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Automated liquid handling systems – Uncertainty of the measurement procedures

Systèmes automatisés de manipulation de liquides – Incertitude des modes opératoires de mesure

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Foreword

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This document was prepared by Technical Committee ISO/TC 48, *Laboratory Equipment*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The examples given in this document are informative and support the requirement found in the ISO 23783 series to perform an estimation of measurement uncertainty when calibrating automated liquid handling systems (ALHS) according to the measurement procedures described in ISO 23783-2. The examples in this document are based on the principles of ISO/IEC Guide 98-3.

Automated liquid handling systems – Uncertainty of the measurement procedures

1 Scope

This document describes the measurement uncertainty analysis of the measurement procedures described in ISO 23783-2, following the approach described in ISO/IEC Guide 98-3.

This document also includes the determination of other uncertainty components related to the liquid delivery process and the device under test (DUT) to estimate the overall measurement uncertainty of delivered volumes by an automated liquid handling system (ALHS).

2 Normative references

ISO 23783-1, *Automated liquid handling systems — Part 1: Vocabulary and general requirements*

ISO/IEC Guide 98-3, *Uncertainty of measurement — Part 3: Guide to the expression of uncertainty in measurement (GUM:1995)*

ISO/IEC Guide 99, *International vocabulary of metrology — Basic and general concepts and associated terms (VIM)*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 23783-1, ISO/IEC Guide 98-3, and ISO/IEC Guide 99 apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

4 General procedure for the uncertainty calculation

The evaluation of measurement uncertainty in this document follows the ISO/IEC Guide 98-3 “Guide to the Expression of Uncertainty in Measurement (GUM).” The method has the following steps:

a) Expressing, in mathematical terms, the relationship between the measurand and its input quantities.

b) Determining the expected value of each input quantity.

c) Determining the standard uncertainty of each input quantity.

d) Determining the degree of freedom for each input quantity.

e) Determining all covariance between the input quantities.

f) Calculating the expected value for the measurand.

g) Calculating the sensitivity coefficient of each input quantity.

h) Calculating the combined standard uncertainty of the measurand.

i) Calculating the effective degrees of freedom of the combined standard uncertainty.

j) Choosing an appropriate coverage factor, k, to achieve the required confidence level.

k) Calculating the expanded uncertainty.

In this document, the uncertainty of the measurement procedure is separated in three different clauses:

— the uncertainty components associated with the measuring system, see Clause 6;

— the uncertainty components associated with the device under test (ALHS), see Clause 7;

— the uncertainty components associated with the liquid delivery process, see Clause 8.

5 Modelling of the measurement

Each measurement procedure has specific uncertainty components associated with the measuring system. These uncertainty components are described in the respective annex for each procedure. See Annex A for the dual-dye ratiometric photometric procedure, Annex B for the gravimetric procedure, and Annex C for the optical image analysis of droplets.

6 Standard uncertainty components associated with the measuring system

6.1 General information on standard uncertainty components estimation

It is possible to experimentally estimate the standard uncertainty of measurement, $u(x)$, for a quantity x, by performing multiple measurements of x under repeatability conditions. This is called a type A evaluation according to ISO/IEC Guide 98-3. The standard deviation of the obtained values is a measure of the repeatability of the measurement. The standard uncertainty associated with x can be a standard deviation based on previous experience (in the case where a single measurement of x is made), or the standard deviation of the mean equal to stdev(x)/sqrt(n) (in the case where x is the average of n readings).

See ISO Guide 98-3:2008, 4.2 for more information on type A evaluation of standard uncertainty.

In addition to repeated measurements, the systematic component of the uncertainty of measurement for a quantity x is estimated by other means. This is called a type B evaluation according to ISO/IEC Guide 98-3. For example, one can obtain information for that estimation by considering the manufacturer's specifications of the ALHS (e.g., resolution, linearity, drift, temperature dependence).

Often the manufacturer's specifications are given in the form of an interval covering the measurement value, with no additional information regarding distribution or coverage. In those cases, the measurement can be assumed to follow a uniform or rectangular distribution. This distribution is characterised by a constant probability inside the interval while the probability outside the interval is zero.

The interval can be used in a type B evaluation to give the variance of x in the form shown in Formula (1):

$$u^2(x) = \frac{(a_+ - a_-)^2}{12} \tag{1}$$

where

$u^2(x)$ is the variance of the variable x;

a_+ and a_- give the upper and lower limits of the interval of the variable x.

The standard uncertainty, $u(x)$, is given as the square root of the variance.

In addition to uniform rectangular, other distributions are also possible when performing type B evaluations. See ISO/IEC Guide 98-3:2008, 4.3 for more information on type B evaluations of standard uncertainty.

6.2 Specific information on standard uncertainty components estimation

Specific information regarding standard uncertainty components particular to the dual-dye ratiometric photometric, gravimetric, and optical volume measurement procedures is given in Annex A, Annex B and Annex C, respectively.

7 Standard uncertainty components associated with the ALHS

7.1 General

Subclauses 7.2 and 7.3 describe uncertainty components, which can influence the operation and performance of the ALHS. Depending on the specific type of ALHS and liquid used, additional uncertainty components can be identified.

7.2 ALHS-type specific influencing parameters

The following parameters can impact the liquid delivery process, depending on the type of ALHS used:

— exchangeable components, e.g., type of tips and others (see ISO 23783-1:2022, 6.8);

— air cushion effects (if applicable, e.g., for air-displacement or liquid-filled piston-operated ALHS with an air gap);

— system liquid effects:

  — dissolved gases in the system liquid,

  — internal dilution effect of the sample with system liquid,

  — temperature sensitivity of the system liquid;

— environmental effects on the deck of the ALHS:

  — rate of air flow,

  — air temperature,

  — relative humidity,

  — barometric pressure;

— vibration effects;

— electrostatic effects.

7.3 Test liquid properties influencing ALHS operation

— surface tension;

— viscosity;

— density;

— content of dissolved gas in the test liquid (formation of micro-bubbles);

— vapor pressure;

— Weber number (contact-free dispensing), see Reference [1].

7.4 Standard uncertainty of ALHS resolution

The standard uncertainty related to the resolution can be determined according to Formula (2):

$$u(res)=\frac{\Delta res}{\sqrt{12}} \tag{2}$$

where

$u(res)$ is the standard uncertainty related to the resolution of the ALHS volume selection device;

Δres is the actual or estimated resolution of the volume selection device of the ALHS.

NOTE 1 The uncertainty related to the resolution of the ALHS is included in the uncertainty budget when the measurements are dependent on the direct reading of the output volume. The uncertainty of the resolution is also included when estimating the uncertainty of the systematic error e_S.

NOTE 2 The physical resolution of an ALHS is not necessarily the resolution displayed in the ALHS' software. For example, different syringe sizes can be mounted on the same stepper motor. The resolution of the stepper motor will translate into various volume resolutions, depending on the size of the syringe mounted on the motor.

7.5 Standard uncertainty of cubic expansion coefficient (optional)

The correction of a measured volume at test temperature to a reference temperature is optional, see ISO 23783-2:2022, Clause 7. If this correction is performed, this subclause applies.

The standard uncertainty related to the cubic expansion coefficient γ is dependent on knowledge of the actual material of the artefact and on the source of the data, which provides an appropriate value. Data from the literature or manufacturer can be used for the expansion coefficient, and this value would be expected to have a relative standard uncertainty of 5 % to 10 % of the expansion coefficient, see Reference [2].

For devices with an air cushion, the thermal effects on the cubic expansion coefficient and the air cushion are entangled and need to be considered in tandem or determined experimentally. The details of this entanglement are beyond the scope of this document.

