FOOD ANALYSIS: THE ROLE OF MEASUREMENT UNCERTAINTY INTERNATIONAL CONFERENCE 2ND IMEKOFOODS

Uncertainty assessment for mycotoxins in food. The example of the metrological approach.

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Abstract – Over the last ten years, the role on measurement uncertainty has been embraced in those sectors where, traditionally, metrology has strived to assert the concept and its applications. Mycotoxins analysis constitutes an example where not many reference guidance are available, thus experts are working to assess, verify and control uncertainty budget coming from the application of their methods. Possible approaches to be used are top down (metrological) or bottom up (holistic approach from the availability of reproducibility data). Examples on how these two approaches may be used in the mycotoxin field will be scrutinized. Moreover, the critical role of the measurement uncertainty in the compliance assessment and decision making in the context of official control will be taken into consideration.

Keywords: uncertainty, mycotoxins,

1. INTRODUCTION

Uncertainty is widely recognized as one of the fundamental parameter to evaluate the reliability of the process of measurement [1]. Uncertainty is defined as "Parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand" (VIM 2008).
In food analysis uncertainty concepts and its use has been growing during the last 10 years and nowadays that standardizations, including legislation, have provided, useful assistance to the users who always feel alone to face with the theory and all the notions around the topic.
A lot of work is still needed and has to be done especially for harmonization, and in the sector of mycotoxins analyses still none specific guidance is available neither from the reference institutions nor from the international standard therefore the sharing of approaches is much more appreciated in order to gather confidence.

2. • APPROACHES FOR UNCERTAINTY

Different approaches are possible to assess the uncertainty, the choice depending on the laboratory expertise, tradition, functions and context. Food analysis experts may have different background and may be asked to assess uncertainty depending on the purpose.

A laboratory may be interested to provide a strict evaluation of each contribution to the measurement, therefore the metrological approach should give the elements and respond to its needs; other laboratories, with less experience, should choose to assess which is the extent of uncertainty, thus a *bottom up* approach would help them in this, only verifying, for example, whether the gross uncertainty of the measurements are included in variability of the method reproducibility.

The *bottom-up* approach is a step-by-step approach, where the uncertainty is calculated from a combination of various components, relating them to building blocks of the overall procedure or to significant error sources. The final uncertainty result of an analytical procedure is determined on

the basis of a detailed uncertainty budget in a series of steps.

On the other hand, the *top-down* approach is an empirical approach where the result is method-dependent. The top down is for routine purposes but does not take systematic errors into account. It is a very practical approach based on validation and Quality Control data already available in laboratory assuming that all factors, which influence measurements, are taken into consideration with the application of the method.

A reference laboratory should provide evidences on the strength and weakness of each approach illustrating the pros and cons also considering the possible over/under estimation that each approach may have.

The Eurachem guidance on the measurement uncertainty gives a perfect guide to be followed for the metrological approach. It has not specific examples on mycotoxins but presents all the crucial elements to calculate the contributions to the uncertainty.

2.1. • Bottom up: metrological approach. Mycotoxin case

The metrological approach is a procedure that takes into account all single elements that may constitute a source of variability. It is especially useful when an in-house method has been developed and a number of measurements have been produced. In these cases, excluding a rough assessment given by the Horwitz equation, the lack of reference methods (either CEN or ISO or AOAC) represent a drawback for the evaluation of the uncertainty.

In the following paragraph, the procedure for the calculation of the measure uncertainty associated to an in-house method for the determination of deoxynivalenol (DON) content in cereals will be illustrated.

The rationale is to start from the method procedure, identify the source of uncertainties, calculate the standard uncertainties that derive from each contribution and finally sum up the contribution to obtain a combined uncertainty u_C. Finally by multipliyng by a proper coverage factor, k, the expanded uncertainty, U_E, is obtained, which represent a measure of the uncertainty to be attributed to the measurement result within a stated level of confidence.

As for starting, and for familiarizing with the procedure, the Eurachem guide envisages a four-step flow diagram where all the key issues that may enter in the assessment are acquired.

The detailed explanation of each step is described in the following paragraphs.

Step 1. Specification of the mesurand. The uncertainty to be assessed is associated to a measurement procedure where the specific mycotoxins (e.g. DON in durum wheat) is measured after the application of the method procedure. Thus, the first step is to write down the exact equation used for the measurement. A clear statement of what is being measured and of the model equation applied for the calculation is the first step.

