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Training Manual on GMO Quantification: Proper Calibration and Estimation of Measurement Uncertainty

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Training Manual on GMO Quantification: Proper Calibration and Estimation of Measurement Uncertainty

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Abstract

The content of this manual is based on the training course that was organised on the premises of the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (Geel, BE) at the end of 2013.

The training manual complements the training course that was intended to improve the quality of measurement results obtained when quantifying genetically modified organisms (GMO) in food and feed. Both, the training course and this manual were developed in line with the current EU GMO legislation [1,2].

The manual is addressed to laboratory managers and practitioners in analytical laboratories who perform GM quantification measurements and use reference materials for calibration, quality control and method validation including in-house verification. It is also intended for analysts who need to assess measurement uncertainties as required by (EC) No 1829/2003 [1], (EC) No 619/2011 [2] and ISO/IEC 17025:2005 [3].

This training document has been written by JRC-IRMM upon request of the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) to further improve the reporting of National Reference Laboratories (NRLs) nominated under Regulation (EC) No 882/2004 [4] and official GMO control laboratories within the EU.

This manual is organised in four chapters covering the proper calibration of PCR methods, the estimation of measurement uncertainty, the establishment of metrological traceability of a measurement result and the way to prove the trueness of measurement results.

The training manual is a didactic support of a previous guidance document that outlines issues related to the estimation of measurement uncertainty (MU) in the GMO sector [5]. The training manual is also in line with the European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs, that is intended for laboratories that are acquiring or are holding a flexible scope of accreditation according to ISO/IEC 17025 [6].

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Glossary

AOAC	AOAC International
AOCS	American Oil Chemists' Society
bp	basepair
cp	copy
Cq	quantification cycle
CRM	certified reference material
EC	European Commission
ENGL	European Network of GMO Laboratories
ERM [®]	Trademark of European Reference Materials
EU	European Union
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
GM(O)	genetically modified (organism)
HGE	haploid genome equivalent
IHCP	Institute for Health and Consumer Protection
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
JRC	Joint Research Centre
LLP	low level presence
LOQ	limit of quantification
MPR	minimum performance requirements
MU	measurement uncertainty
<i>N</i>	number of samples
<i>n</i>	number of measurement replications on the same sample
NRL	National Reference Laboratory
PCR	polymerase chain reaction
qPCR	quantitative (real-time) PCR
QA	Quality Assurance
QC	Quality Control
SI	International System of Units
<i>u</i>	standard uncertainty
<i>U</i>	expanded standard uncertainty
<i>u</i> _{bias}	standard uncertainty associated with bias
<i>u</i> _c	combined standard uncertainty
<i>u</i> _{ip}	standard uncertainty associated with intermediate precision
<i>u</i> _{var,rel}	proportional, relative standard uncertainty associated with measurements above the LOQ
<i>u</i> _r	standard uncertainty associated with repeatability
<i>u</i> _{rel}	relative standard uncertainty
<i>u</i> ₀	constant standard uncertainty contribution associated with measurements at the LOQ
<i>w</i>	mass fraction

Introduction

The European Union has legislation to regulate the placing on the market of food and feed consisting of, containing or produced from GMOs. Food and feed products which contain, consist of or are produced from GMOs in a proportion higher than 0.9 per cent of the food and feed ingredient considered individually or food or feed consisting of a single ingredient, need to be labelled [1]. The labelling threshold is applicable for adventitious presence of GMOs, while GMOs added on purpose need to be labelled independent from a threshold. Additionally feed may contain 0.1 mass per cent of a GM event which was previously authorised in the EU or for which an authorisation process is pending [2].

During the EU authorisation process, the applicant seeking authorisation for a GM event needs to ensure that a reference material for the GM event is available and that an event-specific quantification method has been successfully validated and is published by the EURL-GMFF. Successfully validated methods fulfil the minimum performance criteria laid down by the EURL-GMFF in [7]. As a consequence, (certified) reference materials (CRMs)¹ and validated methods are publically available to GMO testing laboratories for the GM events covered by this guidance document.

The NRLs and the official control laboratories benefit from detailed legislative tools, technical documents or guidance documents and CRMs. However, assessment of the technical implementation of the EU legislation in this field shows that a number of NRLs or official control laboratories have difficulties to properly report and to express their results in an appropriate measurement unit.

Indeed, notwithstanding the overall satisfactory outcome of most comparative testing rounds of the EURL-GMFF, only 58% of participants provided information on measurement uncertainty in a complete and consistent manner in the last comparative testing report EURL-CT-02/13 [8].

The difficulty can be easily understood as there is a lack of coherence between the EU legal requirements and the approaches followed by laboratories when performing the measurement [9, 10]. The estimation of uncertainty associated with the measurement result remains a difficult topic as noticed during the comparative testing campaigns organised by the EURL-GMFF, despite the fact that a number of guidance documents and specific applications notes have been published to help to correctly implement the GMO legislation in Europe [5, 6, 11, 12]. Therefore, the Steering Committee of the European Network of GMO Laboratories (ENGL) asked the JRC-IRMM to organize a training course that should tackle those challenging topics.

This manual is organized in four parts. For each part a summary of the presentation is provided, followed by the presentation itself. Practical exercises and solutions provided during the training course are not included in this training manual. Part I deals with the proper calibration of quantitative PCR. In part II, an approach to estimate measurement uncertainty (MU) is provided. How GMO measurement results can be made traceable is explained in part III. Finally, part IV explains how to check for measurement bias by using CRMs.

¹ Authorisation according to (EC) No 1829/2003 requires the availability of a reference material. The low level legislation for feed (EC) No 619/2011 requires the availability of a certified reference material.

Part I: Calibration of quantitative PCR

Introduction

Quantification of genetically modified (GM) content in food or feed products relies mainly on the detection, amplification and relative quantification of well-defined DNA sequences. The relative quantification is providing a DNA fragment ratio arising from relating the measured amount of a specified DNA sequence to the measured amount of another DNA sequence. The method is based on measuring the amount of specific DNA targets extracted from a sample that can be amplified by an enzymatic chain reaction. The latter process, called quantitative polymerase reaction (qPCR), has been established in Europe as the method of choice to quantify the GM content over the last 15 years. It should be kept in mind that qPCR needs to be calibrated to convert a measured fluorescence signal into a quantity characterising the amount or mass of the DNA fragment of interest. The kind of this quantity is intrinsically determined by the calibrant used.

Hence qPCR needs to be calibrated, and the calibrant used determines the measurement scale as well as the measurement unit in which a test result is reported. The choice of this measurement scale is crucial and changing from one scale to another may introduce a bias and increases the related measurement uncertainty. As an example, the measurement of the temperature with a thermometer calibrated in degrees Celsius provides a temperature which is expressed in degrees Celsius. The measured temperature can easily be converted into another measurement unit as there is a clear mathematical relationship between degrees Celsius and degrees Fahrenheit or Kelvin. For GM quantification such an unequivocal relationship between DNA copy number and mass fraction does not exist.

In this presentation, we describe how to choose the most appropriate calibrant, where those calibrants can be obtained and finally we provide a direct approach to calculate GM content in mass fraction or in DNA copy number ratio. An indirect approach that involves the use of a so-called conversion factor which is applied twice to transform the measurement result into another measurement unit and later back to the measurement unit of the calibrant is also discussed.

What material should I use to establish a calibration curve?

Different types of materials can be used to realise calibration curves for qPCR. The most common materials are either matrix materials from which DNA needs to be extracted or ready-to-use solutions of genomic DNA (gDNA) or even plasmid DNA (pDNA). Indeed, an analyst can decide to extract gDNA from CRMs composed of different mixtures of ground plant materials that have been certified for their GM mass content. DNA is extracted from each individual mixture to generate a calibration curve for the GM event. For the reference gene, chosen as a normaliser in the qPCR assays, the gDNA extracted from the powder material will need to be further diluted in a buffer or in nuclease free water to decrease progressively the amount of reference gene in the assay and, by doing so, generate a calibration curve for the reference gene.

Genetically pure CRMs certified for their GM mass content represent another source of calibrant. Alternatively, ready-to-use solutions of gDNA extracted from genetically pure GM or non-GM materials can also be purchased for some events. For a few GM events, CRMs

certified for their DNA copy number ratio or pDNA certified to have a one-to-one (or 1:1) ratio between the GM event and the reference gene are available.

The choice of the material used for calibration determines how the calibration curves has to be established and which quality criteria should be used to evaluate the validity of those calibration curves.

From where are those materials available?

The availability of CRMs is often insufficiently known. One can, for example, search in the international database for certified reference materials (COMAR) hosted by the German Federal Institute for Materials Research and Testing (www.comar.bam.de). The database provides a list of thousands of certified reference materials (CRMs) produced worldwide by about 220 producers in 25 countries. A number of them are materials certified to contain specific GMOs. The listing of the German Federal Office of Consumer Protection and Food Safety is another source of relevant information as it is especially dedicated to GMO CRMs. From that list, we can conclude that the major GMO CRM providers are the EC-JRC-IRMM and the American Oil Chemists' Society (AOCS). Others, e.g. Eurofins GeneScan, sell extracted gDNA solutions as non-certified reference materials.

How do I make my choice?

To select which is the most appropriate type of certified reference material to generate calibration curves, we recommend using the reference material which is stated in each Commission decision that authorises the placing on the market of products containing, consisting of, or produced from GMOs pursuant to Regulation (EC) No 1829/2003 [1]. For each of such decisions, a reference is provided explaining the accessibility of the reference material. In case of legal dispute, the probability to have a result invalidated because an inappropriate material was used for calibration is low, if a laboratory uses properly the reference material which is referenced in the Commission decision. Those decisions concerning each GM event authorised under Regulation (EC) No 1829/2003 are made available on-line in an EU register of genetically modified food and feed [13].

At this stage, an analyst has made the choice on the reference material that will be used to calibrate the qPCR method for a particular GM event. Next, the analyst has the choice between two calculation approaches, a direct approach or an indirect approach. In the direct approach a CRM with a certified value expressed in one measurement unit is used to report the final result in the same measurement unit. In the indirect approach (or “double conversion approach”) a CRM is also used to establish a calibration curve. However, its certified value is first converted into another measurement unit and eventually converted back into the certified measurement unit to report the final GM content. It is not recommended to convert the measurement unit of a certified value only into another unit, thus performing a so-called ‘single conversion’. Nevertheless, this practice has been already observed in several laboratories.

We will provide examples for both direct and indirect approaches and will start with the direct approach. With the direct approach the calibration can be performed using either CRMs certified for their GM mass fraction or for their DNA copy number ratio.

The direct approach

When CRMs certified for their GM mass fraction (g/kg) are used as calibrant, gDNA needs to be extracted from each CRM using either a DNA extraction method that has been validated by the EURL-GMFF and in-house verified or by an in-house validated DNA extraction method. The amount of extracted DNA can be quantified either by a UV spectrophotometric method using the appropriate molar absorption coefficient [14], or by a fluorometric method such as the PicoGreen® Assay for double stranded DNA or any other preferred method. The extracted DNA concentration is measured to determine the volume of DNA solution needed for the PCR assays targeting the transgene and the reference gene, respectively. It is important to know which amount of DNA has been added in both PCR assays but as the measurement is based on a ratio, a high accuracy of the DNA amount is not required. It is a good practice to verify the intactness or fragmentation status of the gDNA extracted from both calibrant and samples, because high molecular weight DNA extracted from the calibrant will be a sign that the CRM has been stored under appropriate conditions and that the extraction procedure is under control. Equally, high molecular weight DNA extracted from a sample indicates that the sample has not been highly processed. Despite the fact that the PCR targets are often smaller than 200 base pairs, the precise GM quantification can be affected in highly fragmented DNA samples, as shown on artificially degraded soybeans [15].

Once the total DNA concentration has been determined for the reference material and for the samples to be analysed, two calibration curves are made by plotting the number of PCR cycles (C_q values) needed to reach a certain fluorescence level against the logarithm of the amount of DNA in the PCR. The C_q value for the unknown sample is measured and that value is used to calculate the amount of DNA target present in the unknown sample. Ideally a linear regression on the averages of triplicates or on all single data point is calculated to determine two key parameters of the calibration curve: the slope and the Y-intercept. Those two values are needed to convert the C_q values into a DNA amount. The slope and the coefficient of determination are also calculated to verify that the PCR assays are fulfilling the minimum acceptance criteria defined by the EURL-GMFF [16].

The DNA in the PCR assay targeting the reference gene is diluted in buffer or nuclease free water (e.g., dilutions from 150 ng/PCR to 1 ng/PCR are used to establish a calibration curve for the reference gene). To generate a calibration curve for the transgene, 150 ng DNA/PCR extracted from CRMs containing a decreasing amount of GM (e.g., from 50 g/kg to 1 g/kg) are used. To calculate the amount of genetically modified DNA present in the GM assay, an approximation is made. The amount of GM DNA in the assay that has been extracted from a CRM containing 50 g/kg GM (corresponding to 5 % m/m) is arbitrarily considered to be also 5 % in term of GM DNA copies. If, for instance, in 150 ng of DNA 5 % is considered to be genetically modified, this represents 7.5 ng of GM DNA per PCR well. The same proportional approximation is made for the other CRMs that contain a smaller GM mass fraction.

The amounts of transgene and endogene DNA in a DNA solution extracted from an unknown sample are then calculated by converting the measured C_q values into mass values using the two calibration curves and dividing them. The GM mass fraction ratio (transgene versus endogene) is finally multiplied by 100 % to express it as a percentage.

The use of CRMs for the quantification of GMO expressed in DNA copy number ratio is explained in the ERM Application note 5 [11]. A pDNA calibrant containing both the reference and the transgene targets in a 1:1 ratio is used to generate two calibration curves.

For those calibration curves, a starting solution of plasmid is diluted and the serial dilutions are used to generate both calibration curves. The measurement unit on the X-axis in a Cq versus log(copy number) plot is the copy number value of the plasmid. This value is provided on the certificate of the CRM as an indicative value. The quantity of pDNA copies per μL has been determined by UV spectrophotometry. This quantity does not need to be determined with a high accuracy providing that the same calibrant solution is used to generate both calibration curves. It is indeed the ratio of the number of GM targets to reference targets which is certified for the CRM, ensuring that the same amount of GM and reference targets are present in both PCR assays when the same solution of calibrant is used. The results obtained using a CRM certified for its DNA copy number ratio can therefore be used directly to express the result as a copy number ratio.

The indirect approach

For the indirect approach (or the ‘double conversion’ approach), the amounts of DNA in the PCR assay are first converted into DNA copy number taking into account the zygosity of the CRM used for the calibration. The zygosity status of the CRM can be found on the certificate of the CRM. This is a legal requirement under Commission regulation (EU) No 619/2011 for the official control of feed for pending authorisation procedure or for GM material of which the authorisation has expired [2]. This information has been introduced in the legislation because EURL method validations are carried out on genomic DNA and their results have to be converted to approximate mass fractions for estimating if the method meets the 0.1 (m/m) % LOD of the regulation.

To convert “ng” of DNA into “number of GM copies”, not only the zygosity of the material used as calibrant needs to be known, but also the parental origin of the transgene and the relative amount of DNA originating from each of the three main seed tissues. For maize, the seed cover contains two copies of the female genome, but represents only 0.6 % to 3.5 % of the total DNA. The embryo contains one copy of the female and one copy of the male genome, whereas the endosperm contains two copies of the female and one copy of the male genome. The embryo and the endosperm contribute each between 36 to 60 % of the total DNA. A conversion factor can be roughly estimated on the basis of this information and varies around 0.5 (± 0.1) for hemizygous maize. The zygosity of the GM material tested has also to be known. An estimation of the zygosity of the GM material placed on the market is provided in each validation report issued by the EURL-GMFF. This so-called “zygosity of the market” is determined on the control sample provided by the applicant to the EURL-GMFF in charge of the PCR method validation.

The conversion from ng/ μL into haploid genome equivalent (HGE) copies per μL is done by dividing the DNA mass concentration by an average genome mass (i.e. again using an agreed number instead of the genome mass related to the measured samples). The average mass of the genome can be consulted in the RBG Kew Plant DNA C-values database [17].

Having done this, the same approximation as the one used to convert ng of DNA into ng of GM DNA needs to be performed to convert the GM mass fraction into HGE copies of the transgene. In the example provided, 150 ng of DNA extracted from a maize CRM containing 50 g/kg is converted into 54545 HGE copies for the reference gene and 2727 HGE copies of the transgene (representing 5 % of the total number of HGE). This amount needs to be multiplied by the conversion factor taking into account the zygosity of the CRM used (e.g.