7.6 Standard uncertainty associated with air cushion effects (optional)

If applicable, the standard uncertainty related to the air cushion effect $u(\Delta V_{\text{cush}})$ depends on the size of the air cushion that is related to the lifting height in the pipette tip and can be calculated according to Formula (3), which is based on the information given in DKD-R 8-1:2011, 8.7, see Reference [3]:

$$u(\Delta V_{\text{cush}})=\sqrt{\left(u(V\Delta p)\times c_{V\Delta p}\right)^2+\left(u(V\Delta h_r)\times c_{V\Delta h_r}\right)^2+\left(u(V\Delta t_{\text{L}})\times c_{V\Delta t_{\text{L}}}\right)^2} \tag{3}$$

where

$u(\Delta V_{\text{cush}})$ is the standard uncertainty related to the air cushion effect;

$u(V\Delta p)$ is the standard uncertainty attributed to air pressure variation during the tests;

$u(V\Delta h_r)$ is the standard uncertainty attributed to the humidity variation during the tests;

$u(V\Delta t_{\text{L}})$ is the standard uncertainty caused by variation between the test liquid temperature, air temperature and temperature of the ALHS under calibration;

c_i are the sensitivity coefficients related to each uncertainty component.

The variations of each parameter are determined experimentally during the test, and only apply to ALHS which have an air cushion. Variations of these parameters are influenced by the size of the air cushion

relative to the volume of the aspirated test liquid, the test liquid's vapor pressure, and whether the tip has been pre-wetted or not.

The sensitivity coefficients c_i related to the air cushion effect from pressure, relative humidity and temperature can be derived from DKD-R 8-1, see Reference [3].

8 Repeatability and reproducibility of the liquid delivery process

8.1 Repeatability (experimental standard deviation)

Annexes A, B and C allow the determination of the standard uncertainties associated with the respective measurement procedure. To derive the standard uncertainty associated with the liquid delivery process, the experimental standard deviation needs to be included. When the mean delivered volume is the measurand, the standard deviation s_r is divided by the square root of the number of repeated measurements n, as shown in Formula (4):

$$s_r(\bar{V}) = \frac{s_r}{\sqrt{n}} \tag{4}$$

where

$s_r(\bar{V})$ is the standard deviation of the mean volume $\bar{V}$;

s_r is the repeatability standard deviation;

n is the number replicate measurements.

8.2 Reproducibility

The uncertainty related to the reproducibility of $\bar{V}$ (from one test of the ALHS to the next test) also needs to be included. There are several methods to determine this uncertainty contribution:

a) A laboratory can perform experimental studies where the ALHS test is performed multiple times under different reproducibility conditions (see also ISO 23783-1:2022, 3.40 "reproducibility" and ISO 23783-3:2022, 5.3.2 "experiment") and the reproducibility standard deviation of the measurement result $\bar{V}$ is calculated, symbol $s_a(\bar{V})$;

b) If no such information is available, a value for reproducibility of the selected volume can be provided by ALHS manufacturers or third parties. As no further information on the variation of individual measurements is taken into account, a rectangular distribution is suggested.

NOTE Sometimes, the value for reproducibility can be inferred from the declared "accuracy" or "systematic error" of the ALHS. For example, an ALHS with a 5 % specification for accuracy (systematic error) can be used to estimate the reproducibility as 2,9 % according to Formula (1).

The influence of environmental conditions on the reproducibility of ALHS performance can vary depending on the type of ALHS used and needs to be determined experimentally.

9 Combined standard uncertainty of measurement associated with the systematic error of mean volume

According to ISO/IEC Guide 98-3, when the errors of input quantities are uncorrelated, the variance characterising the uncertainty of measurement can be written according to Formula (5):

$$u^2 = \sum_i c_i^2 \times u^2(x_i) \tag{5}$$

where:

u^2 is the variance characterizing the uncertainty of measurement;

$u^2(x_i)$ are the variances associated with each input quantity which contributes to the final result (described by the model);

c_i^2 are the squares of the sensitivity coefficients giving the degree of influence of each individual standard uncertainty.

The sensitivity coefficients can be determined by evaluating the partial derivatives of the measurement equation, by numerical simulations, or by physical experiment. In the case of this technical report, it is possible to obtain explicit functions for many sensitivity coefficients by evaluating the partial derivatives as shown in Clause 10.

In this document, the uncertainty components are described in groups corresponding to Clauses 6, 7 and 8. For the mean volume of a calibration or test, Formula (6) applies.

$$u^2(\overline{V}) = u^2_{\mathrm{MS}}(\overline{V}) + u^2_{\mathrm{ALHS}}(\overline{V}) + u^2_{\mathrm{LDP}}(\overline{V}) \tag{6}$$

where

$u^2(\overline{V})$ is the variance characterising the uncertainty of the mean volume in a test or calibration;

$u^2_{\mathrm{MS}}(\overline{V})$ is the variance characterising the uncertainty due to the measuring system;

$u^2_{\mathrm{ALHS}}(\overline{V})$ is the variance characterising the uncertainty due to the ALHS;

$u^2_{\mathrm{LDP}}(\overline{V})$ is the variance characterising the uncertainty due to the liquid delivery process.

When reporting uncertainty of the mean delivered volume (n replicates), the two liquid delivery process variances (see Clause 8) are combined as shown in Formula (7).

$$u^2_{\mathrm{LDP}}(\overline{V}) = \frac{s_r^2}{n} + s_d^2(\overline{V}) \tag{7}$$

where

s_r is the repeatability standard deviation;

$\frac{s_r^2}{n}$ is the variance of the mean volume due to the repeatability of the ALHS;

$s_d^2(\overline{V})$ is the variance of the mean volume due to test process reproducibility.

10 Sensitivity coefficients

The sensitivity coefficients for the measurement procedure can be derived using any of the following approaches:

a) from the mathematical model of the measurement;

b) experimentally from comparison studies;

c) derived and reported as relative values as percent of the measurand (e.g., EURAMET cg-18 for air density, Reference [4]);

d) available from literature values;

e) numerical simulation.

Sensitivity coefficients specific to each type of measurement procedure (photometric, gravimetric and optical) are given in Annex A, Annex B and Annex C, respectively.

11 Coverage factor *k*

In order to calculate an appropriate coverage factor *k* for a 95 % confidence level (see ISO/IEC Guide 98-3:2008, Annex G), the effective degrees of freedom ν_{eff} are estimated by means of the Welch-Satterthwaite equation as shown in Formula (8):

$$\nu_{\text{eff}} = \frac{u_V^4}{\sum_{i=1}^{n} \frac{u_i^4}{\nu_i}} \tag{8}$$

where

ν_{eff} are the effective degrees of freedom for the measurement;

u_V is the combined standard uncertainty of the measured volume;

u_i is the standard uncertainty of each component;

ν_i are the degrees of freedom of each component.

For 10 or more measurements, *k* can be calculated or *k* = 2 can be used if the individual standard uncertainty values have a similar weight in the combined uncertainty. For less than 10 measurements, *k* is calculated.

12 Expanded uncertainty of measurement associated with the mean volume

The expanded uncertainty of the mean volume $\bar{V}$ is expressed according to Formula (9), where the standard uncertainty is multiplied by the coverage factor *k*.

$$U(\bar{V}) = u(\bar{V}) \times k \tag{9}$$

where

$U(\bar{V})$ is the expanded uncertainty of the mean volume;

$u(\bar{V})$ is the standard uncertainty of the mean volume;

k is the coverage factor.

13 Examples for determining the uncertainty of the volume measurement of ALHS

13.1 Measurement conditions

When reporting measurement results and the associated uncertainty information, it is necessary to understand and interpret the report. A comprehensive list of required and recommended information to be included in the reports is found in ISO 23783-3:2022, Clause 6. The uncertainty related to the reproducibility of $\bar{V}$ (see 8.2) also needs to be included.

For each example in this document, measurement conditions are described in appropriate sub-clauses within the Annexes.

13.2 Results

13.2.1 Standard uncertainty of the ALHS mean volume

The standard uncertainty of the mean delivered volume is calculated according to Formula (10):

$$u(\overline{V}) = \sqrt{u_{\mathrm{MS}}^2(\overline{V}) + u_{\mathrm{ALHS}}^2(\overline{V}) + u_{\mathrm{LDP}}^2(\overline{V})} \tag{10}$$

13.2.2 Expanded uncertainty of the measurement

The expanded uncertainty of the measurement is calculated by multiplying the standard uncertainty of the measurement by the coverage factor k, according to Formula (11). The numerical value of k is reported as part of the result.

$$U(\overline{V}) = u(\overline{V}) \times k \qquad (k = x_k) \tag{11}$$

where x_k is the value of k used in the calculation of the expanded uncertainty of the mean volume.