In the example of the detection of DON in cereals (durum wheat, for example), the content is expressed in µg of toxin per kg of cereal [µg/kg]. For this method taken as an example, the procedure consists of 7 stages with measures of weights and volumes, as shown in Fig 1.

$$W_{DON\_CORR.REC}\left[\frac{\mu g}{kg}\right] = \frac{100}{\mathrm{Re}\,c(\%)}\left(\frac{m_0}{m_s}\cdot\frac{V_1}{V_2}\cdot\frac{V_3}{V_4}\right)$$

Where:
Rec(%) = recovery value, (%)
m_0 = mass of mycotoxin in the test solution from injection (µg)
m_s = mass of the sample, (kg)
V_1 = volume of extraction solvent, (mL)
V_2 = volume the filtered extract used for clean-up, (mL)
V_3 = volume used to recover the purified extract le after evaporation, (mL)
V_4 = injection volume of test solution, (mL)

Procedure. Weighing (wheat sample) → Extraction (volume of extraction solvent) → Purification (volume of extracted sample purified by immunoaffinity clean-up) → Reconstitution (volume of injection solvent) → [HLPC calibration] → Detection (HPLC analysis, injection of extracted sample) → Result.

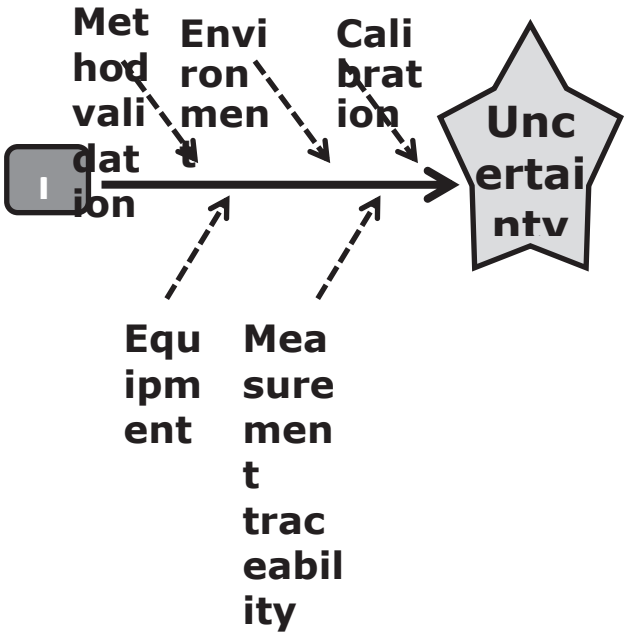
Fig.1. Model equation and procedure for the determination of DON in cereals.

Step 2. Identifying Uncertainty Sources. Starting from the procedure a list of all the possible sources of uncertainty for each part of the process should be identified. At first, it may happen that a long list of sources are registered and only after a critical revision just a number will remain as the important ones. Beside, it also happens that after a critical

revision of the uncertainty procedure, some contributions are definitively negligible.
In this step it may be helpful a breaking the measurement process down and drown a "cause-and-effect" diagram (or fish backbone diagram). In Fig. 2 a), example of fish backbone diagram illustrates the laboratory activities that may represent a source of uncertainty, while in b), the 4 principal sources of uncertainties for the procedure are shown.
Taking into account the sources of uncertainty shown in Fig. 2, the typical sources of uncertainty that affect within the whole procedure for DON analysis are:

- Repeatability (method validation, recovery)
- Weighing operation (mass)
- Volume measurement operation
- Others sources (volume dispensed by injector of HPLC).

a)



b)

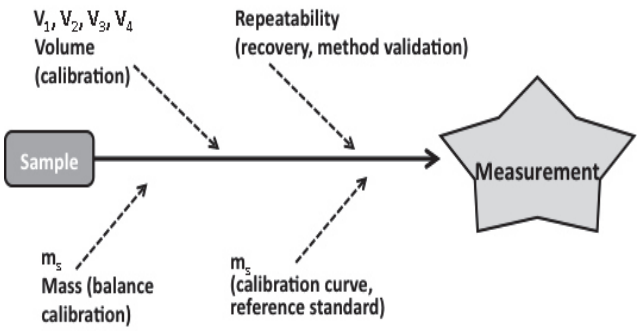


Fig. 2. Fish backbone diagram. a) A diagram illustrating a group of laboratory activities that may contribute with source of uncertainty. b) A diagram showing the 4 principal sources of uncertainties for the procedure.