0.4 in case of a male GM donor) which gives 1091 HGE copies of the transgene. The ratio obtained needs to be converted again to express the result in mass fraction taking into account the zygosity of the sample analysed. Therefore, the ratio is commonly divided by 0.5 in case of a maize sample.

Conclusion

The results obtained by the direct or the indirect approach will be exactly the same, if an identical conversion factor is used for the CRM and for the sample analysed. All the efforts made to convert ng of DNA into copies of HGE are not needed, if the results are expressed in mass fraction. We therefore recommend using one single measurement scale and one measurement unit only to avoid errors introduced by misusing conversion factors. Taking all those considerations into account, it becomes obvious that comparable results will only be obtained if results are calibrated with a common CRM and the results are expressed in the same measurement unit. Therefore, the corresponding Commission Decisions and (EC) 619/2011 guide the analyst to use a specific CRM and the measurement unit mass fraction.

Part II: Estimation of measurement uncertainty

What is measurement uncertainty?

Every measurement result has an uncertainty inseparably associated with it. Measurement uncertainty describes the possible fluctuations of a measurement result, by defining an interval that contains the true value with high probability. In practical terms, the expanded measurement uncertainty is the part of the measurement result that comes after the \pm sign and describes the possible dispersion of quantity values around the true value with a stated probability [18].

Measurement uncertainty is also a measure of the overall measurement performance. It is the quantitative expression of accuracy and, as such, a combination of information on trueness and precision (Figure 1). Trueness describes how close measurement results are to the true value. It is commonly estimated and expressed as bias. Precision, on the other hand, describes the dispersion of results from replicate measurements on the same or similar samples under specified conditions. It is called repeatability, intermediate precision or reproducibility, depending under which variability conditions the measurements were performed, and numerically expressed by statistical parameters such as standard deviation or variance. Measurements performed on one day, in the same lab, on the same instrument and by the same operator are performed under repeatability conditions. If these measurements are, for instance, done on different days, they are performed under intermediate precision conditions. And if measurements are carried out in different labs (possibly also there on different days, in addition by different operators and on different instruments), they are performed under reproducibility conditions.

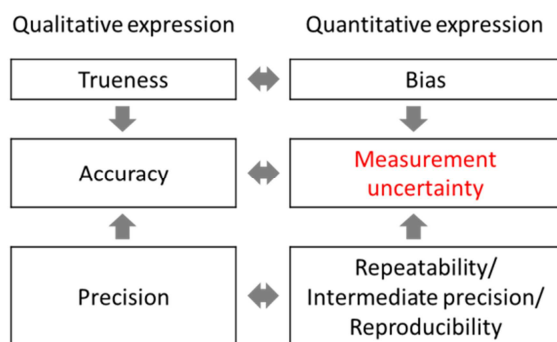


Figure 1: Relationship between measurement uncertainty and measurement performance characteristics.

Who needs measurement uncertainty?

Both parties involved in a measurement need actually to know the measurement uncertainty. The customer needs it to evaluate if the performance of a potential contract laboratory is fit-for-purpose for the foreseen task, to compare results between laboratories, or to check a product for compliance with a legal limit. But also the laboratory performing the measurements needs it, to know the quality of its measurements and to improve it, if necessary, and to comply with ISO/IEC 17025:2005 [3] and/or EU legislation [2].

Where does it come from?

Every single step of an analytical procedure is susceptible to variability. In the case of GMO quantification by real-time PCR, this includes sample preparation, DNA extraction, PCR measurement, and data evaluation. During each step, the operator, the conditions in the laboratory, the instruments or the reagents used will pose a source of variability. This leads to a fluctuation of measurement results, the degree of which is quantified as measurement uncertainty. Measurement uncertainty is associated with a stated value attributed to the GMO content. A change in the measured value may result also in a change of the associated uncertainty [18].

How to estimate measurement uncertainty?

If an unknown sample has been measured for its GM content, the measurement uncertainty associated with this result may be estimated from previous measurements. These data may have been collected during an internal method validation/verification study, during routine testing, as part of internal quality control, or as part of an interlaboratory comparison. Important is that the analytical procedure that is used to measure the unknown sample is entirely the same one that was used for the previous measurements. If the two procedures differ, not all sources of uncertainty will be covered, and measurement uncertainty may be underestimated. Additionally, the mass fraction of the unknown sample should be in the range of the mass fractions of previous measurement results, used to estimate measurement uncertainty. If, for instance, one measurement result is close to the limit of quantification of the measurement procedure and another much higher, there will be different measurement uncertainties associated with them.

Measurement uncertainty is typically estimated as a standard uncertainty. For this, analytical data are collected and used to calculate the standard deviation s . The standard uncertainty u is then estimated as:

$$u = \frac{s}{\sqrt{n}} \quad \text{Equation 1}$$

n : number of repeat measurements

Typically, different sources of uncertainty are estimated separately and summed up (Equation 2), for example the standard uncertainties associated with bias and precision characteristics. The standard uncertainty associated with bias (u_{bias}) is estimated during trueness control (see Part IV, "Use of CRMs to prove laboratory and method performance"). The standard uncertainty associated with precision characteristics can be estimated from previous measurement results. Depending on whether these were obtained under repeatability conditions (*i.e.* measurements performed on a single day) or intermediate precision conditions (*i.e.* measurements performed on different days), this uncertainty component will be called the standard uncertainty associated with repeatability (u_r) or intermediate precision (u_{ip}), respectively. Further details on how to calculate these parameters are provided in Annex II (2.1). Finally, the combined standard uncertainty u_c is calculated by taking the root sum of squares:

$$u_c = \sqrt{u_r^2 + u_{ip}^2 + u_{bias}^2} \quad \text{Equation 2}$$

Standard uncertainties can be expressed in absolute (u) or in relative terms (u_{rel}). Absolute standard uncertainties are expressed in measurement units other than one, for instance as g/kg in case of mass fractions (w). Relative standard uncertainties, however, are dimensionless quantities (also called quantities with the unit one), quantifying the standard uncertainty relative to the average of the measured quantity:

$$u_{rel} = \frac{u}{\text{average}} \quad \text{Equation 3}$$

In Equation 3, u_{rel} is a decimal value. Alternatively, it can be expressed as a percentage value by multiplying with 100 %:

$$u_{rel} = \frac{u}{\text{average}} \cdot 100 \% \quad \text{Equation 4}$$

To combine standard uncertainties, they must be in the same form, either absolute or relative. Although both relative and absolute uncertainties can be expressed in percentages in the case of GMO content (as this is generally reported as relative property), these are not equivalent. For the relative uncertainty value, percentage is connected with a normalized uncertainty (Equations 3 and 4), whereas for the absolute uncertainty value, percentage is connected with a dimensionless quantity, such as mass fraction (w). To easily distinguish relative from absolute uncertainty values and to make sure that only values of the same type are combined, it is highly recommended to express mass fractions, while calculating measurement uncertainty, in g/kg rather than percentage. Since EU legislation expresses mass fractions as percentages [2], a conversion from g/kg to percentage may be done on the final measurement result.

How to report measurement uncertainty?

The combined standard uncertainty u_c (Equation 2) describes an interval around the measurement value (e.g., GMO mass fraction) which accounts for about 68 % of the expected measurement results. By convention, however, measurement uncertainty is reported as the interval that contains the expected value with a high probability, typically 95 %. This is called the expanded uncertainty U . To obtain this, a so-called coverage factor k is used as a multiplier for the combined standard uncertainty:

$$U = k \cdot u_c \quad \text{Equation 5}$$

Typically, a coverage factor of 2 ($k = 2$) can be used, if at least 12 measurement data are available (e.g., from two days each with 6 replicates on independent subsamples) to estimate the uncertainty. This would lead to an interval containing the true value with a level of confidence (probability) of 95 %. By choosing a different coverage factor, a different confidence level can be obtained, for example 99 % if $k = 3$. Therefore, when measurement uncertainty is reported, the coverage factor used to calculate it should always be specified.

When reporting a measurement result as $w \pm U$, the expanded uncertainty U should be rounded first, and then the measured mass fraction w to the same decimal place. The

rounding approach for U is up to the user, though generally, more than 2 non-zero digits will not make sense. Finally, in order to minimize rounding errors, the rounding should only be done on the final, expanded uncertainty.

Practical examples

The GM mass fraction (g/kg) of an unknown sample has been measured by real-time PCR. Two approaches to estimate the expanded measurement uncertainty associated with this measurement value are described in Annex II.

The first approach uses data from an in-house method verification/validation. Different uncertainty contributions associated with repeatability (u_r), intermediate precision (u_{ip}) and bias (u_{bias}) are estimated. These values are combined according to Equation 2, to obtain the combined standard uncertainty (u_c). The final measurement uncertainty is then reported as expanded uncertainty U (Equation 5). Detailed calculations are given in a practical example in Annex II (2.1).

The second approach uses data from routine measurements. Estimations are made of a constant uncertainty contribution associated with measurements at the limit of quantification (LOQ) (u_0), and of a proportional, concentration-dependent uncertainty contribution associated with measurements above the LOQ ($u_{var,rel}$; in other documents called *RSU* [5] or $u_{pro,bias,rel}$ [6]). The two standard uncertainties u_0 and $u_{var,rel}$ are summed up to obtain the combined standard uncertainty (u_c):

$$u_c = \sqrt{u_0^2 + (w \cdot u_{var,rel})^2} \quad \text{Equation 6}$$

w :	measured GM mass fraction of unknown sample
u_0 :	constant standard uncertainty associated with measurements at the LOQ
$u_{var,rel}$:	proportional, concentration-dependent relative standard uncertainty associated with measurements above the LOQ

Again, the final measurement uncertainty is reported as an expanded uncertainty (U ; Equation 5). Also for this approach, detailed calculations are given in a practical example in Annex II (2.2). Besides, it has been described in detail elsewhere [5, 6].

Conclusion

Measurement uncertainty is an inseparable part of each measurement result. It is needed both by the laboratory and the customer. Measurement uncertainty quantifies the fluctuation of measurement results caused by variability in the analytical procedure. It is reported as an expanded uncertainty U with a coverage factor k , corresponding to a particular confidence level for the result. The measurement uncertainty can be estimated based on available measurement data, for example, from a method verification/validation or from routine measurements.

Part III: Traceability of GMO measurement results

In common language the terms traceability and trackability are often confused. In metrology one uses the term 'metrological traceability' to refer to a property of a measurement result, namely that it is for the specified measurand properly linked to the scale of a measurement unit. In contrast to this metrological traceability, traceability is often used in the sense of 'trackability', i.e. connecting a product via the respective production and distribution chains to its origin (e.g., tracking of a food commodity from farm to fork).

Metrological traceability is internationally defined as the 'Property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty' [18]. This definition emphasises already that measurement results without an appropriate uncertainty statement cannot be traceable.

An example taken from our daily life is the purchase of 250 g of sausages at a butcher's shop. Upon weighing the sausages at home, the customer finds only 200 g. It is claimed that both balances (the one of the butcher and the one at home) measure the mass of the sausages. We would trust the measured value on the balance of the butcher, if this balance compares the mass of the sausages with the international prototype kilogram in Paris (because it has been properly calibrated by using intermediate tools such as the national mass prototype and local mass standards). This chain of comparisons establishes the so-called traceability chain. Traceability is therefore a property that makes measurement results meaningful and comparable. It is therefore included in quality standards such as ISO/IEC 17025:2005 [3] and is essential for enabling trade, labelling thresholds, quality control etc.

There are different types of references, i.e. anchor points, for setting up metrological traceability [18]. In the aforementioned example, the reference is the definition of a measurement unit through its practical realisation (the kilogram). The measurement result for the property mass has been obtained by comparing the mass of the sausages with the mass of the kg in Paris. Another reference could be a measurement procedure. Here the procedure defines what is measured. This is often described in a documentary standard. An example would be the dietary fibre content measured, e.g., according to a specific AOAC International method. The third option for a reference is the property value embedded in a Certified Reference Material (CRM). The measurement result is made traceable to the certified value of the CRM. An example is the use of CRM ERM-BF413 for the calibration of the quantification of MON 810 maize in a food sample.

Related to GMO quantification the metrological traceability of a measurement result is ensured via the proper use of a CRM. Unfortunately, the situation in GMO quantification is not ideal, as no independent quality control and calibration materials exist in many cases. Both are only independently available for four GMO events and require the expression of the measurement result as DNA copy number ratio (which is traceable to the mole). However, CRMs certified for their GMO mass fraction (in g/kg) are available for all GMO events approved in the EU. It needs to be noted in this context that measurement results which are traceable to different measurement units cannot be compared.

A measurement result consists of two parts, one specifying the measurand (e.g., mass fraction of MON 810 maize in a particular sample) and the other one the quantity value (e.g., 10.0 ± 1.0 g/kg) with the measurement unit and the associated measurement uncertainty. The

uncertainty is indeed a part of the measurement result and traceability has to be established for both, the measurand (“what has been measured”) and the quantity value (“how much has been measured”).

The identity of GMO-related measurands is ‘structurally’ defined (by two DNA sequences, which are method-independent structural properties of the target molecule). In contrast to this, method-defined identities are often much more difficult to define (e.g., enzyme activities where the measurement procedure with a number of influence parameters defines the measurand).

To achieve traceability of a measurement result, one must link identity and quantity value to a reference. In the case of GMO measurements this is done via the certified quantity value of a GMO CRM. The certified information is providing the identity of the measurand and can be used for calibrating a measurement signal, i.e. for translating it into a quantity value. The calibrant used determines the traceability chain of the measurement result, including the measured identity and the measurement unit.

GMO matrix CRMs are certified using a gravimetric approach leading to certified mass fractions with a low uncertainty, suitable for calibration. Also the use of in-house calibrants, calibrated with a CRM, is possible. In the latter case an additional uncertainty contribution for the additional calibration step needs to be estimated and added. This ensures that the traceability chain to the certified value of the matrix CRM remains valid.

The maintenance of a traceability chain in GMO measurements is less straightforward than in the weighing example mentioned in the beginning. While the use of calibrated balances and pipettes is important and essential, it is impossible to link the quantity value in a sample without additional assumptions to the quantity value in a final extract (e.g., linking the amount of extracted genomic DNA to the amount of gDNA in the seed powder sample which was extracted). For GMO measurements applying PCR, the expression of the measurement result relative to an endogene and the assumption that similar DNA extraction yields can be achieved from GMO- and non-GMO material restore the traceability chain. Here the importance of method validation/verification becomes apparent, while the use of a matrix GMO CRM helps to verify that the traceability of the measurement result is established.

There are three prerequisites which need to be fulfilled to ensure metrological traceability for GMO measurement results. At first, similar extraction yields (similarity is evaluated on the basis of a 95 % confidence level) of transgenic DNA and endogenous DNA, respectively, need to be ensured. This should be checked during method validation/verification. Secondly, the GMO and non-GMO materials used for the production of the GMO CRM had to have similar DNA contents. This had to be checked during GMO CRM development and production. Deviations should be stated by the CRM producer and taken into account when using the CRM. Thirdly, the food and feed products tested would have to contain similar DNA contents in their GMO and non-GMO ingredients. As it is impossible to check for this in unknown samples, the remaining doubt needs to be added to the measurement uncertainty.

Only measurement results linked to the same reference point and using the same measurement scale are comparable. In the case of GMOs, the reference point and the appointed reference material are at the same level. The traceability of the certified value of a reference material has to be stated on the CRM certificate. The traceability chain of a typical GMO quantification measurement result needs to answer three questions: what is measured,

how much is measured and in which measurement unit is this expressed? Using a GMO CRM with a certified value as mass fraction in g/kg for calibration ensures that the measured mass fraction is traceable to an identity (what is measured? – mass fraction of ingredients with specified DNA fragments) and a quantity value (how much is measured and expressed in which measurement unit?).

It is worth to remember that the reference material for implementation of the European labelling threshold laid down in (EC) 1829/2003 and (EC) 619/2011 is appointed by the European Commission in the decision concerning the authorisation [13] and no GMO events are authorised in Europe for which no GMO CRM is available.

Conclusion

Using GMO CRMs in a correct way ensures comparability of the measurement results by establishing and maintaining the traceability chain. The certified value of a GMO CRM certified for the mass fraction of specified GM event is traceable to the identity of this specific GMO event with a unique identifier code and to the quantity value of a mass ratio expressed in kilogram, a unit in the International System of Units (SI).