NOTE 1 This expanded uncertainty is the measurement uncertainty of the calibration as described in ISO 23783-3:2022, 6.1.3 a).

13.2.3 Result of measurement

The overall result of the measurement, including the expanded uncertainty of measurement, can be expressed as shown in Formula (12).

$$V_{\mathrm{M}} = \overline{V} \pm U(\overline{V}) \qquad (k = x_k) \tag{12}$$

V_{M} = 5,014 µl ± 0,012 µl (k = 2)

where V_{M} is the overall result of the measurement including the expanded uncertainty.

NOTE The numerical values are given for illustration only. The particular values for each example are given in each Annex.

13.2.4 Caution regarding use of numerical values in this report

The numerical values of the quantity estimation, standard uncertainty, and sensitivity coefficients are applicable to the specific situation described in each example and are often volume dependent. It is not appropriate to use the values given in this technical report for other situations or volumes.

13.2.5 Remarks on conformity with ISO/IEC Guide 98-3

The term “random error” as used in the ISO 23783 series is equivalent to the term “experimental standard deviation” used in ISO/IEC Guide 98-3.

There is no direct equivalent to “systematic error” e_{S} as used in the ISO 23783 series that is found within ISO/IEC Guide 98-3.

NOTE 1 The term “instrumental bias” is found in ISO/IEC Guide 99 and is similar to “systematic error of measurement” provided the ALHS is considered a liquid measuring instrument and care is taken regarding a positive or negative numerical sign in the result.

To evaluate the uncertainty of the systematic error of measurement, a volume difference V_{D} can be defined, as shown in Formula (13).

$$V_{\mathrm{D}} = V_{\mathrm{S}} - \overline{V} \tag{13}$$

where

V_D is the volume difference between the selected volume and the average of all delivered volumes;

V_S is the selected volume, the volume intended to be delivered;

$\bar{V}$ is the average of all measured volumes.

The uncertainty of this volume difference V_D will include the uncertainty associated with the mean volume $\bar{V}$ and possibly an uncertainty associated with the resolution or setting of the selected volume V_S (see 7.4). The uncertainty of the "volume difference" $u(V_D)$ and the uncertainty of the "systematic error of measurement" $u(e_S)$ are identical provided that the uncertainty intervals are symmetric, as is the case in the examples of this document.

NOTE 2 The "systematic error" $e_S = \bar{V} - V_S$ used within the ISO 23783 series is reversed in sign compared to the volume difference V_D, the "measurement error", and the "instrumental bias" as defined in ISO/IEC Guide 99.

Annex A
(informative)

Dual-dye ratiometric photometric procedure

A.1 Description of the measurement

Absorbance measurements are made in either 96-well or 384-well microplates using a microplate absorbance reader. Typically, the wells of a 96-well microplate have a round cross-section, while those of 384-well microplates have a square-shaped cross section.

Absorbance per unit path length values of the Ponceau S test liquid and copper(II) chloride solutions are determined in cuvettes with 10 mm optical path length using a reference grade spectrophotometer.

The Ponceau S test liquids used in this procedure contain the same concentration of $CuCl_2$ as the $CuCl_2$ solution used as diluent.

The unknown volume of Ponceau S test liquid is delivered into the wells of a microplate. Depending on the amount of Ponceau S test liquid, a non-quantitative amount of $CuCl_2$ can be added to the well. The microplate containing test liquid in its wells is placed on a plate shaker to mix the two liquids and level the meniscus in each well. The microplate is then placed in the absorbance plate reader, and the absorbance at 520 nm and 730 nm is determined for each well. The volume of delivered Ponceau S test liquid is calculated according to Formulae (A.1) to (A.4).

A.2 Modelling the measurement

The amount of test liquid delivered into one well of the microplate is calculated in three steps, according to Formulae (A.1), (A.2) or (A.3), and (A.4).

a) The fill height of the well is equal to the optical path length of the absorbance measurement and is calculated according to Formula (A.1).

$$l = \frac{A_{730}}{a_b} \tag{A.1}$$

where

l is the optical path length (fill height of the well);

A_{730} is the measured absorbance at 730 nm;

a_b is the absorbance per unit path length at 730 nm of the $CuCl_2$ solution.

b) The well geometry of a round well in a microplate can be described by the shape of a truncated cone. The liquid volume contained in such a round well is calculated according to Formula (A.2).

$$V_W = \pi \times l \times \frac{D^2}{4} + \pi \times D \times l^2 \times \frac{\tan\theta}{2} + \pi \times l^3 \times \frac{\tan^2\theta}{3} \tag{A.2}$$

where

V_W is the total liquid volume in the well;

D is the diameter of the well bottom;

θ is the side wall taper angle.

The well geometry of a square-shaped well in a microplate can be described by the shape of a truncated square pyramid. The liquid volume contained in such a square-shaped well is calculated according to Formula (A.3).

$$V_W = l \times w_B^2 + \frac{l^2 \times w_B \times (w_T - w_B)}{h} + \frac{l^3 \times (w_T - w_B)^2}{3 \times h^2} \quad \text{(A.3)}$$

where

w_B is the bottom width of the well;

w_T is the top width of the well;

h is the height of the well.

c) The volume of delivered Ponceau S test liquid is calculated according to Formula (A.4).

$$V_T = V_W \times \left(\frac{a_b}{a_r} \right) \times \left(\frac{A_{520}}{A_{730}} \right) \quad \text{(A.4)}$$

where

V_T is the volume of dispensed test liquid;

a_r is the absorbance per unit path length at 520 nm of the test liquid,

A_{520} is the measured absorbance at 520 nm.

For measurements in round wells forming truncated cones, Formulae (A.1), (A.2), and (A.4) contain six input variables. Four of these inputs are absorbance values, and two are related to the well geometry in the microplate.

For measurements in square-shaped wells, Formulae (A.1), (A.3), and (A.4) contain seven input variables. Four of these inputs are absorbance values, and three are related to the well geometry in the microplate.

A.3 Standard uncertainty components associated with the input quantities to the dual-dye ratiometric photometric procedure

A.3.1 Absorbance per unit path length of $CuCl_2$ solution (a_b)

This solution is prepared according to ISO 23783-2:2022, B.3.4.2 and the absorbance per unit path length will be approximately 0,61 AU/cm (0,061 AU/mm).

According to ISO 23783-2:2022, Table B.1, the spectrophotometer used to measure the absorbance per unit path length has a minimum resolution of 0,000 1 AU, repeatability of 0,000 15 AU, and linearity of 0,000 25 AU. A cuvette of known path length is used, typically 10 mm. For this example, the uncertainty in the knowledge of the cuvette path length is 0,005 mm.

Treating all these uncertainty sources as rectangular distributions, the combined relative standard uncertainty of a_b is 0,002 89 AU/mm for the example given in Table A.1 of this Annex.

A.3.2 Absorbance per unit path length of Ponceau S test liquid (a_r)

Ponceau S test liquids are prepared according to ISO 23783-2:2022, B.3.4.3 and the absorbance per unit path length will vary depending on which of the six test liquids are prepared. For this example, measurements at 1,0 µl, test liquid No. 5 is used, and the absorbance per unit path length will be approximately 185 AU/cm (18,5 AU/mm).

The spectrophotometer used to measure this absorbance has the same resolution, repeatability and linearity as described in A.3.1. The uncertainty of cuvette path length is also the same as in A.3.1. For measurements of high absorbance, a dilution is prepared, with a relative standard uncertainty of 0,007 1 %.

Combining all these uncertainty sources, the relative standard uncertainty of a_r is 0,003 17 AU/mm for the example given in Table A.1 of this Annex.