Step 3. Quantifying Uncertainty. Once clarified which are the actual souces of uncertainty for the final result, each single source is transformed in a measure of variability, i.e. a standard uncertainty; by summing up all the standard uncertainties a combined uncertainty will be calculated. The following paragraphs scrutinize each point to derive and quantify all the standard uncertainty contributions. All the contributions will be considered as relative values in order to have all the addends to be summed up for the final combined uncertainty.

Uncertainty arising from repeatability. The standard uncertainty contribution for repeatability (u_{rep}) can be derived from the in-house study of the method during repeatability experiments. The (u_{rep}) can be estimated by taking the relative standard deviation of the method (RSD) or by dividing the standard deviation of the repeatability assessed for a comparable level (s_{rep}) by the square root of the number of measurements carried out during validation. This is a type A uncertainty, and is calculated as a relative measure:

$$u_{rep\_rel} = \frac{s_{rep}}{\sqrt{n}} \times \frac{1}{x_m}$$

Where: s_{rep} is the repeatability value for the *x* level; *n* is the number of repetitions; x_m is the level to which the repeatability is referred (mean value).
Also the contribution arising from the recovery is considered. The RSD calculated during the in-house method validation step through recovery experiments (homogenised samples spiked with known standard) can be used. The associated standard uncertainty, (u_{Rec}), is estimated by the ratio between the standard deviation associated with the recovery and the square root of the number of determinations used for the calculation. The relative value is the following:

$$u_{rec\_rel} = \frac{s_{rec}}{\sqrt{n}} \times \frac{1}{x_m}$$

Where: s_{rec} is the repeatability value for the level of recovery; *n* is the number of repetitions; x_m is the recovery measured (mean value).

Uncertainty of weighing. The u_{mass} is a type B uncertainty, which can be derived from the balance manufacturer quotation of the linearity (s_{lin}) on the calibration certificate. The linearity contribution is assumed to show a rectangular distribution, thus the square root of 3 is the factor for the standard uncertainty. The contribution for the linearity has to be accounted for twice (tare and gross mass).

The relative standard uncertainty will be:

$$u_{mass\_rel} = \sqrt{2} \times \frac{s_{lin}}{\sqrt{3}} \times \frac{1}{m_s}$$

Where: s_{lin} is the repeatability value for the level of recovery; m_s is the mass of the sample.

Uncertainty of volume measurements. The u_{vol} can be derived by summing contribution for calibration, for temperature variation and repeatability. Considering the negligible contribution of the temperature variation and repeatability, only the calibration contribution will be assessed.

Calibration. Each range of volumes has its calibration uncertainty given by the manufacturer as an expanded uncertainty value, U_E. The calibration contribution is given by dividing the U_E for the factor for triangular distribution (square root of 6). A single contribution is going to be taken into account for each volume measured.

$$u_{vol\_rel} = \frac{U_E}{\sqrt{6}} \times \frac{1}{V}$$

Where: U_E is the value given by the manufacturer; V is the volume measured.

The uncertainty contribution of the calibration curve. The calibration curve that is used for the calculation of the ng in the injected sample, m_0, is constructed by injecting five different concentration levels (x_i) of reference materials, in triplicate. Then, the obtained instrumental signals for each level (y_i obs) are recorded. The statistical treatment of the n data points is based on interpolation of the measures according to the least squares method that allows the knowledge of the coefficients a (slope) and b (intercept) of the curve that best approximates the experimental data.

Assuming that the uncertainties of the values of the abscissa are considerably smaller than the uncertainty on the values of the ordinate, the linear least squares fitting procedure considers the uncertainty for m_0 due to random variation in the signal and not the uncertainty of the calibration standards, nor the inevitable correlations induced by successive dilution from the same stock.