Part IV: Use of CRMs to prove laboratory and method performance

The performance of measurements can be described by using a number of quality parameters. As explained in Part II, precision describes how close repetitive measurements are to one another. The precision of a method can be estimated under repeatability conditions intermediate precision conditions or reproducibility conditions.. In all cases, the assessment does not give an indication on how close the results are to the true value. This difference between the experimental result and a reference value, taken as the true value, is described by the bias. Trueness of a method, meaning the degree of possible bias, can be assessed by using CRMs for which the true value is taken as the certified value. Combination of both precision and trueness of a method defines the accuracy of the method (Figure 1).

Quality Assurance (QA) is supposed to give the customer of a measuring laboratory the confidence that the data that were produced by this laboratory can be trusted and are fit for purpose. It is therefore very important that the laboratory understands for which purpose the data are produced. An example for a failure of this would be to apply a method for which the limit of quantification is above the legal maximum threshold to be controlled. In this sense, QA gives a laboratory also some guidelines on how to carry out certain measurement tasks. Quality Control (QC) defines what a laboratory does on a regular basis to ensure that the data produced are fit for purpose and can be trusted.

When assessing performance characteristics, a distinction should be made between method-related (more correctly: procedure-related) and laboratory-related variations of measurement results. In order to obtain reliable results, both components have to be appropriately considered. A method classified as reliable should have been validated as being fit for purpose and accurate enough. The reliability of the laboratory can be divided into two parts related to the instrumentation (and reagents, other tools) used and to the analyst performing the measurement. The first part covers, among others, the proper functioning of the instrument through adequate calibration and maintenance and the second part the sufficient competence of the analyst through education, experience and training. When all these conditions are met, then one can have confidence in the measurement results. A demonstration of adequate method and laboratory performance is also a prerequisite to obtain laboratory accreditation according to ISO/IEC 17025:2005 [3].

In the case of GMO testing, most of the checking regarding principle method performance is taken care of by the method validation carried out by the EURL-GMFF. It has to be seconded by the in-house verification, which delivers quality characteristics for the combination of the application performance of the validated method and the specific laboratory performance.

There are several ways to ensure adequate method or laboratory performance. The first one is the use of a properly validated method, provided that the laboratory applying it can demonstrate that the method works also appropriately in its premises. This can be achieved through method verification. Then, participation in proficiency testing schemes can show that the performance of the laboratory is reliable. Finally, laboratories can monitor via control charts, whether the method applied is under control. Reference materials are not only useful tools, but are actually required for all these activities, i.e. for method validation, for proficiency testing schemes and for setting up control charts.

Moreover, CRMs can be used as control samples to assess the trueness of the result obtained by the method used. The CRM should be measured as any routine sample and the

measurement result would be expressed including the associated measurement uncertainty. This result is then compared to the certified value and the uncertainty stated on the certificate of the CRM to check whether there is any significant difference. If yes, then the measurement is not under control and an investigation should be made on the causes of the bias in order to eliminate it. If not, then the measurement is under control.

A multistep approach [19] is applied to estimate whether the difference between the own measured value w_m and the certified value w_{CRM} is significant. Firstly, the difference Δ_m between these two values is calculated:

$$\Delta_m = |w_m - w_{CRM}| \quad \text{Equation 7}$$

Then the expanded uncertainty U_{CRM} for the certified material property value is converted into a standard uncertainty u_{CRM} by dividing it with the coverage factor k , taken from the CRM certificate (see Part II, “Estimation of measurement uncertainty”):

$$u_{CRM} = \frac{U_{CRM}}{k} \quad \text{Equation 8}$$

The measurement uncertainty associated with the own measurement result (u_m) is then estimated (see Part II, “Estimation of measurement uncertainty”), either from the method validation/verification, from the within-laboratory standard deviation (intermediate precision) or from the repeatability standard deviation (not that very often, this approach results in an underestimation of the real uncertainty). The uncertainties of the own measurement result and of the certified value are then combined to determine the uncertainty on a potential bias:

$$u_{\Delta_m} = \sqrt{u_m^2 + u_{CRM}^2} \quad \text{Equation 9}$$

Finally, the difference Δ_m is compared with the expanded uncertainty on a potential bias (using a coverage factor of 2 corresponding to a confidence level of 95 %).

If $\Delta_m \leq 2 u_{\Delta_m}$ then the method is not significantly biased. On the other hand, if $\Delta_m > 2 \cdot u_{\Delta_m}$ then the method is significantly biased. In this case, an investigation on the possible causes for the bias should be conducted in order to correct it. The approach of applying a so-called correction factor to ‘numerically correct’ for a bias should be avoided because it requires a thorough study on the way this factor changes over time and in relation to the measured concentration.

On the long run, an adequate method performance can be monitored through quality control charts [20,21]. These charts can be built up from several measurements (minimum 10 measurements) on a quality control material. A typical approach consists in the following: The central line is set as the mean value, the upper and lower warning limits are set as mean value ± 2 times the standard deviation of measurement results on the QC sample (s_{QC}) and the upper and lower control limits are set as mean value ± 3 times s_{QC} .

A method is usually considered to be out of control if one of the following situations occur:

- 1 point above/below control limits (note that, from a statistical point of view, this will occur 1 time out of 100 measurements even if the method is running correctly)
- 2 out of 3 consecutive points between warning and control limits

- 9 consecutive points on the same side of the central line
- 6 or more points in a row steadily increasing or decreasing

The quality control material to be used for this test should be subjected to the same treatment and inserted in the same run as the routine samples. The requirements for this QC material are that the matrix is as close as possible to the matrix of the routine samples (ideal situation: matrix identical), that the same measurand is measured at a similar concentration level, and that the measured target (DNA) is homogeneously distributed in the QC material and stable over time. These requirements are fulfilled when using CRMs as QC materials.

The most important added value of using CRMs for control charts is the possibility to build into the QC charting a trueness assessment. When using a CRM, the control chart is constructed with the certified value as central line. The warning limits are set as 2 times the combined uncertainty (u_c) and the control limits are set as 3 times u_c where

$$u_c = \sqrt{u_{\text{CRM}}^2 + s_{\text{QC}}^2} \quad \text{Equation 10}$$

Obviously, the use of the CRM would slightly increase the domain covered by the warning limits and control limits, which only reflects reality (the corresponding uncertainty is usually unknown and neglected for a non-certified QC material). Consequently, one should only use CRMs with a sufficiently small uncertainty on its certified value. Otherwise the chart may mainly show the uncertainty of the CRM value instead of the variation of the method.

Conclusion

Adequate CRMs are a very powerful tool to prove the performance of analytical methods through assessment of the accuracy of the average of a specific individual series of measurements, bringing evidence of proper laboratory, analyst or instrument performance. The use of CRMs in quality control charts allows not only determining that a method is under control over time but is also giving a trueness dimension to the results.



Calibration of qPCR




Philippe Corbisier

**Joint Research Centre –
Institute for Reference
Materials and Measurements**

*Serving society
Stimulating innovation
Supporting legislation*

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The choice of the measurement scale

What is the temperature ?



Thermometer, calibrated
in Celsius ? in Fahrenheit ? in Kelvin ?

The answer is = 25 ° C

- *The measurement scale is defined by the calibrant*

The choice of the measurement scale

What is the GM content ?



How do I calibrate my qPCR ?

Has the calibrant used a certified value in mass fraction (g/kg) or in copy number ratio (mol/mol)?

- *The calibrant defines the measurement unit*
- *In contrary to the temperature scale, there is no direct mathematical relationship between % m/m and % cp/cp !!!*

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The choice of the calibrant

- What is available ?
 - CRM mixtures certified for their GM mass content (g/kg)
 - individual CRM mixtures

e.g.

CRM	% m/m	Content [g/kg]	Expanded uncertainty
ERM-BF415a	0	<0,4 (blank)	-
ERM-BF415b	0,1	1,0	0,38
ERM-BF415c	0,5	4,9	0,50
ERM-BF415d	1,0	9,8	0,63
ERM-BF415e	2,0	19,6	0,83
ERM-BF415f	5,0	49,1	1,28

- solution of DNA extracted from a material with high GM mass fraction

The choice of the calibrant

- What is available ?
 - Pure CRMs certified for their GM mass content (e.g. > 999 g/kg)
 - CRMs certified for their DNA copy number ratio
 - matrix materials

CRM	Unit	Certified values	Uncertainty
ERM-BF425c (Soya 356043)	Mass fraction	10,0 g/kg	1,1 g/kg
	Copy number ratio	0,85 %	0,11 %
ERM-BF427c (Maize 98140)	Mass fraction	20,0 g/kg	0,8 g/kg
	Copy number ratio	1,75 %	0,13 %

- Plasmids (e.g. ERM-AD415, ERM-AD425)



Where do I find GM CRMs?

- COMAR

<http://www.comar.bam.de/en/>

- Database of the German Federal Office of Consumer Protection and Food Safety (BVL)

http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/nachweis_kontrollen/referenzmaterialien.pdf

will be replaced by : <http://www.euginius.eu/>

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Reference Materials for GMO Detection

Version: 10.12.2012

The table lists publicly available reference materials and sources.

new materials (row shaded in green)

No.	Description	Catalogue Number	Source	GMO	Unique Identifier	Species	Type	Value (% GMO)	Unit	Amount per Unit	Certified
1	Roundup Ready™ soybean (blank)	BF410ak	IRMM / ERM	-	-	soybean	dried soya bean powder	<0,07	mass	1 g	yes
2	Roundup Ready™ soybean	BF410bk	IRMM / ERM	GTS-40-3-2	MON-04032-6	soybean	dried soya bean powder	0,10	mass	1 g	yes
3	Roundup Ready™ soybean	BF410dk	IRMM / ERM	GTS-40-3-2	MON-04032-6	soybean	dried soya bean powder	1,00	mass	1 g	yes
4	Roundup Ready™ soybean	BF410gk	IRMM / ERM	GTS-40-3-2	MON-04032-6	soybean	dried soya bean powder	10,00	mass	1 g	yes
5	Bt-176 maize (blank)	BF411a	IRMM / ERM	-	-	maize	dried maize powder	<0,014	mass	1 g	yes
6	Bt-176 maize	BF411b	IRMM / ERM	E176 Maize	SYN-EV176-9	maize	dried maize powder	0,10	mass	1 g	yes
7	Bt-176 maize	BF411c	IRMM / ERM	E176 Maize	SYN-EV176-9	maize	dried maize powder	0,50	mass	1 g	yes
8	Bt-176 maize	BF411d	IRMM / ERM	E176 Maize	SYN-EV176-9	maize	dried maize powder	1,00	mass	1 g	yes
9	Bt-176 maize	BF411e	IRMM / ERM	E176 Maize	SYN-EV176-9	maize	dried maize powder	2,00	mass	1 g	yes
10	Bt-176 maize	BF411f	IRMM / ERM	E176 Maize	SYN-EV176-9	maize	dried maize powder	5,00	mass	1 g	yes
11	Bt-11 maize (blank)	BF412a	IRMM / ERM	-	-	maize	dried maize powder	<0,012	mass	1 g	yes
12	Bt-11 maize	BF412b	IRMM / ERM	Bt11	SYN-BT011-1	maize	dried maize powder	0,10	mass	1 g	yes
13	Bt-11 maize	BF412c	IRMM / ERM	Bt11	SYN-BT011-1	maize	dried maize powder	0,49	mass	1 g	yes
14	Bt-11 maize	BF412d	IRMM / ERM	Bt11	SYN-BT011-1	maize	dried maize powder	0,98	mass	1 g	yes
15	Bt-11 maize	BF412e	IRMM / ERM	Bt11	SYN-BT011-1	maize	dried maize powder	1,96	mass	1 g	yes



Where do I purchase GM CRMs?

- *The catalogue of AOCS:* <https://secure.aocs.org/crm/>
- *The catalogue of IRMM:* <http://irmm.jrc.ec.europa.eu/Pages/rmcatalogue.aspx>
- *ERM:* <http://www.erm-crm.org/>
 - *Sigma-Aldrich:* <http://www.sigmaaldrich.com/catalog/search/TablePage/9641474>
 - *LGC Standards GmbH:* <http://www.lgcstandards.com>
 - *RTC:* <http://www.RT-Corp.com>

More Reference Materials (NOT CERTIFIED!):

- *Eurofins GeneScan - genomic DNA:* <http://www.eurofins.de/kits-de/gvo-testkits.aspx>

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Legal basis ?

COMMISSION DECISION

of 24 October 2007

authorising the placing on the market of products containing, consisting of, or produced from genetically modified maize NK603xMON810 (MON-00603-6xMON-00810-6) pursuant to Regulation (EC) No 1829/2003 of the European Parliament and of the Council

(notified under document number C(2007) 5140)

(Only the French and Dutch texts are authentic)

(Text with EEA relevance)

(2007/701/EC)



— Reference Material: ERM®-BF413 (for MON-00810-6) and ERM®-BF415 (for MON-00603-6) accessible via the Joint Research Centre (JRC) of the European Commission, the Institute of Reference Materials and Measurements (IRMM) at http://www.irmm.jrc.be/html/reference_materials_catalogue/index.htm

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European Commission

Where do I find the legal basis ?


HEALTH AND CONSUMERS

European Commission > Food > Plant > GMOs > EU Register of authorised GMOs

	PLANTS	CONSUMERS	HEALTH	FOOD	ANIMALS
GMOS	<ul style="list-style-type: none"> Legislation on GMO cultivation Authorisation procedure EU Register of authorised GMOs Safeguards Monitoring after authorisation Co-existence Trans-boundary movement Contained use Non-breeding techniques 				

 **MON-00603-6 x MON-00810-6**


EU register of genetically modified food and feed.

Event	NK603xMON810
Authorisation holder	Monsanto Europe S.A., Scheldelaan 460, Haven 627 - B 2040 Antwerp - Belgium On behalf of Monsanto Company - 800 N. Lindbergh Boulevard - St. Louis, Ms
Products	(1) Foods and food ingredients containing, consisting of, or produced from MC (2) Feed containing, consisting of, or produced from MON-00603-6xMON-001

↓

http://ec.europa.eu/food/dyna/gm_register/index_en.cfm

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- **The direct approach (no conversion)**

use a CRM with a value certified in one measurement unit and express your result in the same measurement unit

e.g. CRM m/m
CRM cp/cp (plasmid calibrant)

- **The indirect approach (double conversion)**

(mis)-use a CRM with a value certified for one measurement unit, convert the certified value into another measurement unit (eventually convert it back into the certified measurement unit)

e.g. CRM m/m

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CRMs certified for their GM mass content (g/kg)

CRM	% m/m	Content [g/kg]	Expanded uncertainty
ERM-BF415a	0	<0,4 (blank)	-
ERM-BF415b	0,1	1,0	0,38
ERM-BF415c	0,5	4,9	0,50
ERM-BF415d	1,0	9,8	0,63
ERM-BF415e	2,0	19,6	0,83
ERM-BF415f	5,0	49,1	1,28

CRMs certified for their GM mass content (g/kg)

- *Extraction of gDNA from each CRM*
- *Quantification of the extracted DNA*
 - *UV absorbance (ISO 21571:2005 annex B)*
 - *PicoGreen*
- *Intactness/Fragmentation status of the gDNA from both calibrant & sample*
- *Calibration curves (GM- & reference gene-specific targets)*



Extraction of the DNA

A DNA extraction method validated by the EURL-GMFF
and in-house verified
OR
use your in-house validated DNA extraction method



<http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx>

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European Union Reference Laboratory for GM Food and Feed

European Commission - JRC - IJRC - JRC-81, GMFF

Status of dossiers

EURL GMFF Home

Legal texts

Tasks and dates

Guidance documents

Status of dossiers

Network database

Capacity building

ENIG

Emerging/Undetected GMFs

Contact

EURL GMFF validation process

The following table lists the EURL GMFF validation process carried out within the frame of the Regulation (EC) No 1829/2003, providing details on the current status of the validation process.

The following table provides information about additional validation studies conducted by the EURL GMFF in support to notifications submitted according to Directive 2002/68/EC, about GMO authorized in the EU, notifications submitted according to Directive 2002/100/EC and approvals issued by the European Food Safety Authority (EFSA).