A.3.3 Measured absorbance at 520 nm (A_{520})

Absorbance at 520 nm is measured using an absorbance microplate reader with the minimum performance requirements of ISO 23783-2:2022, Table 4. Photometric resolution is 0,001 AU with trueness of 0,005 AU in the range of 0 AU to 1,0 AU.

In this example, the measured absorbance at 520 nm is 0,593 AU with a standard uncertainty of 0,002 94 AU.

A.3.4 Measured absorbance at 730 nm (A_{730})

Absorbance at 730 nm is measured using the same absorbance microplate reader described in A.3.3.

In this example, the measured absorbance at 730 nm is 0,369 AU with a standard uncertainty of 0,002 94 AU.

A.3.5 Uncertainty components of the geometric dimensions of microplate wells (D, θ)

Geometric dimensions of the microplates are needed so that the path length of a filled well can be used to calculate the total volume in each well of the microplate. Geometric dimensions can be obtained from manufacturer's drawings, direct measurement of plates (e.g., by a coordinate measuring machine), or other means.

In this example, a 96-well microplate is used, and Formula (A.2) applies. The well diameter D and taper angle θ each carry uncertainty.

In this example, the diameter D is 6,359 mm and the taper angle θ is 0,021 6 radians. The experimentally determined uncertainty in D is 0,010 5 mm and the uncertainty in θ is 0,000 855 radians.

A.3.6 Other uncertainty components

The fill height l is calculated according to Formula (A.1) and the uncertainty in fill height is based on A_{730} (see A.3.4.) and a_b (see A.3.1). Uncertainty in the fill height is included as part of the mathematical analysis of the contribution from these two input variables (A_{730} and a_b) and the sensitivity components in A.4, so there is no need to include fill height as a row in Table A.1.

The natural constant π is found in Formula (A.2). In principle, the uncertainty of this constant is zero. In practice, the uncertainty of π is limited by internal rounding within the calculator. With modern software, the uncertainty contribution due to internal digital rounding is negligible.

A.4 Sensitivity coefficients

For the example provided in Table A.1, there are six input variables. Sensitivity of each input variable can be expressed as a formula which is derived from an analysis of partial derivatives for the Formulae in A.2. The variables found in these formulae are all described in A.2.

The sensitivity of the test volume to errors in A_{520} is given in Formula (A.5):

$$\frac{\partial V_T}{\partial A_{520}} = \frac{V_T}{A_{520}} \tag{A.5}$$

Formula (A.6) gives the sensitivity of the test volume to errors in the absorbance per unit path length of the Ponceau S test liquid.

$$\frac{\partial V_T}{\partial a_r} = \frac{-V_T}{a_r} \tag{A.6}$$

The sensitivity of the test volume to errors in A_{730} is given in Formula (A.7):

$$\frac{\partial V_T}{\partial A_{730}} = \frac{V_T}{A_{730}} \times \left\{ \frac{\left[\frac{D^2}{4} + \frac{D \times \tan\theta \times A_{730}}{a_b} + \frac{\tan^2\theta \times A_{730}^2}{a_b^2} \right]}{\left[\frac{D^2}{4} + \frac{D \times \tan\theta \times A_{730}}{2 \times a_b} + \frac{\tan^2\theta \times A_{730}^2}{3 \times a_b^2} \right]} - 1 \right\} \tag{A.7}$$

Formula (A.8) gives the sensitivity of the test volume to errors in the absorbance per unit path length at 730 nm of the $CuCl_2$ solution.

$$\frac{\partial V_T}{\partial a_b} = \frac{V_T}{a_b} \times \left\{ 1 - \frac{\left[\frac{D^2}{4} + \frac{D \times \tan\theta \times A_{730}}{a_b} + \frac{\tan^2\theta \times A_{730}^2}{a_b^2} \right]}{\left[\frac{D^2}{4} + \frac{D \times \tan\theta \times A_{730}}{2 \times a_b} + \frac{\tan^2\theta \times A_{730}^2}{3 \times a_b^2} \right]} \right\} \tag{A.8}$$

Formulae (A.9) and (A.10) are sensitivities of the test volume to the bottom diameter D and taper angle θ of microplates which form a truncated cone.

$$\frac{\partial V_T}{\partial D} = \frac{\pi}{2} \times \left(\frac{A_{520}}{a_r} \right) \times \left[D + \frac{A_{730} \times \tan\theta}{a_b} \right] \tag{A.9}$$

$$\frac{\partial V_T}{\partial \theta} = \pi \times \frac{A_{520}}{a_r} \times \frac{A_{730}}{a_b} \times \sec^2\theta \times \left[\frac{D}{2} + \frac{2 \times A_{730} \times \tan\theta}{3 \times a_b} \right] \tag{A.10}$$

NOTE The symbol sec refers to the trigonometric function secant which is the multiplicative inverse of the cosine.

A.5 Example for determining the uncertainty of the volume measurement of an ALHS with the dual-dye ratiometric photometric procedure

A.5.1 Measurement conditions

The measurement conditions for this example are as follows:

— twelve-fold measurement of a selected volume V_S of 1 µl of test liquid (Ponceau S test liquid No. 5, according to ISO 23783-2:2022, Annex B), delivered by a single-channel pipetting ALHS;

NOTE Twelve replicates were used in this test to fill one row of a 96-well microplate.

— spectrophotometer meeting the minimum requirements of ISO 23783-2:2022, Table B.1;

— absorbance microplate reader meeting the minimum requirements of ISO 23783-2:2022, Table 4;

— thermometer and other measuring instruments meeting the requirements of ISO 23783-2:2022, Table 5;

— mean volume: $\overline{V}$ = 1,060 µl;

— random error of measurement (standard deviation, n = 12): s_r = 0,025 9 µl;

— standard deviation of the mean $s_r\left(\overline{V}\right) = s_r / \sqrt{12}$ = 0,007 5 µl;

— systematic error of measurement: $e_S = \overline{V} - V_S$ = +0,060 µl.

The determination of the uncertainty for these conditions is given in Tables A.1 and A.2. Contributions from the optional uncertainty components described in 7.5 and 7.6 are not included in this example.

Table A.1 — Measuring system standard uncertainty

Uncertainty component	Unit	Symbol	Estimation	Distribution	Standard uncertainty	Sensitivity coefficient	Uncertainty contribution µl	Degrees of freedom	Percent contribution %
Absorbance per path $CuCl_2$	AU/mm	a_b	0,061	normal	$2{,}89\times10^{-3}$	$7{,}05\times10^{-1}$	$2{,}04\times10^{-3}$	∞	8,9
Absorbance per path Ponceau S	AU/mm	a_r	18,5	normal	$3{,}17\times10^{-3}$	$5{,}73\times10^{-2}$	$1{,}82\times10^{-4}$	∞	0,1
Absorbance at 520 nm of mixture in well	AU	A_{520}	0,593	rectangular	$2{,}94\times10^{-3}$	1,79	$5{,}26\times10^{-3}$	∞	59,4
Absorbance at 730 nm of mixture in well	AU	A_{730}	0,369	rectangular	$2{,}94\times10^{-3}$	$1{,}16\times10^{-1}$	$3{,}43\times10^{-4}$	∞	0,3
Well diameter	mm	D	6,359	normal	$1{,}05\times10^{-2}$	$3{,}27\times10^{-1}$	$3{,}43\times10^{-3}$	3 167	25,2
Taper angle	radians	θ	0,021 6	normal	$8{,}55\times10^{-4}$	1,99	$1{,}70\times10^{-3}$	3 167	6,2
Measuring system standard uncertainty[a]	**µl**	u_{MS}					**$6{,}83\times10^{-3}$**	**>10 000**	**100**

[a] Measuring system standard uncertainty is the square root of a summation of all values in this table according to Formula (5).