The uncertainty on the m_0 value is given by the following formula that considers un-weighted data and a calibration curve obtained by 15 measurements (5 levels in triplicate).

$$u_{cal} = \left[\frac{\left(\frac{S_{y}}{x} \right)}{a} \right] \times \sqrt{\left\{ \frac{1}{m} + \frac{1}{15} + \left[\frac{\left(x_{pred} - x_m \right)^2}{\sum (x^2) - (\sum x_i)^2 / n} \right] \right\}}$$

Where: $S_{x/y}$ is the residual for the i^{th} point; p is the number of measurement for m_0; x_{pred} is the estimated value; x_m the mean of xi values; a is the slope of the calculated best fit.

$$S_{x/y} = \sqrt{\frac{\sum \left(y_{pred} - y_{obs} \right)^2}{(n-2)}}$$

Where: $S_{x/y}$ is the residual; y_{obs} the observed instrumental response; y_{pred} is the value of the predicted signal from the calibration line.

The relative standard uncertainty associated with the linear regression line for m_0, u_{cal_rel}, is calculated:

$$u_{cal\_rel} = u_{cal} \frac{1}{m_o}$$

Step 4. Calculating the combined standard uncertainty and expanded uncertainty

After the estimation of individual components of uncertainty expressed as relative standard uncertainties, the combined standard uncertainty u_{c_rel} is calculated by the following formula:

$$u_c(w)/w = u_{c\_rel} = \sqrt{\left(u_{rep\_rel}\right)^2 + \left(u_{rec\_rel}\right)^2 + \left(u_{mass\_rel}\right)^2 + 4 \cdot \left(u_{vol\_rel}\right)^2 + \left(u_{cal\_rel}\right)^2}$$

The combined standard uncertainty value for w in [µg/kg], $u_c(w)$, is obtained by multiplying w for the relative uncertainty value.

$$u_c(w) = u_{c\_rel} \times w$$

Finally, the combined standard uncertainty $u_c(w)$ is multiplied by the chosen coverage factor in order to obtain the expanded uncertainty, U_E.

The expanded uncertainty is required to provide an

interval, which may be expected to encompass a large fraction of the distribution of values, which could reasonably be attributed to the measurand.

$$U_E = u(w) \times k$$

Horwitz equatio

2.2. Horwitz-Thompson equation

The linearised Horwitz function as expressed below

$$\log_{10} \sigma_H = 0.8495 \times \log_{10} c - 1.6990$$

is a generalisation about the reproducibility standard deviation (expressing inter-laboratory precision), therefore it can be used as a measure of uncertainty.

Horwitz equation [4] represents one of the first empirical parameters to be used for quality control laboratories, and to be used in proficiency testing results.

The concept of the equation is that it may be used as a reference for the interlaboratory precision measurement so that, considering this variation the largest possible, a laboratory has to verify its performances, and compare and assess the appropriateness of the value.

Therefore Horwitz equation is a reference for the interlaboratory precision and a number of international organization such as Union of Pure and Applied Chemistry [2], Nordic Analytical Committee [3], and the European Committee for Standardization (CEN), among others, have been cited the equation as an appropriate reference.

It is known that the function is slightly pessimistic at high concentrations (above 10% m/m) and so at low trace concentrations. Below about 10 ppb, there is a tendency for an invariant RSD of about 20-25%. This is because a method with a higher RSD would hardly provide any useful quantitative information.

The equation is commonly used in food analysys and more specifically in mycotoxin analyses provided that the Thompson adjustment is considered [5]. In fact, some of the mycotoxins, namely the aflatoxins and ochratoxin A, are present within a range of values where the Horwitz equation fails. In these cases the following functions are suggested for the σ_H assessment:

σ_H =0 22c if c <1.2 10^{-7} (1)

σ_H =0 22c $^{0.8495}$ if 1.2 $10^{-7} \leq c \leq$ 0.138 (2)

σ_H = 0,01c if c > 0.138 (3)

The Hurwitz reproducibility calculation is sometimes used by the laboratory as an estimate of uncertainty when scarce data have been provided or when still small experience has been acquired on the procedures for uncertainty assessment. However the Horwitz approach can represent a guidance value of a maximum budget uncertainty.

4. CONCLUSIONS

Much work has been already done by the laboratories of the food sector to improve their the performance in terms of quality control and technical expertise, and a lot has been done, yet, for the adoption of procedures for uncertainty calculation when this parameter was hardly taken into consideration not long time ago. Today the majority of laboratory, especially those of the official control, and most important, the risk managers have acquired the concept behind; nevertheless the community of experts in the laboratory still experience a lot of difficulties for the definition and calculation of uncertainty in defined situations. The harmonization of procedures, the consensus of views and the sharing of knowledge would help in familiarize with the theories and soften the concept and facilitate the uncertainty assessment.

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