Genetic materials submitted in support to notifications submitted under Directive 2002/68/EC

European Commission information on GM authorizations, approvals and other

Information about the notifications submitted in the context of Directive 2002/68/EC

Opinions of the EFSA Scientific Panel on Genetically Modified Organisms

Last update: 06/11/2013

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Determine the quantity of the extracted DNA

- UV spectrometric method (ISO 21571:2005 (E) Annex B)

$$\rho_{DNA} = F \times (OD_{260} - OD_{320}) * \delta \quad (1)$$

ρ_{DNA} = mass concentration in $\mu\text{g/mL}$

F = dilution factor

OD_{260} = absorbance at 260 nm

OD_{320} = absorbance at 320 nm

δ = molar absorption coefficient in $\mu\text{g/mL}$

δ = 50 $\mu\text{g/mL}$ for dsDNA and 37 $\mu\text{g/mL}$ for ssDNA

under denaturing conditions



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CRMs certified for their GM mass content (g/kg)

- *Extraction of gDNA from each CRM*
- *Quantification of the 'total' gDNA*
 - *UV absorbance (ISO 21571:2005 annex B)*
 - *PicoGreen*
- *Intactness/Fragmentation status of the gDNA from both calibrant & sample*
- *Calibration curves (GM- & reference gene-specific targets)*

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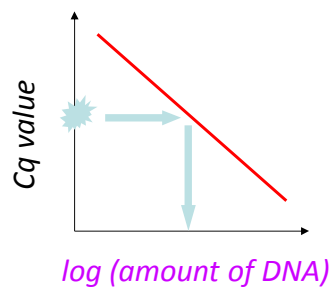


CRMs certified for their GM mass content (g/kg)

- *Extraction of gDNA from each CRM*
- *Quantification of the 'total' gDNA*
 - *UV absorbance (ISO 21571:2005 annex B)*
 - *PicoGreen*
- *Intactness/Fragmentation status of the gDNA from both calibrant & sample*
- *Calibration curves (GM- & reference gene-specific targets)*

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A calibration curve is made by plotting the number of PCR cycles (Cq value) needed to reach a certain fluorescence level against the log (amount of DNA) in the PCR.



The Cq value for the unknown sample is measured and that value is used to calculate the amount of DNA present in the unknown sample

The calibration curve

X-axis = $\log(\text{mass concentration})$

Y-axis = Cq values

Linear regression on the averages of triplicates or on all data

$$y = a \cdot \log(x) + b \quad (2)$$

a = slope

x = mass concentration

b = Y-intercept

y = Cq value

The calibration curve

How to transform Cq values into a mass concentration ?

$$y = a * \log(x) + b \quad (2)$$



Calculate the mass concentration (x) when the Cq (y) is measured

$$x = 10^{(y-b)/a} \quad (3)$$

The calibration curve

In MS Excel

LOG (x)

SLOPE(known y's, LOG (known x's))

INTERCEPT(known y's, LOG (known x's))

To calculate the efficiency

(POWER(10,(-1/a))-1) * 100

To calculate the mass concentration from the Cq values

POWER(10,((Cq - b)/a))

The parameters of the calibration curve

JRC Scientific and Technical Reports



Verification of analytical methods for GMO testing when implementing interlaboratory validated methods

Guidance document from the European Network of GMO Laboratories (ENGL)

Prepared by the ENGL working group on "Method Verification"



EUR 24967 EN - 2011



Amplification efficiency (slope)

$$\mathcal{E} = (10^{(-1/\text{slope})} - 1) \times 100 \quad (4)$$

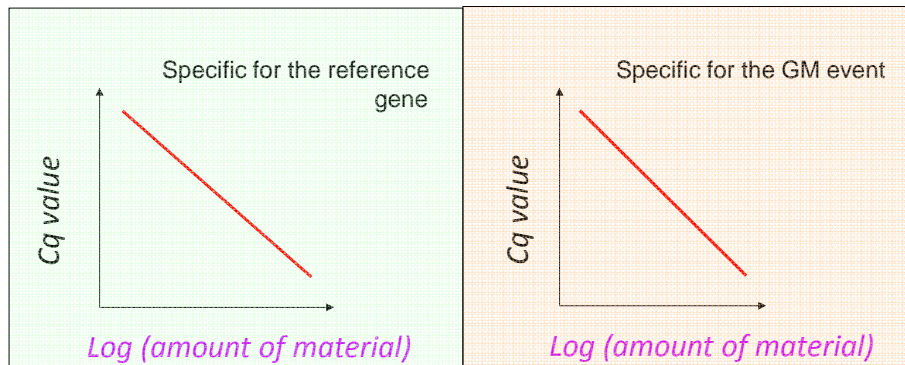
$$-3,6 \leq \mathcal{E} \leq -3,1$$

$$90 \% \leq \mathcal{E} \leq 110 \%$$

Coefficient of determination

$$R^2 \text{ shall be } \geq 0,98$$

- 1 PCR assay specific for the reference gene
 - 1 PCR assay specific for the GM event
- 2 calibration curves are needed assuming different PCR efficiencies for the reference gene and the GM event



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2. The choice of the calibrant
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 - Example : CRM mixtures
 - Example : diluted gDNA
 - 2.1.2 Calibration with a CRM (cp/cp)
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Example: A series of CRMs m/m is used for calibration

	Reference gene		50 g/kg (5%)		GM gene	
	DNA ng/PCR	Cq	DNA ng/PCR	Cq	GM DNA ng/PCR	Cq
50 g/kg	150	23,2	150	23,20	7,5	28,32
20 g/kg	150	23,1	35	25,30	3,0	30,52
10 g/kg	150	23,2	10	27,10	1,5	32,64
5 g./kg	150	23,1	5	28,12	0,75	33,90
1 g/kg	150	23,0	1	30,70	0,15	35,72
Unknown sample:			37,50	← 25,20	0,11	← 34,82

$$\rightarrow 0,11 / 37,50 * 100 = 0,3 \text{ m/m}$$

or 3 g/kg

A CRM with a value certified for mass is used for calibration and results are directly expressed in % m/m

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Example

Dilution series of DNA extracted from a CRM (m/m) is used for calibration (50 g/kg)

Reference gene		50 g/kg (5%)	GM gene	
DNA ng/PCR	Cq		GM DNA ng/PCR	Cq
150	23,20	↓ diluted	7,5	28,32
35	25,30		3,0	30,52
10	27,10		1,5	32,64
5	28,12		0,75	33,90
1	30,70		0,15	35,72
Unknown sample	37,50 ← 25,20			0,11 ← 34,82

This is simple !



$$\longrightarrow 0,11 / 37,50 * 100 = 0,3 \% \text{ m/m or } 3 \text{ g/kg}$$

A CRM with a value certified for mass is used for calibration and results are directly expressed in % m/m



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Example



Plasmid calibrants certified for their copy number ratio

Application Note 5



November 2007

Use of Certified Reference Materials for the quantification of GMO in DNA copy number ratio

This application note provides guidance on the correct use of European Reference Materials certified for their GM (genetically modified) copy number fraction of a specific GM event. The details given below refer particularly to the use of the CRMs ERM-BF413d and ERM-AD413 and the upcoming CRMs certified for copy number ratio.

Author: Philippe Corbisier
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www.erm-crm.org

A CRM with a value certified for cp number ratio is used for calibration and results are directly expressed in % cp/cp

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Example



Plasmid calibrants certified for their copy number ratio

	Reference gene		GM gene	
	DNA cp/PCR	Cq	GM DNA cp/PCR	Cq
diluted ↓	500000	20,67	50000	25,19
	100000	23,00	10000	27,62
	10000	26,36	1000	30,89
	5000	27,29	100	35,12
	1000	29,19	25	35,67
Unknown sample	17007	← 25,44	50	← 35,30

This is also simple !



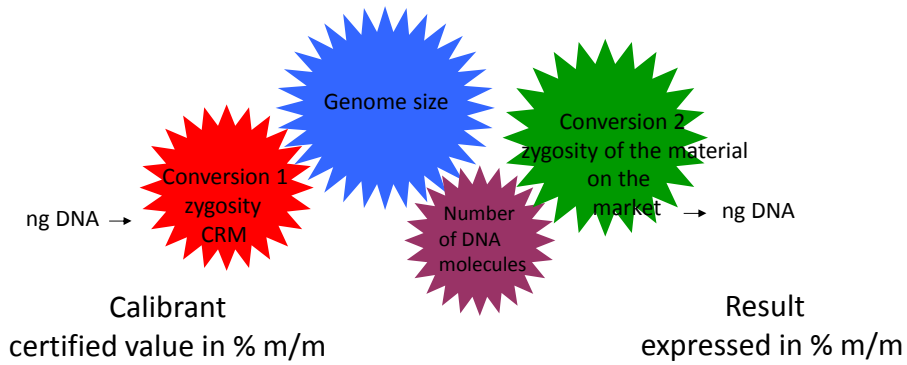
➔ $50 / 17007 * 100 = 0,3 \% \text{ cp/cp}$

A CRM with a value certified for copy number ratio is used for calibration and results are directly expressed in % cp/cp

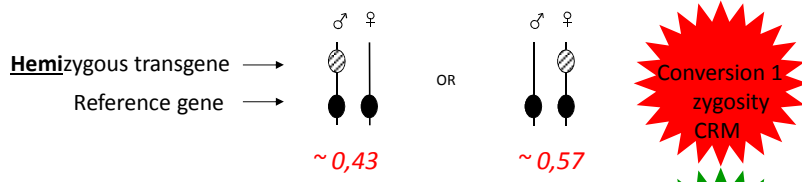
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• The indirect approach



Conversion factor



MAIZE	Part	Genotype	% of total DNA
	Cover	♀ ♀	0.64 -3.51 % of total DNA
	Endosperm	♀ ♀ ♂	36.3 -59.4 % of total DNA
	Embryo	♀ ♂	38.6 -62.24 % of total DNA



$$\text{Conversion factor} = X \text{ m/m} * (0,5 \pm 0,167 * Y)$$

X = GM content in m/m %
 Y = contribution of the endosperm
 Y varies in function of the variety ~40 %



Where do I find the conversion factors ?

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

zygosity of the CRM

3. The information accompanying the certified reference material shall include information on the breeding of the plant which has been used for the production of the certified reference material and on the zygosity of the insert(s). The certified value of the GMO content shall be given in mass fraction and, where available, in copy number per haploid genome equivalent. (HGE)



***Zygosity is provided in the certificate of Analysis of the CRM
(Conversion factors are not provided)***

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Example: Maize DAS-40278-9

zygosity of the CRM → conversion factor?



DESCRIPTION OF THE MATERIAL

ERM-BF433d is one of four DAS-40278-9 maize powder certified reference materials (CRMs) containing different mass fractions of this genetically modified maize. ERM-BF433d has been produced from whole seeds of non-modified maize and genetically modified DAS-40278-9 maize, both supplied by Dow AgroSciences (DAS, Oxon, UK). According to the information provided by Dow AgroSciences the genetically modified donor for the hemizygous DAS-40278-9 maize event was the male parent. In accordance with

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Where do I find the conversion factors ?



When results are primarily expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EU-RL.

zygosity of the market material

*Zygosity is indicated in each validation report of the EURL
(Conversion factors are not provided)*

→ *Technical guidance from the EURL-GMFF*

Example: Maize DAS-40278-9

zygosity of the market material → conversion factor 1 ?



Table 6. Summary of dPCR analysis conducted on the DAS-40278-9 and *hmg* targets in the positive control sample.

Mean ratio (DAS-40278-9 / <i>hmg</i>)	0.991
Standard deviation	0.067
RSD_r (%)	6.8
Standard error of the mean	0.017
Upper 95% CI of the mean	1.028
Lower 95% CI of the mean	0.954

In conclusions, the 95% confidence interval (CI) spans around 1 and therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous and a single-copy reference target, for an alpha = 0.05.

Hence:

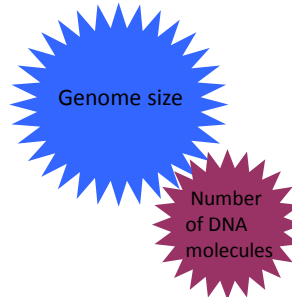
GM % in DNA copy number ratio = GM % in mass fraction



- The mass of the species

Query the RBG Kew Plant DNA C-values database
 Bennett MD, Leitch IJ. 2012. Plant DNA C-values database (release 6.0, Dec. 2012)
<http://www.kew.org/cvalues/>

e.g. *Zea mays*: mass of 1 genome = $2,73 \cdot 10^{-3}$ ng



- Conversion from ng/μL into HGE (haploid genomic equivalent) cp/μL

$$\text{HGE cp number [HGE cp/}\mu\text{L]} = \frac{\text{DNA mass concentration [ng/}\mu\text{L]}}{\text{Genome mass [ng/HGE cp]}}$$

e.g. in 50 ng/μL of *Zea mays* → we have $50 / (2,73 \cdot 10^{-3}) = 18315$ HGE cp of the *Zea mays* genome per μL.



Example: 50 g/kg maize CRM is used for calibration

Conversion 1: zygosity of the CRM (e.g. 0,4)

	Reference gene			GM gene		
	DNA ng/PCR	HGE cp/PCR	Cq	HGE cp/PCR	HGE cp/PCR	Cq
dilution 1	150	54545	23,20	2727	1091	28,32
dilution 2	35	12727	24,80	637	255	30,52
dilution 3	10	3636	26,82	182	73	32,64
dilution 4	5	1818	28,18	90	36	33,90
dilution 5	1	364	30,00	17,5	7	35,72
Unknown sample		12903 ← 25,00			16 ← 34,82	

Conversion 2 (from HGE cp to mass fraction): zygosity of the market (e.g. 0,5)

→ $16 / 12903 \cdot 100 = 0,1\%$ HGE cp $0,12 / 0,5 = 0,2\%$ m/m (or 2 g/kg)



Quantification approaches



Not recommended

- The direct approach
RESULT = 0,3 % m/m
- The indirect approach
RESULT = 0,2 % m/m

$$0,2 * 0,5/0,4 = 0,3 \text{ % m/m}$$

≈ zygosity of the market material/zygosity of the CRM

→ Both values are approximate values

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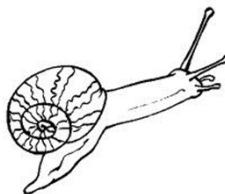
Conclusions

- *Use one single measurement scale*
→ *Favorize a direct approach for the qPCR*
- *Mention the measurement unit used*
- *Add the measurement uncertainty* (part 2)
- *Indicate the traceability of your result* (part 3)
- *Be confident in the results obtained* (part 4)



Thank you for your attention

Please mark the GMO





Content

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Estimation of Measurement Uncertainty




Oliver Zobell

**Joint Research Centre –
Institute for Reference
Materials and Measurements**

*Serving society
Stimulating innovation
Supporting legislation*



Joint Research Centre



Content

1. About measurement uncertainty
 - 1.1. What is measurement uncertainty (MU)?
 - 1.2. Who needs it?
 - 1.3. Where does it come from?
 - 1.4. How to estimate it?
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Definition of measurement uncertainty

ISO Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM):

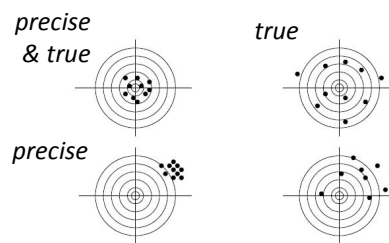
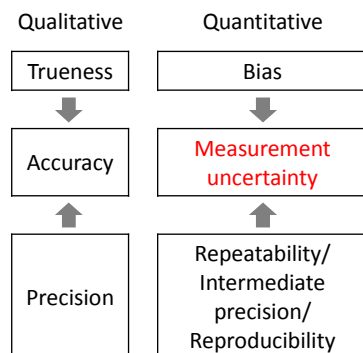
“A parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand.”

Measurement uncertainty is

- ... an integral part of the result of a measurement.
- ... a measure of the accuracy of a measurement.
- ... a statistical parameter which describes the possible fluctuation of a measurement result.
- ... an interval that covers the true value with high probability.
- ... the number after \pm .

Measurement performance

Measurement performance characteristics:



1. *About measurement uncertainty*
 - 1.1. *What is measurement uncertainty (MU)?*
 - 1.2. **Who needs it?**
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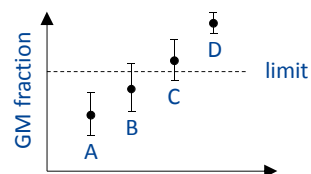
Who needs measurement uncertainty?

The **customer** needs it...

- ... to evaluate the fitness-for-purpose of a laboratory.
- ... to compare results between laboratories.
- ... to check a product for compliance with a legal limit.

The **laboratory** needs it...

- ... to know its quality of measurement.
- ... to improve its quality of measurement.
- ... to be accredited for ISO 17025.