Table A.2 — Combined standard uncertainty of the mean volume

Uncertainty component	Unit	Symbol	Distribution	Standard uncertainty	Sensitivity coefficient	Uncertainty contribution µl	Degrees of freedom	Percent contribution %
Measuring system standard uncertainty [a]	µl	u_{MS}	normal	$6{,}83\times10^{-3}$	1	$6{,}83\times10^{-3}$	10 000	3
ALHS standard uncertainty [b]		u_{ALHS}				see footnote b		
Experimental standard deviation of the mean [c]	µl	$s_r(\bar{V})$	normal	$7{,}48\times10^{-3}$	1	$7{,}48\times10^{-3}$	11	3
Reproducibility of the calibration [c]	µl	$s_d(\bar{V})$	normal	$4{,}14\times10^{-2}$	1	$4{,}14\times10^{-2}$	5	94
Standard uncertainty of the mean delivered volume	**µl**	$u(\bar{V})$	**normal**	**n/a**	**n/a**	$4{,}26\times10^{-2}$	**6**	**100**

[a] Measuring system standard uncertainty is taken from Table A.1.

[b] Included in the reproducibility of the calibration $s_d(\bar{V})$. The reproducibility study was performed over the course of several days and captured the uncertainty components described in Clause 7.

[c] Standard deviation of the mean and reproducibility of the calibration are described in Clause 8. Values for both are from unpublished experimental data provided by Artel. These components describe the standard uncertainty of the liquid delivery process, u_{LDP}, which has a value of $4{,}20\times10^{-2}$ µl in this example [see also Formula (7)].

A.5.2 Results

A.5.2.1 Standard uncertainty of the ALHS mean volume

The standard uncertainty of the mean delivered volume is calculated according to Formula (10):

$$u(\bar{V}) = 0{,}042\ 6\ \mu l$$

NOTE See Table A.2 for the source of the 0,042 6 µl value.

A.5.2.2 Expanded uncertainty of the measurement

The expanded uncertainty of the measurement is calculated by multiplying the standard uncertainty of the measurement by the coverage factor *k*, according to Formula (11). In this example, a coverage factor of (k = 2,45) is used based on the six effective degrees of freedom calculated and shown in Table A.2. The value of k can vary (see Clause 11).

$$U(\bar{V}) = 0{,}042\ 6\ \mu l \times 2{,}45$$

$$U(\bar{V}) = 0{,}10\ \mu l \qquad (k = 2{,}45)$$

A.5.2.3 Result of measurement

The overall result of the measurement, including the expanded uncertainty of measurement, can be expressed according to Formula (12):

$$V_M = 1{,}06\ \mu l \pm 0{,}10\ \mu l \qquad (k = 2{,}45)$$

A.5.2.4 General remarks

For the example given in Table A.2, the uncertainty associated with the day-to-day reproducibility of the ALHS being tested comprises 94 % of the overall test uncertainty. This is typical of many (but not all) ALHS calibrations where the overall calibration uncertainty is dominated by the repeatability and reproducibility of the liquid delivery process.

The numerical values of the quantity estimation, standard uncertainty, and sensitivity coefficients in this example apply only to this example and are not applicable to other uncertainty calculations. Other numerical values will result in different uncertainty budgets.

Annex B
(informative)

Gravimetric procedure

B.1 Description of the measurement

In the gravimetric measurement procedure, a quantity of test liquid is delivered by the instrument under calibration (ALHS) into a vessel that will be weighed on a balance. Ambient conditions are recorded so that the liquid density and air density can be determined and, consequently, the delivered volume can be calculated from these data.

Furthermore, the influence of possible evaporation and possible temperature difference of the ALHS from the reference calibration temperature are taken into consideration as corrections in the mathematical model of the volume determination.

The following variations of the gravimetric procedure are addressed in this document:

— single-channel delivery into one well;

— regression analysis.

B.2 Modelling the measurement

B.2.1 General gravimetric formula

The general formula for calculation of the volume at the test temperature from the weighing value of the delivered test liquid as described in ISO 4787 is given by Formula (B.1). The determination of the mass of the test liquid (m_{measured}) depends on the type of gravimetric measurement method, e.g., single-channel analysis, analysis of deliveries into multiple wells, or gravimetric regression analysis.

$$V_{\text{L}} = (m_{\text{measured}}) \times \frac{1}{\rho_{\text{L}} - \rho_{\text{A}}} \times \left(1 - \frac{\rho_{\text{A}}}{\rho_{\text{B}}}\right) \tag{B.1}$$

where

V_{L} is the calculated volume of test liquid at the test temperature, in ml;

m_{measured} is the mass of the delivered test liquid, in g;

ρ_{A} is the density of air, in g/ml (see Formula (B.4) below);

ρ_{B} is the actual or assumed density of the weights used to calibrate the balance, in g/ml;

ρ_{L} is the density of the test liquid at the test temperature, in g/ml.

NOTE Stainless steel weights of density 7,95 g/ml or 8,0 g/ml are typically used for balance calibration.

B.2.1.1 Single-channel analysis

For the single-channel gravimetric analysis, the mass of the test liquid is determined according to Formula (B.2).

$$m_{\text{measured}} = m_{\text{L}} - m_{\text{E}} + m_{\text{evap}} \tag{B.2}$$

where

m_L is the balance indication of the weighing vessel after liquid delivery, in g;

m_E is the balance indication of the weighing vessel before liquid delivery, in g ($m_E = 0$ in case the balance was tared with the weighing vessel);

m_{evap} is the estimated mass lost to evaporation during the weighing process, in g.

B.2.1.2 Regression analysis

For the gravimetric regression analysis, the mass of the test liquid is determined according to Formula (B.3).

$$m_{measured} = m_{after} - m_{before} \quad \text{(B.3)}$$

where

m_{after} is the mass calculated by linear regression of the recorded balance readings after the delivery of test liquid, extrapolated backward to the time of delivery (t_{del});

m_{before} is the mass calculated by linear regression of the recorded balance readings before the delivery of test liquid, extrapolated forward to the time of delivery (t_{del}).

Detailed modelling of the regression analysis method is described in B.2.6.

B.2.2 Air density

Air density can be calculated as described in CIPM-2007[5] and densities calculated this way will have a smaller uncertainty than the two alternatives given in this clause.

The simplified formula for the air density, given in Formula (B.4), can be used at temperatures between 15 °C and 27 °C, barometric pressure between 600 hPa and 1 100 hPa, and relative humidity between 20 % and 80 %.

$$\rho_A = \frac{1}{1\ 000} \times \frac{0{,}348\ 48 \times p - 0{,}009 \times h_r \times e^{(0{,}061 \times t_A)}}{t_A + 273{,}15} \quad \text{(B.4)}$$

where

ρ_A is the air density, in g/ml;

t_A is the ambient temperature, in °C;

p is the barometric pressure, in hPa;

h_r is the relative air humidity, in %.

Another commonly used formula for air density is described in Spieweck's work, see Reference [6].

B.2.3 Test liquid density

The density of the test liquid can be measured to varying degrees of accuracy using calibrated hydrometers, pycnometers, or oscillation type density meters. Liquid density changes with temperature, therefore it is necessary to either measure density at the test temperature or apply a temperature correction when the test temperature differs from the temperature of density measurement. An example of temperature corrections to measured liquid density is given in Reference [7].

When pure water is used as a calibration liquid for density measurement devices, or is used as the test liquid for ALHS testing, the density of pure water can be calculated from formulae given in the literature. Formula (B.5) given by Tanaka[8] can be used (suitable for measurements conducted between 0 °C and 40 °C).

$$\rho_W = a_5 \times \left[1 - \frac{(t_W + a_1)^2 \times (t_W + a_2)}{a_3 \times (t_W + a_4)} \right] \quad \text{(B.5)}$$

where

ρ_W is the density of water, in g/ml;

t_W is the water temperature, in °C;

a_1 –3,983 035 °C;

a_2 301,797 °C;

a_3 522 528,9 $(°C)^2$;

a_4 69,348 81 °C;

a_5 0,999 974 950 g/ml.

