (for the conversion from copy number ratio into mass fraction)
F_n	factor used to estimate s from a range, depending on the number of measurements)
C_{CRM}	certified mass fraction of a CRM`
df	degrees of freedom
d_i	difference between results of sample i
k	coverage factor
n	number of independent measurements (based on independent extraction replicates)
N	number of samples
MS_{within}	mean square within (used to calculate the average of all variances between the sample
$MS_{between}$	mean square between (used to calculate the average of all variances within the sample
s	standard deviation
s_r	repeatability standard deviation (related to within-day variation)
$s_{between}$	standard deviation related to between-day variation
s_{ip}	intermediate precision standard deviation
u	standard uncertainty
u_{bias}	standard uncertainty of the bias
u_c	combined standard uncertainty
u_{CRM}	standard uncertainty of the certified value (of a CRM)
u_r	repeatability standard uncertainty
U	expanded standard uncertainty
U_{CRM}	expanded standard uncertainty of the certified value (of a CRM)
%	denoting parameters expressed relative

ANNEX II: Definitions applicable to GMO analysis

bias

Difference between mean measured value from a large series of test results and an accepted reference value (a certified or nominal value). The measure of trueness is normally expressed in term of bias [20].

combined standard uncertainty

standard uncertainty of the result of a measurement when that result is obtained from the values of a number of other quantities, equal to the positive square root of a sum of terms, the terms being the variances or covariances of these other quantities weighted according to how the measurement result varies with changes in these quantities [12]

expanded uncertainty (*U*)

The expanded uncertainty is the interval within which the value if the measurand is believed to lie with a higher level of confidence. *U* is obtained by multiplying the combined standard uncertainties by a coverage factor *k*. The choice of the factor k is based on the level of confidence desired (adopted from [13]).

intermediate precision

The standard deviation of test results obtained within the one laboratory under intermediate precision conditions, which resemble the variations occurring within one laboratory.

limit of quantification (LOQ)

The limit of quantification of an analytical procedure is the lowest amount or concentration of analyte in a sample, which can be quantitatively determined with an acceptable level of precision and accuracy (modified from [29]).

measurement uncertainty (MU)

Parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand [30].

method verification

Provision of objective evidence that a laboratory can adequately operate a method, achieving the performance requirements for the sample matrices to which the method is being applied [31].

repeatability standard deviation (RSDr)

The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time (adopted from [15]).

standard uncertainty (*u*)

Uncertainty of the result of measurement expressed as a standard deviation [12].

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[29] ISO/FDIS 24276 (2005): International Standard, Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – General requirements and definitions

[30] ISO/IEC Guide 99:2007: International vocabulary of metrology — Basic and general concepts and associated terms (VIM). The HTML version of JCGM 200, on which ISO/IEC Guide 99:2007 is based, can be found at https://bit.ly/3dK4xkY.

[31] Weitzel, M L J, Lee S M, Smoot M, Viafara N, Brodsky M (2007): ALACC guide: How to meet ISO 17025 requirements for method verification; https://bit.ly/3cKGKA1

ANNEX III: Example – Estimation of MU using data obtained on routine samples

Illustrative data set of 15 routine samples containing different content levels of GTS-40-30-2 soya, analysed twice with two independent DNA extractions. The results were used to estimate the constant uncertainty contribution (α) and the proportional contribution (β).

Results sorted by increasing GMO content measured.

All values are expressed in g/kg (except sample # and relative difference).

Sample	$C_{1,i}$	$C_{2,i}$	$\lvert d_i \rvert$
1	1.04	1.01	0.03
2	1.55	1.47	0.08
3	1.42	1.70	0.28
4	1.77	1.74	0.03
5	2.20	3.20	1.00
6	2.95	2.54	0.41

The replicates of the routine data sets show differences in their relative standard variation. In two cases (samples 5 and 9) the variability threshold of 33 % recommended in [32] (in Annex VII) is exceeded. Whilst measures against inhomogeneity should be taken, results obtained on samples with a complex matrix should still be retained.