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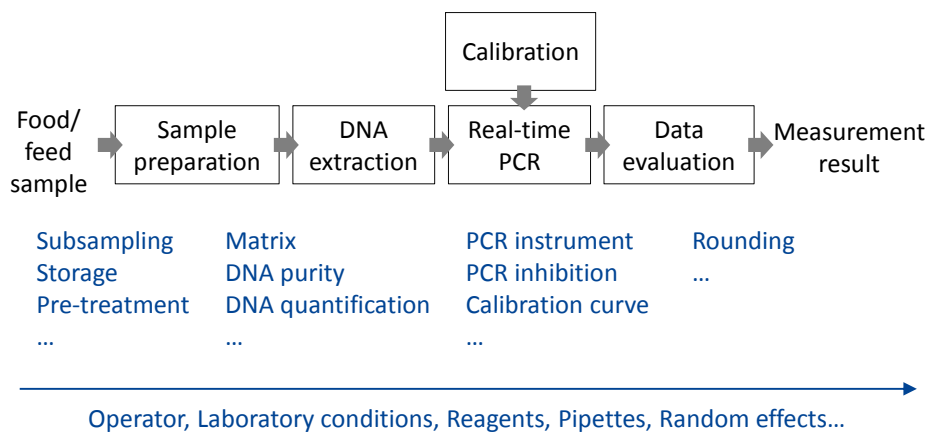
2.1. Estimation from in-house method validation/verification data

2.2. Estimation from routine measurement data

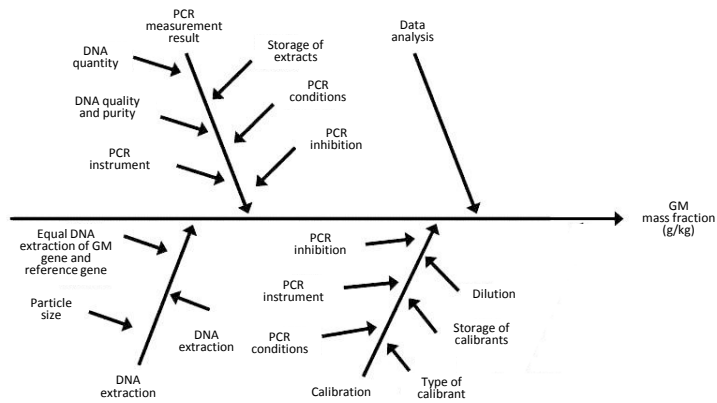
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Sources of uncertainty



Sources of uncertainty



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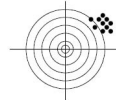
2.2. Estimation from routine measurement data

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Know your measurement

- What is the measurand: what do I measure?
- Does my measurement have a bias?



→ Annex IV "Use of certified reference materials to prove laboratory and method performance"

Definitions

Standard uncertainty (u):

- uncertainty of the result of a measurement
- often calculated via a standard deviation (s)

$$u = \frac{s}{\sqrt{n}} \quad n = \text{number of measurements}$$

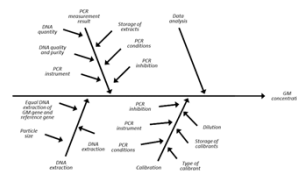
Combined standard uncertainty (u_c):

- combination of several uncertainty contributions

$$u_c = \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2 + u_5^2}$$

Bottom-up approach to estimate combined standard uncertainty

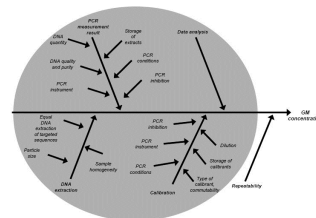
1. Draw a cause-effect (fishbone / Ishikawa) diagram.
2. Note the equation for the calculation of the analytical result.
3. Estimate standard uncertainty for each contributing component.
4. Combine standard uncertainties:



$$u_c = \sqrt{u_1^2 + u_2^2 + u_3^2 + u_4^2 + u_5^2}$$

Top-down approach to estimate combined standard uncertainty

1. Perform a number of measurements or collect existing analytical data:
 - Method validation/verification
 - Internal quality control data
 - Interlaboratory comparison
2. Calculate standard deviations.
3. Estimate combined uncertainty for the whole analytical procedure.



Bottom-up vs. top-down approach

	Bottom-up	Top-down
Low risk of missing important factors		✓
Relatively simple and fast		✓
Good guide for method improvement	✓	

Combined approach (bottom-up + top-down) to estimate u_c

1. Collect existing analytical data.
2. Estimate different contributing standard uncertainties for the complete analytical procedure, e.g.:
 - uncertainty associated with repeatability (u_r)
 - uncertainty associated with intermediate precision (u_{ip})
 - uncertainty associated with bias (u_{bias})
3. Combine standard uncertainties:

$$u_c = \sqrt{u_r^2 + u_{ip}^2 + u_{bias}^2}$$

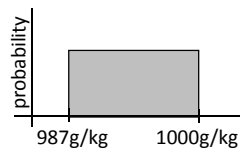
Uncertainties from other sources

... may require conversion:

- Purity of a CRM (e.g. ≥ 987 g/kg)

→ any value in interval 987-1000 g/kg equally likely:

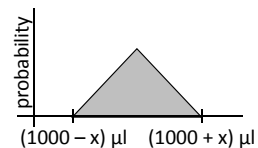
→ rectangular distribution → $u = \frac{(1000-987)/2 \text{ g/kg}}{\sqrt{3}}$



- Volume pipetted with a pipette (e.g. $1000 \pm x$ μ l)

→ nominal value more likely:

→ triangular distribution → $u = \frac{x \mu\text{l}}{\sqrt{6}}$



Absolute and relative uncertainties

Uncertainties can be expressed in absolute values (u) or

in relative values (u_{rel}):

- **Relative** = dimensionless quantity; uncertainty relative to the average:

$$u_{\text{rel}} = \frac{u}{\text{average}} \quad \text{e.g. } 0,059 \text{ (as a decimal)}$$

$$\text{or: } u_{\text{rel}} = \frac{u}{\text{average}} \times 100 \% \quad \text{e.g. } 59 \% \text{ (as a percentage)}$$

- **Absolute** = quantity in measurement units, in this case mass fraction (w):

in g/kg e.g. 100 g/kg

or: in % e.g. 10 %

Absolute and relative uncertainties

Uncertainties can be expressed in absolute values (u) or
in relative values (u_{rel}):

- **Relative** = dimensionless quantity; uncertainty relative to the average:

$$u_{\text{rel}} = \frac{u}{\text{average}} \quad \text{e.g. } 0,059 \text{ (as a decimal)}$$

$$\text{or: } u_{\text{rel}} = \frac{u}{\text{average}} \times 100 \% \quad \text{e.g. } 59 \% \text{ (as a percentage)}$$

- **Absolute** = quantity in measurement units, in this case mass fraction (w):

in g/kg e.g. 100 g/kg

or: in % e.g. 10 %

Express mass fraction w in g/kg, not %

- When **combining uncertainties**, all uncertainties must be in the **same form**, relative or absolute.
- Both relative and absolute uncertainties can be expressed in %.
-> **easy to confuse** relative with absolute uncertainties
- Therefore, it is strongly recommended to...
 - ... express mass fractions (w) in **g/kg** instead of % during calculations.
 - ... perform the conversion from g/kg to % only on the final, reported measurement result, if it is required that a measured GM mass fraction is reported in %.

Rounding of standard uncertainties

- Rounding only on final, reported uncertainty (expanded uncertainty)
- To minimize rounding errors, standard uncertainties should have at least one digit more than the final, expanded uncertainty.

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Definitions

Coverage factor (k):

- numerical factor used as multiplier for u_c
- used to convert a standard deviation into a confidence interval

Expanded uncertainty (U):

- interval that contains a large fraction of the expected data

$$U = k \times u_c$$

Expanded uncertainty U

- Conversion of a standard deviation (combined standard uncertainty) to a confidence interval (expanded uncertainty):

$$U = k \times u_c \quad k = \text{coverage factor}$$

- Recommended level of confidence = 95%
→ $k = 2$, if at least 6 independent sub-samples measured on 2 days
- For a 99% level of confidence: $k = 3$


 European Commission


 European Commission


 European Reference Materials

JOINT RESEARCH CENTRE
 Institute for Reference Materials and Measurements


CERTIFICATE OF ANALYSIS

ERM® - BF433d

MAIZE POWDER		
	Mass Fraction	
	Certified value ²⁾ [g/kg]	Uncertainty ³⁾ [g/kg]
DAS-40278-9 maize ¹⁾	100	8

1) Genetically modified maize DAS-40278-9 with the unique identifier DAS-40278-9.
 2) The certified value is based on the masses of mixed dried genetically modified DAS-40278-9 maize powder and of dried non-modified maize powder, taking into account their respective purity with regard to DAS-40278-9 maize and their respective water content. The certified value is traceable to the SI.
 3) The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.


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Certified uncertainty of a CRM property value

... requires conversion before using it as a component in a combined measurement uncertainty:

For example:

- CRM with a certified value of (100 ± 9) g/kg⁽¹⁾
 - ⁽¹⁾ The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$.
- The standard uncertainty u_{CRM} is calculated as follows:

$$U_{\text{CRM}} = k \cdot u_{\text{CRM}} \Rightarrow u_{\text{CRM}} = \frac{U_{\text{CRM}}}{2} = 4,5 \text{ g/kg}$$


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Rounding of expanded uncertainties

- Rounding system up to the user
Generally, more than 2 non-zero digits will not make sense.
- First round the expanded uncertainty, then the measured fraction to the same decimal place.
e.g. average measured fraction = 100,42 g/kg
if $U = 9,3$ g/kg
→ measurement result = $(100,4 \pm 9,3)$ g/kg

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Estimation of measurement uncertainty from in-house method verification data

- A sample has been measured for its GM mass fraction (g/kg).
- The measurement uncertainty associated with this result is estimated from available in-house verification data.

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Uncertainties estimated during in-house method verification

... concerning only the **individual measurement**

→ u_r = uncertainty associated with repeatability

... a particular **measurement series**

→ u_{ip} = uncertainty associated with intermediate precision

... all measurements with a particular **method** and by a certain **laboratory**:
deviation from a reference value (method bias, lab bias)

→ u_{bias} = uncertainty associated with bias

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Repeatability (u_r)

- Covers effects on individual measurement
- Does not include effects on standard curve preparation, performance of instrument, reagents...
- Estimate u_r directly from method verification. Use ANOVA to calculate s_r (separating repeatability from day-to-day effect):

$$s_r = \sqrt{MS_{within}}$$

$$u_r = \frac{s_r}{\sqrt{n}} \quad n = \text{number of replicates / day}$$

- As relative standard uncertainty: $u_{r,rel} = \frac{u_r}{average}$

Intermediate precision (u_{ip})

- Covers series-to-series or day-to-day effects
- Does not include effects from instrument type, laboratory...
- u_{ip} estimated from method verification. Use ANOVA to calculate s_{ip} (separating day-to-day effect from repeatability influence):

$$s_{ip} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \quad n = \text{replicates/day (during validation)}$$

$$u_{ip} = \frac{s_{ip}}{\sqrt{N}} \quad N = \text{measurement days}$$

- As relative standard uncertainty: $u_{ip,rel} = \frac{u_{ip}}{average}$

Intermediate precision (u_{ip})

- If $MS_{\text{between}} < MS_{\text{within}}$:
 - > value under the square root becomes negative
 - > s_{ip} cannot be calculated
 - > calculate maximum hidden between-day-variation (s_{ip}^*) instead:

$$s_{ip}^* = \sqrt{\frac{MS_{\text{within}}}{n}} \times \sqrt{\frac{2}{N \times (n-1)}}$$

n = replicates/day (during validation)

N = measurement days

Bias (u_{bias})

- How certain am I that the method gives on average the correct result?
- Bias determination by measurements of a CRM:

$$u_{\text{bias,rel}} = \sqrt{\frac{s_{\text{bias,rel}}^2}{n_{\text{bias}}} + \left(\frac{u_{\text{CRM}}}{w_{\text{CRM}}}\right)^2}$$

$s_{\text{bias,rel}}$ = relative standard deviation of trueness measurements

n_{bias} = number of measurements for trueness determination

u_{CRM} = certified standard uncertainty of CRM value (absolute, in g/kg)

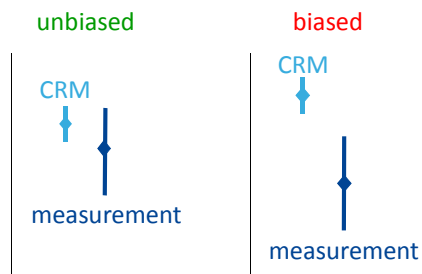
w_{CRM} = certified value of the CRM (absolute, in g/kg)

Measurement uncertainty

- **Combined** standard uncertainty: $u_{c,rel} = \sqrt{u_{r,rel}^2 + u_{ip,rel}^2 + u_{bias,rel}^2}$
 - Formula is only applicable if trueness check reveals no bias!
 - Use relative uncertainties
 - Repeatability alone underestimates uncertainty
- **Expanded** standard uncertainty: $U_{rel} = k \times u_{c,rel}$
 $k = \text{coverage factor}$
- Measurement uncertainty for result w :
 $w \text{ (g/kg)} \times U_{rel} = U$
- **Result** = $w \pm U \text{ g/kg}$

Bias check

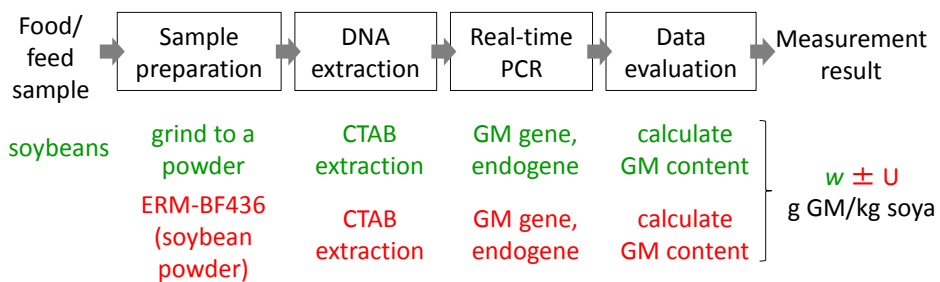
Bias must also be checked by using a CRM:



→ Day 2: Use of certified reference materials to prove laboratory and method performance (G. Auclair)

Calculation example

- Measure GM content in a **soybean sample** (g/kg).
- Estimate associated measurement uncertainty based on **in-house method verification data**.



Experiment details

Unknown soybean sample:

- DAS-44406-6 content measured on 2 days in 3 independent extraction replicates (N = 2, n = 3)
- average GM mass fraction = 85,3 g/kg (DAS-44406-6 soya / total soya)

Method verification:

- measurement of DAS-44406-6 content in soybean CRM ERM[®]-BF436e
- 5 independent extractions (n = 5), repeated on 5 days (N = 5)

Bias check: no bias found.

Method verification data

Repeated measurements of ERM®-BF436e with a certified DAS-44406-6 mass fraction of $(100,0 \pm 9,0)$ g/kg:

g/kg	Day 1	Day 2	Day 3	Day 4	Day 5	Average (g/kg)
Rep 1	113,1	111,8	99,3	94,6	113,6	96,4
Rep 2	103,2	90	115,7	97,5	112,7	
Rep 3	87,8	66,9	93	86,5	103,7	
Rep 4	110,4	82,1	82,3	73,9	89,9	
Rep 5	120,5	84,3	88,2	86,5	103,2	

→ Perform one-way ANOVA to separate repeatability (variation between replicates) from intermediate precision (variation between days).