B.2.4 Estimating evaporation

For single-channel analysis (see B.2.1.1), the mass lost to evaporation is estimated by using data of one or more evaporation trials. These trials are accomplished by duplicating the weighing process while delivering no test liquid. The mass lost to evaporation creates a difference between an initial weight and final weight and is used to determine the value of m_{evap} which is added to the measurement result according to Formula (B.2).

For the regression method (see B.2.1.2), the mass lost to evaporation is automatically compensated within the measurement method and does not need to be estimated independently.

B.2.5 Modelling the gravimetric single-channel method

Combining Formulae (B.1) and (B.2) results in Formula (B.6) for calculating the delivered volume. The mass values m_L and m_E are balance indications, and the evaporation value m_{evap} is experimentally estimated as described in B.2.4.

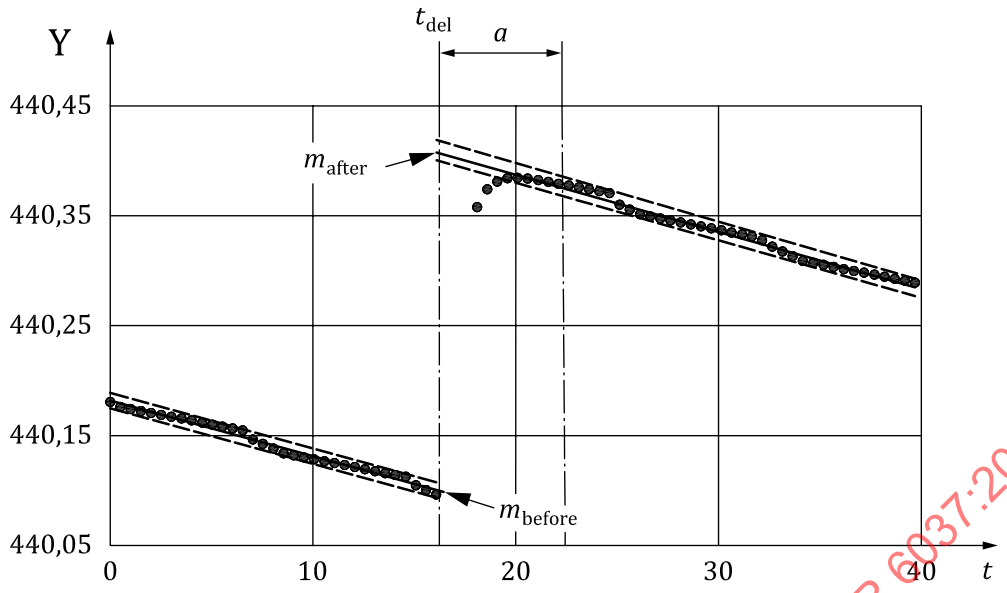
$$V_L = (m_L - m_E + m_{evap}) \times \frac{1}{\rho_L - \rho_A} \times \left(1 - \frac{\rho_A}{\rho_B} \right) \quad \text{(B.6)}$$

B.2.6 Modelling the gravimetric regression method

Combining Formulae (B.1) and (B.3) results in Formula (B.7) for calculating the delivered volume. The two mass values (m_{before} and m_{after}) are not read directly from the balance indication but instead are calculated from a series of balance indications using a regression analysis. In this case, the effect of evaporation is included in the calculated mass after delivery, and therefore the evaporation term is omitted.

$$V_L = (m_{after} - m_{before}) \times \frac{1}{\rho_L - \rho_A} \times \left(1 - \frac{\rho_A}{\rho_B} \right) \quad \text{(B.7)}$$

Figure B.1 illustrates the graph of a typical measurement with the gravimetric regression method.



Key

●	balance readouts
———	linear regression
– – – –	confidence interval
a	settling time of the balance (mass recorded during this time is not used in calculations)
t	time in s
Y	mass in mg

Figure B.1 — Graph of a typical measurement - The linear regressions of the recorded mass before (m_{before}) and after (m_{after}) test liquid delivery (t_{del}) shown as a function of time

Assuming a constant evaporation rate, the mass has a linear relationship to time as shown in Formula (B.8). By taking the random measurement error into account, the balance readings I_i can be expressed as shown in Formula (B.8).

$$I_i = m_i + \varepsilon_i = B_0 - B_1 \times t_i + \varepsilon_i \quad \text{(B.8)}$$

where

I_i is the i-th balance readout, in mg;

ε_i is the random error between the linear regression model and the i-th balance reading, in mg;

m_i is the i-th mass reading of the balance, in mg;

t_i is the time at which the i-th mass reading was acquired, in s;

B_0 is the regression parameter B_0 (see Formula (B.10), in mg;

B_1 is the regression parameter B_1 (see Formula (B.11), in mg/s.

To determine the regression parameters B_0 and B_1, the least-squares method is applied, which is a technique commonly used in regression analysis. The method seeks to minimize the sum of the squared residuals ε_i as shown in Formula (B.9):

$$\sum_{i=1}^{n} \varepsilon_i^2 = \sum_{i=1}^{n} (I_i - B_0 - B_1 \times t_i)^2 \tag{B.9}$$

The regression parameters B_0 and B_1 can be calculated using Formulae (B.10) and (B.11):

$$B_0 = \frac{\sum_{i=1}^{n} t_i^2 \times \sum_{i=1}^{n} I_i - \sum_{i=1}^{n} t_i \times \sum_{i=1}^{n} t_i I_i}{n \times \sum_{i=1}^{n} t_i^2 - \left(\sum_{i=1}^{n} t_i\right)^2} \tag{B.10}$$

$$B_1 = \frac{n \times \sum_{i=1}^{n} t_i I_i - \sum_{i=1}^{n} t_i \times \sum_{i=1}^{n} I_i}{n \times \sum_{i=1}^{n} t_i^2 - \left(\sum_{i=1}^{n} t_i\right)^2} \tag{B.11}$$

The linear regression parameters ($B_{0,\text{before}}$ and $B_{1,\text{before}}$), as well as ($B_{0,\text{after}}$ and $B_{1,\text{after}}$) are determined according to Formulae (B.10) and (B.11) based on two series of balance readouts before and after liquid delivery. The first series for ($B_{0,\text{before}}$ and $B_{1,\text{before}}$) starts when the data acquisition starts and ends at t_{del}. The second series starts at (t_{del} + settling time a) and ends when the data acquisition ends.

The settling time a of the balance is the time the balance needs to reach the steady state again after loading (e.g. impinging drop from an ALHS). The settling time is based on the balance manufacturer's data sheet; typically, 6 s to 20 s.

To calculate the masses m_{after} and m_{before} at time t_i based on the linear regression analysis, it has to be distinguished between two cases, the time before and after the test liquid delivery t_{del}, calculated by linear regression, as shown in Formulae (B.12) and (B.13):

For $t_i \geq t_{\text{del}}$:

$$m_{\text{after}}(t_i) = B_{0,\text{after}} + B_{1,\text{after}} \times t_i \tag{B.12}$$

For $t_i \leq t_{del}$:

$$m_{\text{before}}(t_i) = B_{0,\text{before}} + B_{1,\text{before}} \times t_i \tag{B.13}$$

where

$m_{\text{after}}(t_i)$ is the projected mass value at the time i, based on the linear regression of post-delivery mass values;

$m_{\text{before}}(t_i)$ is the projected mass value at the time i, based on the linear regression of pre-delivery mass values;

$B_{0,\text{after}}$ is the linear regression parameter B_0 after the delivery;

$B_{0,\text{before}}$ is the linear regression parameter B_0 before the delivery;

$B_{1,\text{after}}$ is the linear regression parameter B_1 after the delivery;

$B_{1,\text{before}}$ is the linear regression parameter B_1 before the delivery;

t_{del} is the time of the test liquid delivery.