6 samples at "low" GM content (Note, that the separation into 'low' and 'high' content is artificial, aiding the estimation of the various uncertainty components. The continuity of the data causes, in the worst case, that the constant uncertainty contribution (α) is overestimated, whilst omitting the estimation of α is likely to result in an underestimation of the overall uncertainty.)

$|d_i| = |C_{1,i} - C_{2,i}|$
$|\overline{d}| = \sum_1^6 |d_i|/6$ = 0.31 g/kg (average difference)

n = 2 (replicates) ➔ F_n = 1.128 (cf. Table 1)
$\alpha = |\overline{d}| / F_n$ = 0.31 / 1.128 = **0.27 g/kg**

[32] ENGL (2014) Guidelines for sample preparation procedures in GMO analysis, Publications Office of the European Union, LU, EUR 27021 EN, ISBN 978-92-79-44704-4; https://gmo-crl.jrc.ec.europa.eu/ENGL/docs/WG-SPP-Final-Report.pdf

Sample	$C_{1,i}$	$C_{2,i}$	$\overline{C_i}$	$\lvert d_i \rvert$	$\lvert d_i \rvert_{rel}$
7	2.80	3.40	3.10	0.60	0.19
8	3.47	4.14	3.81	0.67	0.18
9	4.00	6.00	5.00	2.00	0.40
10	6.98	7.00	6.99	0.02	0.00
11	9.98	9.31	9.65	0.67	0.07
12	14.93	16.71	15.82	1.78	0.11
13	20.86	17.33	19.09	3.53	0.18
14	17.50	22.02	19.76	4.52	0.23
15	28.00	25.00	26.50	3.00	0.11

9 samples at **"high" content**

$\overline{C_i} = (C_{1,i} + C_{2,i}) / 2$ (average content for sample *i*)

$|d_i|_{rel} = |d_i| / \overline{C_i}$ (relative difference for sample *i*)

$\overline{|d|_{rel}} = \sum_1^9 |d_i|_{rel}/9 = 0.16$ (average relative difference)

n = 2 (replicates) ➔ F_n = 1.128

$\beta = \overline{|d|_{rel}} / F_n$ = 0.16 / 1.128 = **0.15**

The CRM ERM-BF410d containing GTS 40-30-2 was used for bias control.

Six independent measurements were carried out, and the following results (C_i) were obtained, expressed in g/kg.

C_i	11.0	10.9	12.1	11.2	10.7	10.9

- Uncertainty contribution due to bias (u_b):

C_{CRM} = 10.0 g/kg

U_{CRM} = 1.6 g/kg (k = 2) (expanded uncertainty)

u_{CRM} = 1.6 / 2 = 0.8g/kg

n = 6

$\overline{C_i}$ = 11.1 g/kg (average result for CRM)

s_{Ci} = 0.50 g/kg (standard deviation)

$u(\overline{C_i}) = s_{Ci}/\sqrt{n}$ = 0.50 / √6 = 0.20 g/kg

bias: $|b| = |\overline{C_i} - C_{CRM}| = |11.1 - 10.0|$ = 1.1 g/kg

$u_{bias} = \sqrt{u_{CRM}^2 + u(\overline{C_i})^2}$ = √ [0.8² + 0.2²] = **0.83 g/kg**

- No significant bias detected, since $|b| < 2\ u_b$ (1.1 < 2 * 0.83)

A content of 15.0 g/kg of GTS 40-30-2 was measured in the unknown sample. • Combined uncertainty (u_c)	C = 15.0 g/kg (measured result for the unknown sample) $u_c = \sqrt{[\alpha^2 + (\beta * C)^2 + u_b^2]} = \sqrt{[0.27^2 + (0.15*15)^2 + 0.83^2]}$ = **2.41 g/kg** • It can be concluded that, in this illustrative example, the major contributor to the combined uncertainty is $\beta * C$, which is heavily influenced by the value of C.
• **Expanded uncertainty (U)**	**$U = 2 * u_c = 2 * 2.41 = 4.82$ g/kg, rounded to 4.9 g/kg** • Uncertainties are rounded in a way that the uncertainty introduced by the rounding corresponds to 3-30 % of the uncertainty.
• The expanded uncertainty is rounded to two significant figures and the final result to be reported is:	**Mass fraction of GTS 40-30-2 in soya:** **15.0 ± 4.9 g/kg (k =2)** • The same number of significant digits should be given for the value and its uncertainty.