ANOVA

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Column 1	5	535	107	153,525
Column 2	5	435,1	87,02	264,837
Column 3	5	478,5	95,7	164,015
Column 4	5	439	87,8	84,23
Column 5	5	523,1	104,62	91,367

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1711,794	4	427,9486	2,822977	0,0524259	2,866081
Within Groups	3031,896	20	151,5948			
Total	4743,69	24				

Calculate u_r and u_{ip}

- **Repeatability** (within-day variation):

$$u_r = \frac{\sqrt{MS_{\text{within}}}}{\sqrt{n}} = \frac{\sqrt{151,59}}{\sqrt{3}} = 7,11 \text{ g/kg} \quad \text{relative} \quad \rightarrow \quad u_{r,rel} = \frac{7,11 \text{ g/kg}}{96,4 \text{ g/kg}} = 0,0738$$

- **Intermediate precision** (between-day variation):

$$s_{ip} = \sqrt{\frac{MS_{\text{between}} - MS_{\text{within}}}{n}} = \sqrt{\frac{427,95 - 151,59}{5}} = 7,43 \text{ g/kg}$$

$$u_{ip} = \frac{s_{ip}}{\sqrt{N}} = \frac{7,43}{\sqrt{2}} = 5,25 \text{ g/kg} \quad \text{relative} \quad \rightarrow \quad u_{ip,rel} = \frac{5,25 \text{ g/kg}}{96,4 \text{ g/kg}} = 0,0545$$

Calculate u_{bias}

- Method verification data obtained with ERM®-BF436e, certified fraction $w = (100,0 \pm 9,0) \text{ g/kg}$

→ use only data from day 1 to estimate u_{bias} →

- According to the certificate, $k = 2$:

$$\rightarrow u_{CRM} = \frac{u_{CRM}}{2} = \frac{9,0}{2} = 4,5 \text{ g/kg}$$

- Bias:

$$u_{bias,rel} = \sqrt{\frac{s_{bias,rel}^2}{n_{bias}} + \left(\frac{u_{CRM}}{w_{CRM}}\right)^2} = \sqrt{\frac{0,1158^2}{5} + \left(\frac{4,5}{100,0}\right)^2} = 0,0686$$

g/kg	Day 1
Replicate 1	113,1
Replicate 2	103,2
Replicate 3	87,8
Replicate 4	110,4
Replicate 5	120,5
Average (g/kg)	107,0
s_{bias} (g/kg)	12,39
$s_{bias,rel}$	0,1158

Measurement result

- **Combined** standard uncertainty :

$$u_{c,rel} = \sqrt{u_{r,rel}^2 + u_{ip,rel}^2 + u_{bias,rel}^2} = \sqrt{0,0738^2 + 0,0545^2 + 0,0686^2} \\ = 0,1146$$

- **Expanded** uncertainty:

$$U_{rel} = k \times u_{c,rel} = 2 \times 0,1146 = 0,2292$$

- **Measurement result** soybean sample:

$$w \pm (w \times U_{rel}) = 85,3 \pm (85,3 \times 0,2292) \text{ g/kg} = 85,3 \pm 19,6 \text{ g/kg} \\ = \boxed{85 \pm 20 \text{ g/kg (DAS-44406-6 soya / total soya)}}$$

1. About measurement uncertainty

- 1.1. What is measurement uncertainty (MU)?
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- 1.3. Where does it come from?
- 1.4. How to estimate it?
- 1.5. How to report it?

2. Practical examples

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- 2.2. Estimation from routine measurement data

3. Conclusions

4. Recommended reading



Estimation of measurement uncertainty from routine measurement data

- A sample has been measured for its GM mass fraction (g/kg).
- The measurement uncertainty associated with this result is estimated from available measurement data of routine samples.

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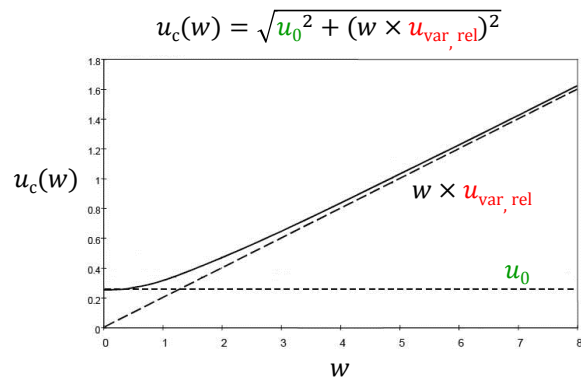
Important considerations

- Independent results needed on **≥ 15 samples**
- Be aware of range of **sample matrices** and **GM levels** for routine measurements: newly measured, unknown sample must be included.
- Use smallest no. of extraction replicates (**$n = 2$**) for internal quality control measurements, to maximise sample number.
- Use same experimental setup to measure unknown sample and routine samples: **same no. of extraction and PCR replicates**
- Add newly available routine sample data to **update** measurement uncertainties (and remove old ones, *e.g.* older than one year).
- Include a **bias control** through measurement of a CRM.

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Intermediate precision: Constant & proportional uncertainty contribution

Overall uncertainty has a constant and a proportional component:



$u_{\text{var,rel}} = u_{\text{pro,bias,rel}}$ in *Guidance document on measurement uncertainty for GMO testing laboratories*
= *RSU* in *European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs*

Fig. E.5.1, *Quantifying Uncertainty in Analytical Measurement 3rd Ed. (2012)*

Constant contribution u_0

- To estimate the uncertainty component that is **not dependent on the level of analyte** (for example at the LOQ, 6 lowest fractions).

- $d_i = |w_{i,1} - w_{i,2}|$ = absolute difference between replicates

$$\bar{d} = \frac{\sum d_i}{N} = \text{average absolute difference (range) between replicates}$$

$N = 6$ (samples with lowest fraction)

- Estimate of **absolute** intermediate precision (s_0):

$$s_0 = \frac{\bar{d}}{d_2} = \frac{\bar{d}}{1,128} = u_0$$

d_2 = factor for estimating s from the average absolute difference (range) btw. replicates (see *Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories, 2012*)

Proportional contribution - part 1: $u_{\text{pro,rel}}$

- To estimate the uncertainty component that is **proportional to the level of analyte** (above the LOQ, at higher fractions).

- $\overline{w}_i = \frac{w_{i,1} + w_{i,2}}{2}$ = average of replicates

$$d_i = |w_{i,1} - w_{i,2}| = \text{absolute difference between replicates}$$

$$d_{i,\text{rel}} = \frac{d_i}{\overline{w}_i} = \text{relative difference (range) between replicates (as a decimal)}$$

$$\overline{d_{i,\text{rel}}} = \frac{\sum d_{i,\text{rel}}}{N} = \text{average relative difference between replicates}$$

N = number of samples with high fraction

- Estimate of **relative** intermediate precision ($s_{\text{pro,rel}}$):

$$s_{\text{pro,rel}} = \frac{\overline{d_{i,\text{rel}}}}{d_2} = \frac{\overline{d_{i,\text{rel}}}}{1,128} = u_{\text{pro,rel}} \quad d_2 = \text{factor for estimating } s$$

Proportional contribution - part 2: $u_{\text{bias,rel}}$

- Perform a bias control by measurement of a CRM
- Estimate uncertainty associated with bias:

$$u_{\text{bias,rel}} = \sqrt{\frac{s_{\text{bias,rel}}^2}{n_{\text{bias}}} + \left(\frac{u_{\text{CRM}}}{w_{\text{CRM}}}\right)^2}$$

$s_{\text{bias,rel}}$ = relative standard deviation of bias measurements

n_{bias} = number of measurements for bias determination

u_{CRM} = certified standard uncertainty of CRM value (absolute, in g/kg)

w_{CRM} = certified value of the CRM (absolute, in g/kg)

= same equation as in Example 1 (using method validation data)



Measurement uncertainty

- **Constant** standard uncertainty contribution: $u_0 = s_0$
- **Proportional** standard uncertainty contribution:

$$u_{\text{var,rel}} = \sqrt{u_{\text{pro,rel}}^2 + u_{\text{bias,rel}}^2}$$

- **Combined** standard uncertainty:
- **Expanded** uncertainty: $U = u_c \times k$

$$u_c = \sqrt{u_0^2 + (w \times u_{\text{var,rel}})^2}$$

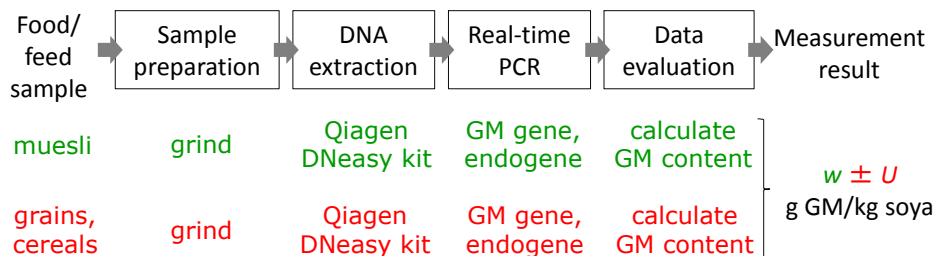
k = coverage factor

- **Result** = $w \pm U$ g/kg



Calculation example

- Measure NK603 GM maize mass fraction in a **muesli sample** (g/kg).
- Estimate associated measurement uncertainty based on **routine sample data**.



Experiment details

Unknown muesli sample:

- NK603 maize mass fraction (g/kg) measured in duplicate ($n = 2$)
- average mass fraction = 46,8 g/kg (NK603 maize / total maize)

Routine data from 15 various samples:

- measurement of NK603 maize mass fraction (g/kg)
- 2 independent extraction replicates ($n = 2$) measured for each sample

Bias check:

- 6 measurements of NK603 maize mass fraction (g/kg) in ERM®-BF415f ($n = 6$)
- no bias found

Routine measurement data

Measurement results obtained on routine samples ($n=2$)

analysis nr.	$w_{i,1}$ (g/kg)	$w_{i,2}$ (g/kg)	average w_i (g/kg)	d_i (g/kg)	average d (g/kg)	$d_{i,rel}$	average $d_{i,rel}$	
u_0	10	0,14	0,16	0,15	0,16	0,02	0,13	
	7	0,16	0,15	0,16		0,01		0,06
	3	0,33	0,29	0,31		0,04		0,13
	5	0,61	0,58	0,60		0,03		0,05
	6	1,53	1,29	1,41		0,24		0,17
	13	1,93	2,56	2,25		0,63		0,28
$u_{pro,rel}$	2	4,30	4,25	4,28	0,05	0,01	0,06	
	12	5,16	5,60	5,38	0,44	0,08		
	1	9,56	10,28	9,92	0,72	0,07		
	9	12,85	14,08	13,47	1,23	0,09		
	14	14,40	13,86	14,13	0,54	0,04		
	11	26,37	29,64	28,01	3,27	0,12		
	8	27,94	27,20	27,57	0,74	0,03		
	15	56,38	60,98	58,68	4,60	0,08		
4	71,26	75,65	73,46	4,39	0,06			

Calculate u_0 and $s_{\text{pro,rel}}$

- u_0 estimated from 6 results with lowest fractions (at LOQ):

$$u_0 = s_0 = \frac{\bar{d}}{d_2} = \frac{\bar{d}}{1,128} = \frac{0,16}{1,128} = 0,14 \text{ g/kg}$$

- $u_{\text{pro,rel}}$ estimated from remaining results at higher fractions (above LOQ):

$$s_{\text{pro,rel}} = \frac{\overline{d_{i,rel}}}{d_2} = \frac{\overline{d_{i,rel}}}{1,128} = \frac{0,06}{1,128} = 0,053 = u_{\text{pro,rel}}$$

-> next: $u_{\text{pro,rel}}$ combined with $u_{\text{bias,rel}}$ to calculate the proportional part of the standard uncertainty ($u_{\text{var,rel}}$)

Data bias check

- Bias check with ERM[®]-BF415f, certified fraction = (49,1 ± 1,3) g/kg:

→ no bias found

→ use data to estimate $s_{\text{bias,rel}}$ and $u_{\text{bias,rel}}$

Measurement of ERM [®] -BF415f (n=6)				
analysis nr.	w (g/kg)	average w (g/kg)	s_{bias} (g/kg)	$s_{\text{bias,rel}}$
1	51,0			
2	48,9			
3	52,1			
4	51,2	50,3	1,35	0,027
5	49,7			
6	48,8			

- According to the certificate, $k = 2 \rightarrow u_{\text{CRM}} = \frac{u_{\text{CRM}}}{2} = \frac{1,3}{2} = 0,65 \text{ g/kg}$

Calculate $u_{\text{bias,rel}}$ and $u_{\text{pro,bias,rel}}$

- Relative standard uncertainty associated with bias:

$$u_{\text{bias,rel}} = \sqrt{\frac{s_{\text{bias,rel}}^2}{n_{\text{bias}}} + \left(\frac{u_{\text{CRM}}}{w_{\text{CRM}}}\right)^2} = \sqrt{\frac{0,027^2}{6} + \left(\frac{0,65}{49,1}\right)^2} = 0,017$$

- Proportional standard uncertainty contribution:

$$u_{\text{var,rel}} = \sqrt{u_{\text{pro,rel}}^2 + u_{\text{bias,rel}}^2} = \sqrt{0,053^2 + 0,017^2} = 0,056$$

Measurement uncertainty

- Combined** standard uncertainty :

$$u_c = \sqrt{u_0^2 + (w \times u_{\text{var,rel}})^2} = \sqrt{0,14^2 + (46,8 \times 0,056)^2} = 2,62 \text{ g/kg}$$

- Expanded** uncertainty:

$$U = k \times u_c = 2 \times 2,62 = 5,24 \text{ g/kg}$$

- Measurement result** muesli sample:

$$w \pm U = 46,8 \pm 5,2 \text{ g/kg (NK603 maize / total maize)}$$

1. *About measurement uncertainty*
 - 1.1. *What is measurement uncertainty (MU)?*
 - 1.2. *Who needs it?*
 - 1.3. *Where does it come from?*
 - 1.4. *How to estimate it?*
 - 1.5. *How to report it?*
2. *Practical examples*
 - 2.1. *Estimation from in-house method validation/verification data*
 - 2.2. *Estimation from routine measurement data*
3. **Conclusions**
4. *Recommended reading*

Conclusions

Measurement uncertainty...

... is an **inseparable** part of each measurement result.

... is **needed** both by the laboratory and the customer.

... quantifies the **fluctuation** of measurement results caused by variability in the analytical procedure.

... is reported as an **expanded** uncertainty U with a coverage factor k , corresponding to a particular confidence level.

... can be estimated based on **available measurement data**, for example from method verification/validation or routine measurements.

1. *About measurement uncertainty*
 - 1.1. *What is measurement uncertainty (MU)?*
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4. **Recommended reading**

Recommended reading

Review on measurement uncertainty:

- Meyer, V.R. (2007) Measurement uncertainty, J. Chromatogr. A 1158,15

General guidance for estimating measurement uncertainty:

- "GUM": Guide to the expression of uncertainty in measurement, ISO/IEC Guide 98-3:2008
- "QUAM": EURACHEM/CITAC Guide CG4: Quantifying Uncertainty in Analytical Measurement, 3rd ed. 2012
- ISO/TS 21748:2004: Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation
- Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories, v3.1, Nordtest, 2012
- <http://www.nordtest.info>; <http://www.measurementuncertainty.org/>

Specific guidance for estimating measurement uncertainty in GMO testing:

- Guidance document on measurement uncertainty for GMO testing laboratories, EUR 22756EN, ISBN 978-92-79-05566-9
- European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs, 2nd version, EUR 26547 EN, ISBN 978-92-79-35936-1


European Commission


Traceability of GMO Measurement Results

Stefanie Trapmann
**Joint Research Centre –
Institute for Reference
Materials and
Measurements**

*Serving society
Stimulating innovation
Supporting legislation*



Joint Research Centre


European Commission

Traceability vs trackability

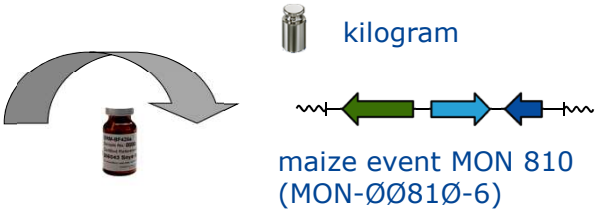
Metrological traceability

- Linking of a measurement result to a unit and an identity

Analysis report

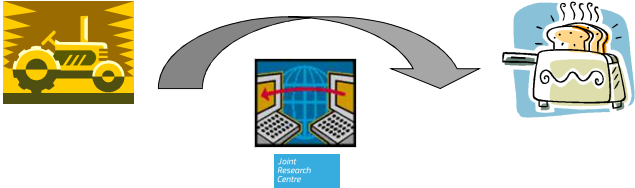
Samples # 458 contains 8 ± 2 g/kg ($k = 2$) of genetically modified MON 810 maize.

The measurement result is traceable to the SI through the certified value of CRM ERM[®]-BF413ck, which was used for calibration.



Trackability

- Tracking a commodity, e.g. from farm to fork



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Outline



- What is metrological traceability?
- Link between traceability and calibration?
- How to maintain traceability?
- How to interpret the traceability statement of GMO CRMs?

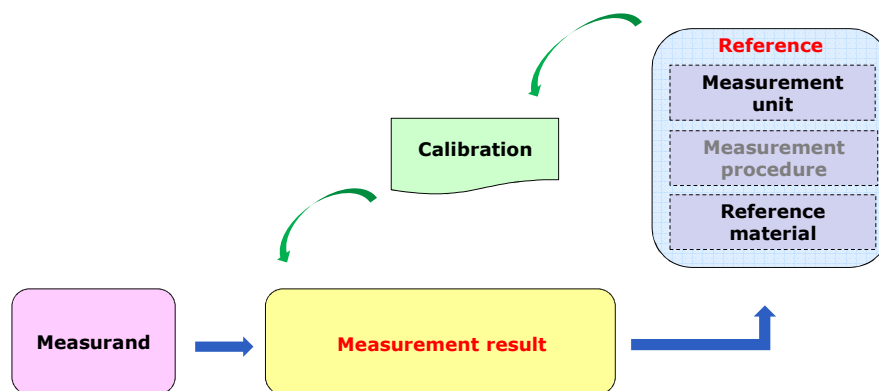
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Definition



'Property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty.'