B.3 Standard uncertainty components associated with the measuring system

B.3.1 Standard uncertainty of weighing

The general standard uncertainty $u(m)$ related to an individual weighing value m is calculated according to Formula (B.14):

$$u(m)=\left[u^2(m_{\text{measured}})+u^2(\delta m)+u^2(m_{\text{cal}})\right]^{1/2} \quad \text{(B.14)}$$

where

$u^2(m)$ is the variance associated with the standard uncertainty related to the weighing value m, in g;

$u^2(m_{\text{measured}})$ is the variance associated with the standard uncertainty related to the balance indication of the weighing vessel, in g;

$u^2(\delta m)$ is the variance associated with the drift of the balance, in g;

$u^2(m_{\text{cal}})$ is the variance associated with the standard uncertainty related to the calibration of the balance, in g.

Note 1 The uncertainty of the balance indications can be estimated according to Reference [9] or Reference [4] at the value corresponding to the selected volume.

The uncertainty of the balance indications can be taken from the balance calibration certificate if the expanded uncertainty in use is expressed. Otherwise, it can be calculated by using the uncertainty at calibration and including non-corrected errors, as well as possible drift and environmental effects to balance sensitivity.

The uncertainty calculation for the weighing is determined considering that the weighing vessel is not removed during the test. Additional uncertainties can arise if the vessel is removed from the balance.

Note 2 The correlations found in mass measurements are, in the case of this gravimetric measurement procedure, negligible.

B.3.1.1 Single-channel analysis

For the single-channel gravimetric analysis, the uncertainty of the mass of the test liquid is determined according to Formula (B.15):

$$u(m_{\text{measured}})=\left[u^2(m_{\text{L}})+u^2(m_{\text{E}})+u^2(m_{\text{evap}})\right]^{1/2} \quad \text{(B.15)}$$

where

$u^2(m_{\text{L}})$ is the variance associated with the standard uncertainty related to the balance indication of the weighing vessel after test liquid delivery, in g;

$u^2(m_{\text{E}})$ is the variance associated with the standard uncertainty related to the balance indication of the weighing vessel before test liquid delivery, in g;

$u^2(m_{\text{evap}})$ is the variance associated with the estimated mass lost to evaporation during the weighing process, in g.

B.3.1.2 Regression analysis

Though the linear regression method accounts for evaporation, it also introduces an error in the calculation of $m_{\text{after}}(t_{\text{del}})$ and $m_{\text{before}}(t_{\text{del}})$ due to the back projection of the mass value to time t_{del}. Since both,

$m_{\text{after}}(t_{\text{del}})$ and $m_{\text{before}}(t_{\text{del}})$, are projected values in time, it is imperative to account for the uncertainties that come from the prognostic calculation, denoted $u(m_{\text{after}}(t_{\text{del}}))$ and $u(m_{\text{before}}(t_{\text{del}}))$. The standard uncertainty $u(m_{\text{measured}})$ related to the weighing value (m_{measured}) is calculated as shown in Formula (B.16):

$$u(m_{\text{measured}}) = \left[u^2(m_{\text{after}}(t_{\text{del}})) + u^2(m_{\text{before}}(t_{\text{del}}))\right]^{1/2} \quad \text{(B.16)}$$

where

$u^2(m_{\text{after}}(t_{del}))$ is the variance associated with the standard uncertainty of the mass calculated by linear regression of the recorded balance readings after the delivery, extrapolated to the time of delivery t_{del} in g;

$u^2(m_{\text{before}}(t_{del}))$ is the variance associated with the standard uncertainty of the mass calculated by linear regression of the recorded balance readings before the delivery, extrapolated to the time of delivery t_{del} in g;

Since linear regression was used here to determine the mass at the time of delivery, the uncertainty of this method must be determined. The uncertainty of m_{after} is larger, since due to the settling time the time span of the back projection to t_{del} is larger than for m_{before}. For determining the uncertainty denoted $u(m_{\text{after}}(t_{\text{del}}))$ and $u(m_{\text{before}}(t_{\text{del}}))$, derived from the regression analysis, the confidence interval has been applied. This confidence interval covers the expectation value under a probability of (1 – α) at time t_{del} and the half-width of the confidence interval is calculated according to Formulae (B.17), (B.18), and (B.19):

$$C_I = m_{\text{after}} \pm t_{1-\alpha/2,n-2} \times s_y \times \sqrt{\frac{1}{n} + \frac{(t-\bar{t})^2}{(n-1)\times\sum_{i=1}^{n}(t-\bar{t})^2}} \quad \text{(B.17)}$$

where

C_I is the half-width of the confidence interval;

$\bar{t}$ is the average time of n measurement points;

s_y is the standard deviation of the random error ε_i;

$t_{1-\alpha/2,n-2}$ is the statistical t-value with n–2 degrees of freedom.

$$\bar{t} = \frac{1}{n}\sum_{i=1}^{n} t_i \quad \text{(B.18)}$$

$$s_y = \frac{\sum_{i=1}^{n}\varepsilon_i^{\,2}}{n} = \frac{\sum_{i=1}^{n}(I_i - B_0 - B_1 \times t_i)^2}{n} \quad \text{(B.19)}$$

The uncertainty $u(m_{\text{after}})$ is the half-width of the confidence interval. The calculation for $u(m_{\text{before}})$ follows the same principle.

B.3.1.3 Standard uncertainty of balance drift

Drift in the balance calibration can be evaluated by comparing the as-left calibration of the balance with the as-found calibration at its next calibration interval. For more details regarding balance drift, see Reference [4].

B.3.1.4 Standard uncertainty of the calibration of the balance

The uncertainty of the balance indications can be taken from the balance calibration certificate if the expanded uncertainty in use is expressed. Otherwise, it can be calculated by using the uncertainty at

calibration and including non-corrected errors, as well as possible drift and environmental effects to balance sensitivity.

The uncertainty calculation for the weighing is determined considering that the weighing vessel is not removed during the test. Additional uncertainties can arise if the vessel is removed from the balance.

B.3.2 Standard uncertainty of temperature

The standard uncertainty $u(t_L)$ related to the temperature t_L of the test liquid and the ALHS is calculated according to Formulae (B.20) and (B.21):

$$u(t_L)=\left[u^2(t_L)+u^2(\delta t_s)\right]^{1/2} \quad \text{(B.20)}$$

where

$u(t_L)$ is the standard uncertainty related to the temperature of the test liquid and ALHS;

$u^2(t_L)$ is the variance associated with the temperature of the test liquid;

$u^2(\delta t_s)$ is the variance associated with the difference between the test liquid temperature and ALHS temperature.

$$u(t_L)=\left[\left(\frac{U_{TMS}}{k}\right)^2+u^2(r_{TMS})+u^2(\delta t)\right]^{1/2} \quad \text{(B.21)}$$

where

U_{TMS} is the expanded uncertainty of the calibration of the temperature measuring system used to measure the temperature of the test liquid, in °C;

k is the coverage factor used in the calculation of the expanded uncertainty of the temperature measuring system calibration;

$u^2(r_{TMS})$ is the variance associated with the standard uncertainty related to the resolution of the used temperature measuring system;

$u^2(\delta t)$ is the variance associated with the standard uncertainty caused by possible drift and ageing of the temperature measuring system after its calibration.

B.3.3 Standard uncertainty of test liquid density

Both examples of gravimetric analyses in this document use water as test liquid. The standard uncertainty related to the density of the test liquid, i.e., water, is calculated according to Formulae (B.22) to (B.24). When the test liquid is not pure water, the uncertainties related to liquid density and thermal expansion are determined by other means, and different constants apply.

The standard uncertainty $u(\rho_w)$ related to the water density ρ_w is calculated according to Formula (B.22):

$$u(\rho_W)=\left[u^2(\rho_{W,form})+u^2(\delta\rho_W)+u^2(\rho_{W,t})\right]^{1/2} \quad \text{(B.22)}$$

where

$u(\rho_W)$ is the standard uncertainty related to the density of water, in g/ml;

$u^2(\rho_{W,form})$ is the variance associated with the standard uncertainty contributed by Formula (B.5). When using pure water, the standard uncertainty contributed by Formula (B.5) has the value of $4{,}5\times10^{-7}$ g/ml (see Reference [8]);

$u^2(\delta\rho_W)$ is the variance associated with the standard uncertainty related to the water purity, in g/ml;

NOTE 1 If the quality of the water is of grade 3 according to ISO 3696, this uncertainty contribution can be considered negligible. More information on how to estimate this uncer-tainty contribution can be found in References [8] and [2].