ANNEX IV: Example – Estimation of MU using in-house method verification data using CRMs

A soybean sample is measured on one day in three independent extractions for compliance testing with EC No 1829/2003 [6] for the GMO event DAS-44406-6. A mean GMO mass fraction of 85.3 g/kg (DAS-44406-6 / total soya) is measured.

During the method verification for the DAS method, repeated measurements were carried out on the CRM ERM –BF436e. The certified mass fraction is provided.

Five independent extracts (replicates) of the CRM were analysed every day, for five days (n_{days} = 5).

The results provided below are expressed in g/kg.

	Day1	Day2	Day3	Day4	Day5
Rep1	113.1	111.8	99.3	94.6	113.6
Rep2	103.2	90	115.7	97.5	112.7
Rep3	87.8	66.9	93	86.5	103.7
Rep4	110.4	82.1	82.3	73.9	89.9
Rep5	120.5	84.3	88.2	86.5	103.2

➔ average: 96.43 g/kg

N = 1 day

C = **85.3 g/kg** (n = 3) (measured result for the sample)

C_{CRM} = 100.0 g/kg (certified mass fraction)

U_{CRM} = 9.0 g/kg; k = 2 (expanded uncertainty of the certified value)

u_{CRM} = 4.5 g/kg (standard uncertainty)

Note: Measuring on different days ensures that the intermediate precision can be properly evaluated. The same effect can be reached using different PCR plates, individually calibrated.

ANOVA: Single Factor

Source of Variation	*SS*	*df*	*Mean square (MS)*	*F*	*P-value*	*F crit*
Between Groups	1711.8	4	427.95	2.8230	0.0524	2.8661
Within Groups	3031.9	20	151.59			
Total	4743.7	24				

- Intermediate precision

$s_r = \sqrt{MS_w}$ = √151.59 = 12.31 g/kg (repeatability standard deviation)

$s_{between} = \sqrt{\frac{MS_b - MS_w}{n}}$ = √[(427.95 - 151.59)/5] = 7.43 g/kg

$s_{ip} = \sqrt{s_r^2 + s_{between}^2}$ = √[12.31^2 + 7.43^2] = 14.38 g/kg

The unknown soybean sample was measured on 1 day in 3 independent extractions; • Uncertainty contribution due to between and within group variation:	$u = \sqrt{\frac{s_r^2}{m} + s_{between}^2}$ = √[(12.31²/3) + 7.43²] = **10.29 g/kg**
The uncertainty of the bias can be estimated from the method validation data (see results obtained for CRM ERM-BF436e, day 1; *n* = 5 replicates). • Uncertainty contribution due to bias (u_b):	$\overline{C_{day1}}$ = 107.0 g/kg (average) $s(C_{day1})$ = 12.4 g/kg (standard deviation) $u(C_{day1}) = s(C_{day1})/\sqrt{n}$ = 12.4 / √5 = 5.54 g/kg bias: $\|b\| = \|\overline{C_i} - C_{CRM}\|$ = |107.0 – 100.0| = 7.0 g/kg $u_{bias} = \sqrt{u_{CRM}^2 + u(\overline{C_{day1}})^2}$ = √[4.5² +5.54²] = **7.14 g/kg** • No significant bias detected, since $\|b\| < 2 * u_b$ (7 < 2*7.14)
• Combined uncertainty (u_c)	$u_c = \sqrt{(u^2 + u_{bias}^2)} = \sqrt{(10.29^2 + 7.14^2)}$ = **12.52 g/kg**
• Expanded uncertainty (U)	$U = 2 * u_c = 2 * 12.52$ = **25.04 g/kg** (k = 2)
• The expanded uncertainty is rounded to two significant figures and the final result to be reported is:	**Mass fraction of DAS-44406-6 in soya:** **85 ± 25 g/kg (*k* =2)**

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