ISO/IEC Guide 99:2007, International vocabulary of metrology – Basic and general concepts and associated terms (VIM)

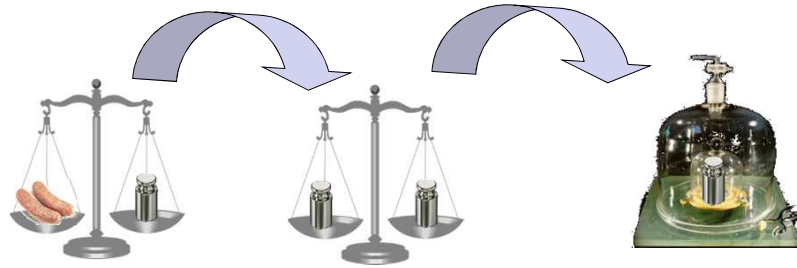


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Traceability chain



Imagine, you buy 250 g of sausages at the local butcher. Upon weighing at home, you find only 200 g.
Is there a problem?



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Importance



Traceability ...

- Is a property that makes results meaningful, hence, it is included in quality standards such as ISO/IEC 17025:2005

5.6 Measurement traceability

5.6.2.1 Calibration (RMs)

5.6.2.2 Testing (RMs)

5.6.3 Reference standards and reference materials (RMs)

- Is essential for:
 - Trade agreements
 - Labelling thresholds
 - Quality control

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Metrological references



Reference

- Definition of a measurement unit through its practical realisation
Example: 5 kg
- Measurement procedure (documentary standard)
Example: dietary fibre measured according to AOAC method X
- Reference material (via the certified value)
Example: 4.9 ± 1.0 g MON 810 maize / kg maize in CRM ERM-BF413ck

Measurement unit

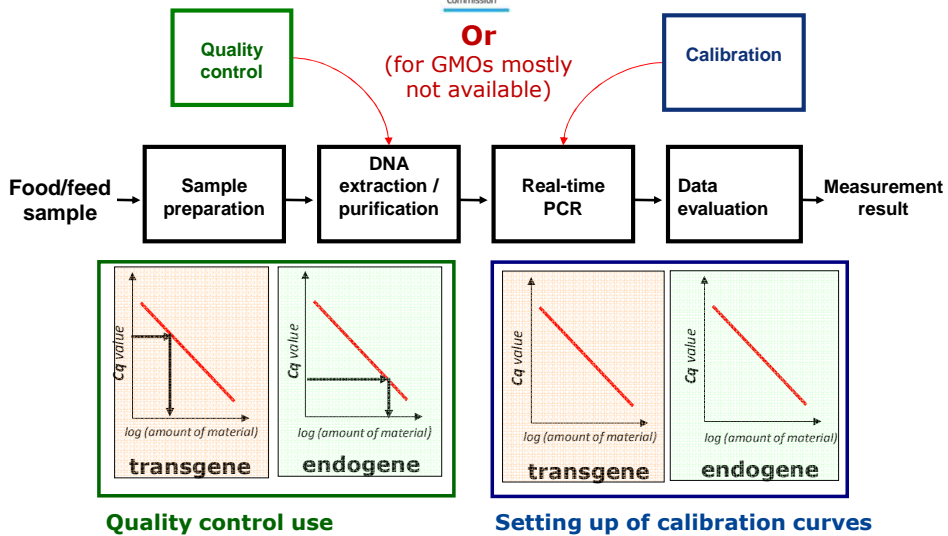
Measurement procedure

Reference material

ISO/IEC Guide 99:2007 (VIM3)


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Use of the CRM





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Situation in the GMO area



European Commission


<p>Measurement unit</p> <p>[mol/mol] DNA copy number ratio</p>		<p>Availability</p> <p>Quality control material and independent calibrant only available <u>for 4 GMO events</u></p>
<p>[g/kg] mass fraction</p>		<p>Matrix materials <u>for all events</u> approved in the EU available</p>

Legal requirement to report in mass fractions




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Traceability – certified value



European Commission

MON 810 maize mass fraction: 4.9 ± 1.0 g/kg




Traceability
of a certified value

Identity
(measurand)

- Structurally defined**
e.g. GMO CRMs
- Operationally defined**
e.g. enzyme activity CRMs

Quantity value
(number & unit)

SI traceability in the case of GMO CRMs (certified value expressed in g/kg) requires that all equipment (e.g. balance) is calibrated to SI

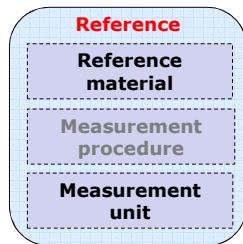


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Traceability – measurement result



To achieve traceability of a measurement result, one must link identity and quantity value to a reference



In the case of GMO measurements this is done via the certified value of a GMO CRM, linking **identity of the measurand** and **calibrating the measured quantity value.**

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Calibration



The calibrant used determines the traceability chain of the measurement result, the measured identity and the measured quantity:

Analysis report

Samples # 458 contains 8 ± 2 g/kg ($k = 2$) of **genetically modified MON 810 maize**.

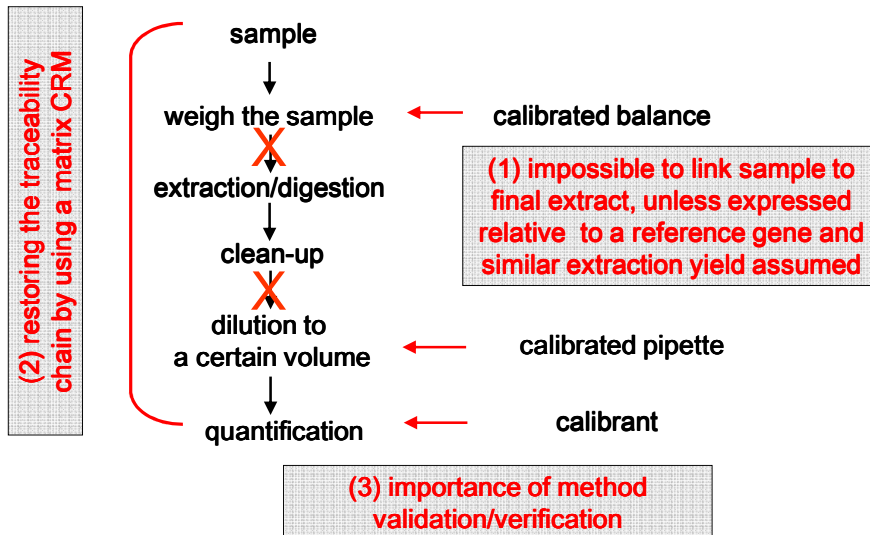
The measurement result is traceable to the SI through the certified value of CRM ERM[®]-BF413ck, which was used for calibration.

Notes:

- GMO matrix CRMs are certified using a gravimetric approach leading to certified mass fractions with a low uncertainty, suitable for calibration.
- Use of in-house calibrants, calibrated with a CRM, is possible. The additional uncertainty contribution needs to be estimated and added.

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Maintaining the traceability chain



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Prerequisites DNA content



Prerequisite (1):
similar extraction yield* of transgenic DNA and endogenous DNA

to be checked during method validation/verification

Prerequisite (2):
similar DNA content* of the GMO material and non-GMO materials used for the GMO CRM

to be checked during GMO CRM development, deviations to be stated and to be taken into account when using the CRM

Prerequisite (3):
similar DNA content of the GMO and non-GMO material in the food and feed products

Impossible to be checked for unknown samples, part of the measurement uncertainty

* Similarity is evaluated on the basis of a 95 % confidence level

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Comparability (I)

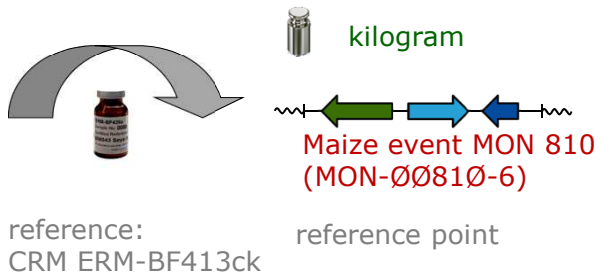


- Only measurement results linked to the same reference point are comparable

Analysis report

Samples # 458 contains 8 ± 2 g/kg ($k = 2$) of genetically modified MON 810 maize.

The measurement result is traceable to the SI through the certified value of CRM ERM[®]-BF413ck, which was used for calibration.



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Comparability (II)

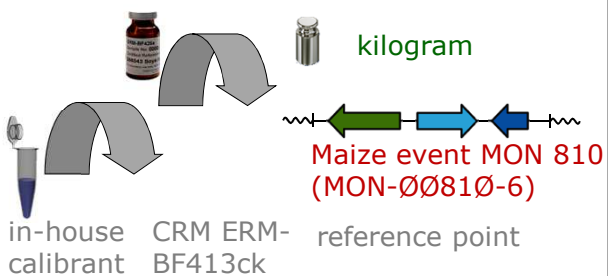


- This link can be short or longer, resulting in higher uncertainties

Analysis report

Samples # 458 contains 8 ± 2 g/kg ($k = 2$) of genetically modified MON 810 maize.

The measurement result is traceable to the SI through the certified value of CRM ERM[®]-BF413ck, which was used for calibration.



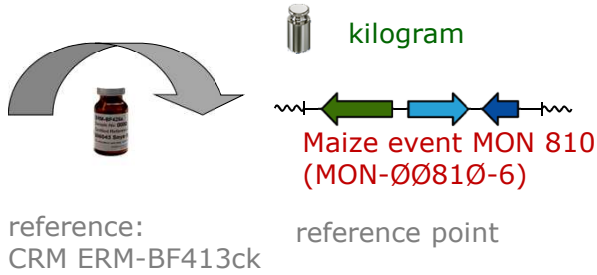
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Comparability (III)



- In the case of GMOs, reference point and appointed reference material are at the same level.

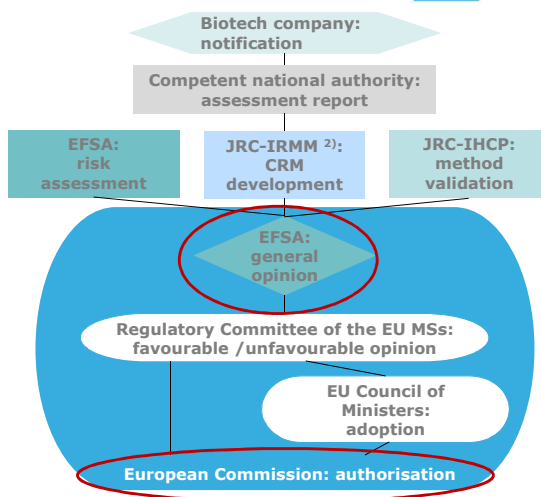
Analysis report
 Samples # 458 contains 8 ± 2 g/kg ($k = 2$) of genetically modified MON 810 maize.
 The measurement result is traceable to the SI through the certified value of CRM ERM[®]-BF413ck, which was used for calibration.



- Reference material for implementation appointed by the EC in the decision concerning the authorisation

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GMO authorisation ¹⁾



EFSA general opinion:

The applicant informs about the available reference material.

EC authorisation decision:

The accessibility of the reference material for implementation is specified

¹⁾ for commercial release (placing on the market) for a maximum of 10 years (renewable)

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²⁾ or another Reference Material Producer



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EU Register of authorised GMOs

http://ec.europa.eu/food/dyna/gm_register/index_en.cfm



keyword search
 e.g. for MON 810
 maize
 details


 European Commission



**Reference Material:
 ERM-BF413k
 accessible via the Joint
 Research Centre (JRC)
 of the European
 Commission, the
 Institute for Reference
 Materials and
 Measurements (IRMM)
 at**
<https://irmm.jrc.ec.europa.eu/rmcatalogue>

CRMs and traceability



Traceability

of a certified value

- Traceability of the assigned value of a certified reference material has to be stated on the CRM certificate
- Using the GMO CRMs in a correct way ensures comparability of the measurement results

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CRM and ERM



- GMO CRMs offered by IRMM carry all the trademark ERM® (European Reference Materials are certified materials, which undergo uncompromising peer evaluation and offer highest quality and reliability.)
- ERM® is a brand name (trademark), the term CRM is defined by ISO Guide 30.
- There is no restriction or specific authorisation of ERMs for the European market only.

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**Certified value
identity**



CERTIFICATE OF ANALYSIS

ERM®- BF429b

COTTON SEED POWDER		
	Mass fraction	
	Certified value ²⁾ [g/kg]	Uncertainty ³⁾ [g/kg]
T304-40 cotton ¹⁾	10.0	1.3

1) Mass fraction of T304-40 cotton (unique identifier code BCS-GHØØ4-7) based on the masses of genetically modified T304-40 cotton seed powder and non-modified cotton seed powder and their respective water content.

2) The certified value is traceable to the International System of Units (SI).

3) The certified uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) with a coverage factor $k = 2$, corresponding to a level of confidence of about 95 %.

1) Mass fraction of T304-40 cotton (unique identifier code BCS-GHØØ4-7) based on the masses of genetically modified T304-40 cotton seed powder and non-modified cotton seeds powder and their respective water content.



**Certified value
traceability of the
quantity value**



CERTIFICATE OF ANALYSIS

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COTTON SEED POWDER		
	Mass fraction	
	Certified value ²⁾ [g/kg]	Uncertainty ³⁾ [g/kg]
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2) The certified value is traceable to the International System of Units (SI).



Certified value and expanded uncertainty



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COTTON SEED POWDER		
	Mass fraction	
	Certified value ²⁾ [g/kg]	Uncertainty ³⁾ [g/kg]
T304-40 cotton ¹⁾	10.0	1.3

1) Mass fraction of T304-40 cotton (unique identifier code BCS-GH004-7) based on the masses of genetically modified T304-40 cotton seed powder and non-modified cotton seed powder and their respective water content.

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Summary



- **GMO CRMs** certified for their mass fraction are available to set up the traceability chain
- **GMO measurements** using as calibrants matrix GMO CRMs certified for their mass fraction are traceable to the SI Unit kg and the identity of the specific GMO event
- In-house calibrants are traceable to the same **reference point** if they are calibrated against such a GMO CRM, taking into account the additional uncertainty components.
- **Measurement results** which are traceable to different measurement units are **not comparable**.
- **Measurement results** obtained by using as calibrant the GMO CRM with a certified value in mass fraction correctly are per default **comparable**.

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Thank you for your attention!




Use of CRMs to Prove Laboratory and Method Performance



Guy Auclair


**Joint Research Centre –
Institute for Reference
Materials and Measurements**

*Serving society
Stimulating innovation
Supporting legislation*



Outline

- **Introduction**
 - terminology
 - quality control
- **Laboratory and method performance**
 - importance to prove the performance
 - use of CRMs
- **Tools and techniques**
 - quality control sample
 - quality control chart
 - proficiency testing schemes



Terminology

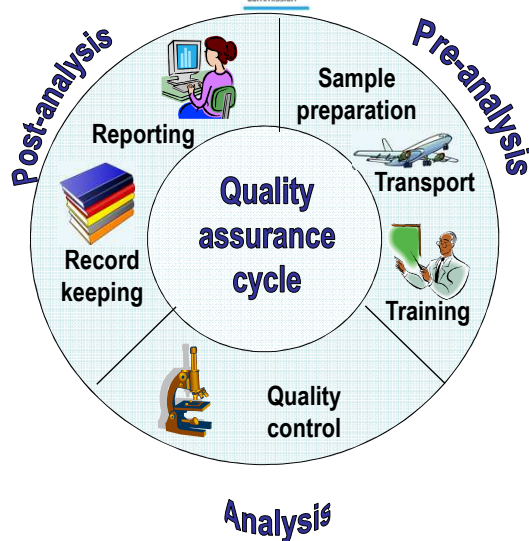


- Precision:** Closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions
- Intermediate precision:** Condition of measurement, out of a set of conditions that includes the same measurement procedure, same location, and replicate measurements on the same or similar objects over an extended period of time, but may include other conditions involving changes
- Bias:** Estimate of a systematic measurement error
- Trueness:** Closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value
- Accuracy:** Closeness of agreement between a measured quantity value and a true quantity value of a measurand

ISO/IEC Guide 99:2007 International vocabulary of metrology – Basic and general concepts and associated terms (VIM)

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Quality assurance Quality control



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Quality in analytical measurements



Quality:

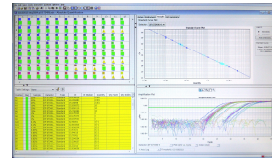
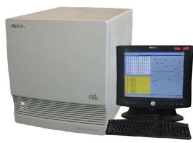
- To deliver in time a product or service that meets the specification agreed with the customer
- Satisfying customer requirements
- Fitness for purpose
- Getting it right the first time
- *Degree to which a set of inherent characteristics fulfils requirements* ¹⁾
- ...

Quality is about satisfying a customer!

¹⁾ ISO 9000:2005 Quality management systems – Fundamentals and vocabulary

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Laboratory and method performance (I)



Lab technician and instrumentation

(Standard) method

Measurement result



Competence (training) and calibration?



Validated and fit for purpose?



Reliable and traceable?

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Laboratory and method performance (II)



Competence ¹⁾: Appropriate education, training, experience, etc

Validation ²⁾: *Process of defining an analytical requirement, and confirming that the method under consideration has performance capabilities consistent with what the application requires*

Reliable: Sound and accurate ³⁾ result?

Proficiency of a laboratory to provide, based on a certain method, data of the required quality

¹⁾ ISO 9001:2001 Quality management systems – Requirements

²⁾ EURACHEM Guide 1998: The fitness for purpose of analytical methods

³⁾ ISO 5725:1994 Accuracy (trueness and precision) of measurement methods and results

Importance to prove performance (I)



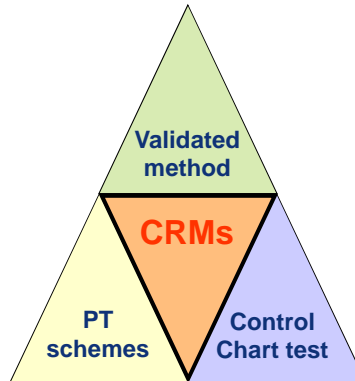
Why?

- **Customer can trust the analytical results from a laboratory**
- **Comparability of measurement results**
- **Accreditation to an international standard**
(e.g. ISO/IEC 17025:2005)

Importance to prove performance (II)



How?



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Use of CRMs as control samples



EURL
European Union Reference Laboratory
for GM Food & Feed

CRM

Certificate of analysis

Best estimate of "true" value
and measurement uncertainty

???

Certified value
and uncertainty

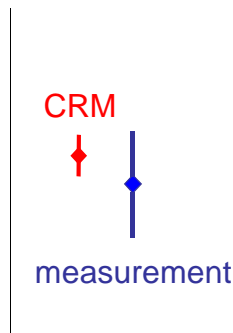
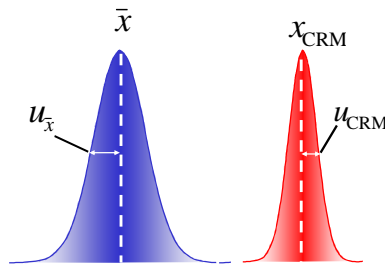
Is the difference between mean measured and certified value significant ?

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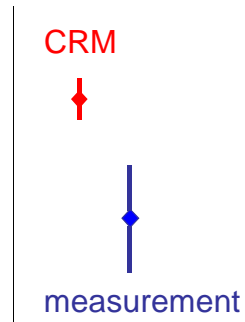
Comparison of measured and certified value (I)



test results certified value



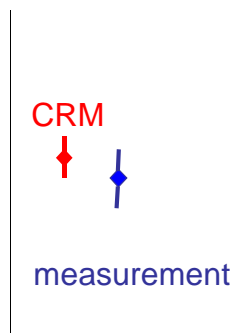
unbiased



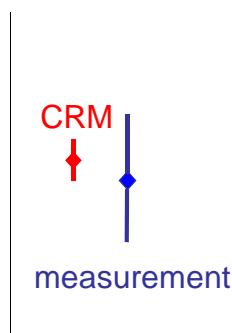
biased

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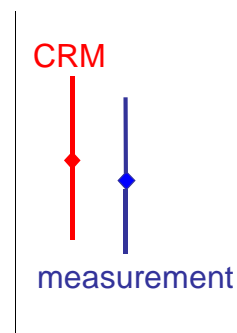
Comparison of measured and certified value (II)



much information about potential bias

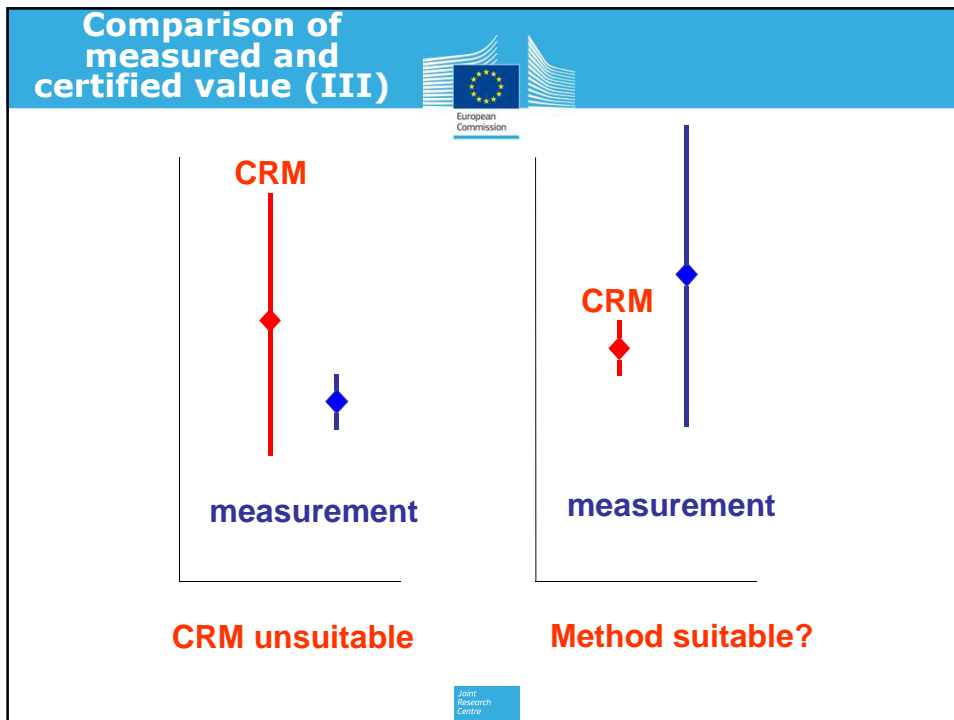


less information about potential bias




little information about potential bias

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Comparison of measured and certified value (IV)




Multi-step approach:

1. Determine difference (Δ_m) between mean measured value (c_m) and certified (true) value (c_{CRM})

$$\Delta_m = |c_m - c_{CRM}|$$

2. Convert expanded uncertainty (U_{CRM}) of c_{CRM} into standard uncertainty (u_{CRM})

$$u_{CRM} = \frac{U_{CRM}}{k}$$



Comparison of measured and certified value (V)



3. Estimate measurement uncertainty (u_m)
 - a) Method validation (bottom-up or top-down)
 - b) Within-laboratory standard deviation (intermediate precision)
⇒ *Quality control chart based on CRMs*
 - c) Repeatability standard deviation
⇒ *Often underestimation of the real uncertainty ($n \geq 6$)*

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Comparison of measured and certified value (VI)



4. Estimate the combined uncertainty (u_{Δ_m})

$$u_{\Delta_m} = \sqrt{u_m^2 + u_{CRM}^2}$$

5. Compare Δ_m with $2 \cdot u_{\Delta_m}$

$$\text{if } \Delta_m \leq 2u_{\Delta_m}$$

Method not significantly biased!

$$\text{if } \Delta_m > 2u_{\Delta_m}$$

Method significantly biased!

ERM® Application Note 1, <http://www.erm-crm.org>

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Comparison of measured and certified value (VII)



Method significantly biased!

Investigate the cause for the bias, correct and measure again!

Method not significantly biased!

NOTE:

- All attempts shall be made to eliminate the bias.
- If the bias cannot be eliminated, the lab might consider to calculate a correction factor (and include the uncertainty linked to this correction into the uncertainty). This is only possible if
 - (a) the bias is systematic over time (*control chart*)
 - (b) the bias proved to be constant or relative to the measured concentration (*difficult to investigate*)

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Certified value



CERTIFICATE OF ANALYSIS

ERM[®]- BF433d

MAIZE POWDER

	Mass Fraction	
	Certified value ²⁾ [g/kg]	Uncertainty ³⁾ [g/kg]
DAS-40278-9 maize ¹⁾	100	8

1) Genetically modified maize DAS-40278-9 with the unique identifier DAS-40278-9.

2) The certified value is based on the masses of mixed dried genetically modified DAS-40278-9 maize powder and of dried non-modified maize powder, taking into account their respective purity with regard to DAS-40278-9 maize and their respective water content. The certified value is traceable to the SI.

3) The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95% estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

confidence

coverage factor

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Coverage factor



ERM® materials, coverage factor is given straight away from the certificate ($k = 2$)

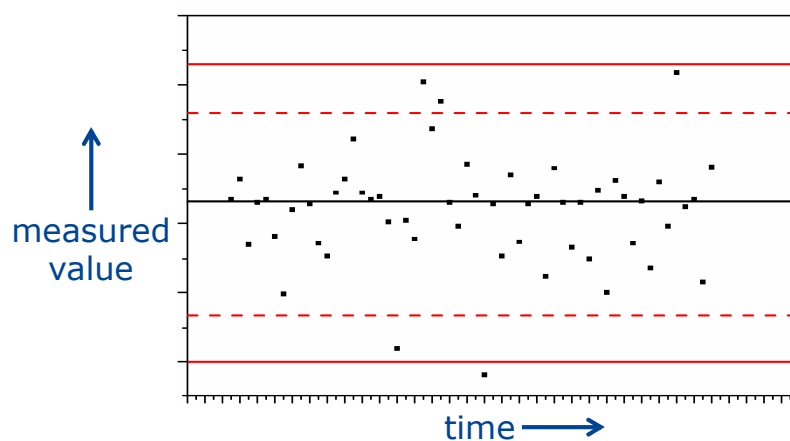
$$u_{\text{CRM}} = \frac{U_{\text{CRM}}}{k}$$

For other materials when value is given as $c_{\text{CRM}} > \text{xx.x \%}$ (rectangular distribution)

$$u_{\text{CRM}} = \frac{100 - c_{\text{CRM}}}{\frac{2}{\sqrt{3}}}$$

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Quality control chart (I)



Acceptable method performance?

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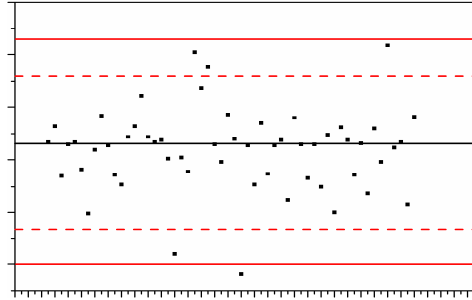
Quality control chart (II)



Control limits

Shewhart control chart (most popular)

- **Central line**
= mean value (min $n = 10$)
- **Upper/lower warning limits**
= mean value $\pm 2 \cdot s_{QC}$
- **Upper/lower control limits**
= mean value $\pm 3 \cdot s_{QC}$



Out-of-control

- 1 point above/below control limits
- 2 out of 3 consecutive points between warning and control limits
- 9 consecutive points on the same side of the central line
- 6 or more points in a row steadily increasing or decreasing
 \Rightarrow based on ISO 7870-2:2013; other interpretations exist

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Quality control chart (III)



Quality control materials

- **Material inserted into the run alongside the test material and subjected to exactly the same treatment**
- **Requirements for QCM:**
 - matrix identical or as close as possible to matrix of test material
 - same measurand and similar concentration
 - homogeneous
 - stable over time

ISO 7870-2:2013 Shewhart control charts
IUPAC harmonized guidelines for internal quality control in analytical chemistry laboratories, 1995

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Quality control chart (IV)



CRMs

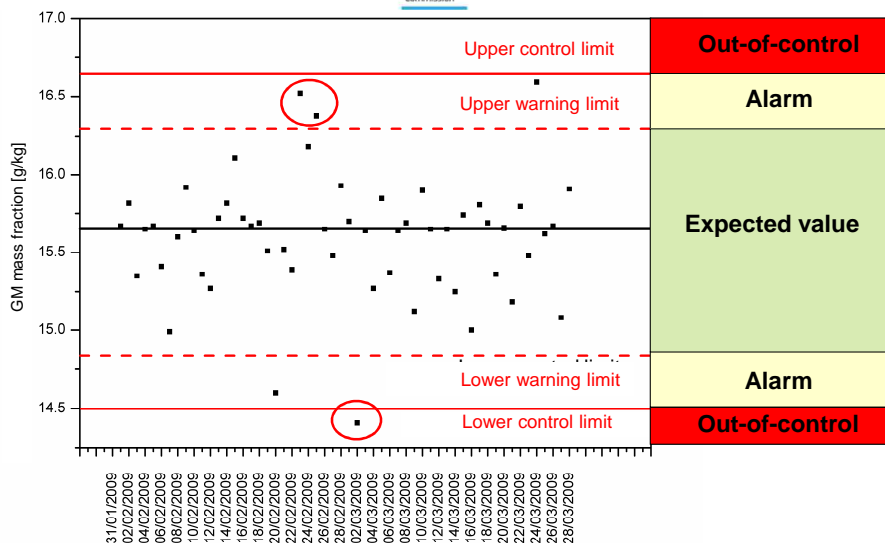
- Adds trueness assessment (evaluation see "Control sample")
- No need to set-up quality control chart again after running out of QCM
- Set-up like a normal quality control chart
 - certified value is used as expected value
 - warning limits are $2 \cdot u_c$ (u_c = combined uncertainty)
 - control limits are $3 \cdot u_c$

$$u_c = \sqrt{u_{\text{CRM}}^2 + s_{\text{QC}}^2}$$

ISO 7870-2:2013 Shewhart control charts
IUPAC harmonized guidelines for internal quality control in analytical chemistry laboratories, 1995

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Quality control chart (V)



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Proficiency testing (PT) schemes



WHAT?

- Comparison of laboratory's results with those of other laboratories

HOW?

- Regular circulation of homogeneous samples, sent by a coordinator of an independent testing body to the different participating laboratories
- Samples (normally) analysed by the laboratory's method of choice

WHY?

- To assess and demonstrate the reliability of produced data
- Important for accreditation

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Conclusions



CRMs are powerful tools that can prove the performance of measurement methods by:

- Assessing the accuracy on average or for an individual measurement
- Checking laboratory, analyst and instrument performance
- Giving quality control charts an added trueness dimension

NOTE:

Inappropriate CRMs & incorrect use destroy all these advantages!

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Thank you for your attention!

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EUR 26889 EN – Joint Research Centre – Institute for Reference Materials and Measurements

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Author(s): P. Corbisier, O. Zobell, S. Trapmann, G. Auclair and H. Emons

Luxembourg: Publications Office of the European Union

2014 – 110 pp. – 21.0 x 29.7 cm

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doi:10.2787/10085

Abstract

The content of this manual is based on the training course that was organised on the premises of the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (Geel, BE) at the end of 2013.

The training manual complements the training course that was intended to improve the quality of measurement results obtained when quantifying genetically modified organisms (GMO) in food and feed. Both, the training course and this manual, were developed in line with the current EU GMO legislation.

The manual is addressed to laboratory managers and practitioners in analytical laboratories who perform GM quantification measurements and use reference materials for calibration, quality control and method validation including in-house verification. It is also intended for analysts who need to assess measurement uncertainties as required by (EC) No 1829/2003 [1], (EC) No 619/2011 [2] and ISO/IEC 17025:2005.

This training document has been written by JRC-IRMM upon request of the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) to further improve the reporting of National Reference Laboratories (NRLs) nominated under Regulation (EC) No 882/2004 [] and official GMO control laboratories within the EU.

This manual is organised in four chapters covering the proper calibration of PCR methods, the estimation of measurement uncertainty, the establishment of metrological traceability of a measurement result and the way to prove the trueness of measurement results.

The training manual is a didactic support of a previous guidance document that outlines issues related to the estimation of measurement uncertainty (MU) in the GMO sector []. The training manual is also in line with the European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs, that is intended for laboratories that are acquiring or are holding a flexible scope of accreditation according to ISO/IEC 17025.

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*Serving society
Stimulating innovation
Supporting legislation*