$u^2(\rho_{W,t})$ is the variance associated with the standard uncertainty of the water temperature, which depends on the expansion coefficient of the water β, see Formula (B.23), g/ml.

$$u(\rho_{W,t}) = u(t_W)\times\beta\times\rho_W \tag{B.23}$$

where

$u(\rho_{W,t})$ is the standard uncertainty of the density of water at the test temperature;

$u(t_W)$ is the standard uncertainty related to the water temperature;

β is the thermal expansion coefficient of water;

ρ_W is the density of water.

The expansion coefficient of water β can be estimated as it is described in Reference [10] and shown in Formula (B.24).

$$\beta = (-0{,}117\,6\times t_W^2 + 15{,}846\times t_W - 62{,}677)\times10^{-6}\ {}^{\circ}C^{-1} \tag{B.24}$$

where t_W is the water temperature.

B.3.4 Standard uncertainty of air density

The standard uncertainty $u(\rho_A)$ related to the air density ρ_A is calculated according to OIML R 111-1:2004, section C.6.3.6[11] as shown in Formula (B.25):

$$u(\rho_A) = \rho_A\times\left[\left(\frac{u_{p_A}(\rho_A)}{\rho_A}\times u(p_A)\right)^2 + \left(\frac{u_{t_A}(\rho_A)}{\rho_A}\times u(t_A)\right)^2 + \left(\frac{u_{h_r}(\rho_A)}{\rho_A}\times u(h_r)\right)^2 + \left(\frac{u_{form}(\rho_A)}{\rho_A}\right)^2\right]^{1/2} \tag{B.25}$$

For the simplified air density given by Formula (B.4), the relative standard uncertainty due to Formula (B.4) is $u_{form} = 2{,}4\times10^{-4}$.

B.3.5 Standard uncertainty of weights density

The standard uncertainty $u(\rho_B)$ related to the weights density ρ_B is obtained by the value presented in the calibration certificate of the set of reference weights used in the analytical balance calibration. Alternatively, the uncertainties corresponding to the used weight class according to OIML R 111-1[11] can be used.

NOTE If EURAMET cg-18 (Reference [4]) or ASTM E898 (Reference [9]) are used for the calibration of the balance, the standard uncertainty $u(\rho_B)$ related to the weights density ρ_B is already taken into account and any further consideration is not needed.

B.4 Sensitivity coefficients

The sensitivity coefficients c_i in Formula (5) are calculated as partial derivatives using formulae in this annex to result in Formulae (B.26) through (B.29).

The sensitivity coefficient c_m related to the balance indication $m_{measured}$ is calculated according to Formula (B.26):

$$\frac{\partial V_L}{\partial m_{measured}} = \frac{1}{\rho_L - \rho_A} \times \left(1 - \frac{\rho_A}{\rho_B}\right) \tag{B.26}$$

The sensitivity coefficient c_{ρ_L} related to the test liquid density ρ_L is calculated according to Formula (B.27):

$$\frac{\partial V_L}{\partial \rho_L} = -1 \times m_{measured} \times \left(1 - \frac{\rho_A}{\rho_B}\right) \times \left(\frac{1}{\rho_L - \rho_A}\right)^2 \tag{B.27}$$

The sensitivity coefficient c_{ρ_A} related to the density of air ρ_A is calculated according to Formula (B.28):

$$\frac{\partial V_L}{\partial \rho_A} = (m_{measured}) \times \frac{(\rho_B - \rho_L)}{(\rho_L - \rho_A)^2 \times \rho_B} \tag{B.28}$$

The sensitivity coefficient c_{ρ_B} related to the density of the standard weights ρ_b is calculated according to Formula (B.29):

$$\frac{\partial V_L}{\partial \rho_B} = m_{measured} \times \frac{\rho_A}{(\rho_L - \rho_A) \times \rho_B^2} \tag{B.29}$$

The sensitivity coefficients for the standard deviation of the repeatability, the reproducibility, and the resolution are 1 in this case.

B.5 Examples for determining the uncertainty of the volume measurement of the ALHS

B.5.1 Example of testing a single channel ALHS with multiple replicates

B.5.1.1 Measurement conditions

The measurement conditions for this example are as follows:

— 12-fold measurement of a selected volume V_S of 200 µl of test liquid (water), delivered by a single-channel dispensing ALHS;

— a 4-place balance meeting the minimum requirements of ISO 23783-2:2022, Table 3;

— thermometer and other measuring instruments meeting the requirements of ISO 23783-2:2022, Table 5;

— mean volume: $\bar{V}$ = 199,192 µl;

— random error of measurement (standard deviation, n = 12): s_r = 0,576 µl;

— standard deviation of the mean $s_r\left(\overline{V}\right) = s_r / \sqrt{12}$ = 0,166 µl;

— systematic error of measurement: $e_S = \overline{V} - V_S$ = -0,808 µl.

The determination of the uncertainty for these conditions is given in Tables B.1 and B.2. Contributions from optional uncertainty components described in 7.5 and 7.6 are not included in this example.

Table B.1 — Measuring system standard uncertainty of the single channel example

Uncertainty component	Unit	Symbol	Estimation	Distribution	Standard uncertainty	Sensitivity coefficient	Uncertainty contribution µl	Degrees of freedom	Percent contribution %
Weighing (measured) [a]	mg	$m_{measured}$	200	normal	$2{,}0\times10^{-1}$	1,004	$2{,}01\times10^{-1}$	∞	99,5
Weights density	g/ml	ρ_B	7,95	rectangular	$2{,}89\times10^{-2}$	$3{,}81\times10^{-3}$	$1{,}10\times10^{-4}$	∞	0
Water density	g/ml	ρ_W	0,997 4	rectangular	$6{,}75\times10^{-5}$	$2{,}02\times10^{2}$	$1{,}36\times10^{-2}$	∞	0,5
Air density	g/ml	ρ_A	0,001 2	rectangular	$1{,}20\times10^{-5}$	$1{,}76\times10^{2}$	$2{,}11\times10^{-3}$	∞	0
Measuring system standard uncertainty	**µl**	$\boldsymbol{u_{MS}}$					$\mathbf{2{,}01\times10^{-1}}$	∞	**100**

[a] Weighing (measured) includes initial weighing, final weighing, and variation in evaporation at k = 1.

Table B.2 — Combined standard uncertainty of the single channel example

Uncertainty component	Unit	Symbol	Distribution	Standard uncertainty	Sensitivity coefficient	Uncertainty contribution µl	Degrees of freedom	Percent contribution %
Measuring system standard uncertainty [a]	µl	u_{MS}	normal	$2{,}01\times10^{-1}$	1	$2{,}01\times10^{-1}$	∞	7
ALHS standard uncertainty [b]	µl	u_{ALHS}				see footnote b		
Experimental standard deviation of the mean [c,e]	µl	$s_r(\bar{V})$	normal	$1{,}66\times10^{-1}$	1	$1{,}66\times10^{-1}$	11	5
Reproducibility of the calibration [d,e]	µl	$s_d(\bar{V})$	normal	$6{,}94\times10^{-1}$	1	$6{,}94\times10^{-1}$	5	88
Standard uncertainty of the mean delivered volume	**µl**	$u(\bar{V})$	**normal**	**n/a**	**n/a**	**0,741**	**6**	**100**

[a] Measuring system standard uncertainty is taken from Table B.1.

[b] Included in the reproducibility of the calibration $s_d(\bar{V})$.

[c] Standard deviation of the mean is described in Clause 8. Value for repeatability of the 12 replicate measurements is supplied by Artel.

[d] Reproducibility of the mean is based on 6 calibrations over six different days, resetting the ALHS power each day. Data provided by Artel.

[e] Standard deviation of the mean and reproducibility of the calibration are described in Clause 8. These components describe the uncertainty of the liquid delivery process, uLDP [see also Formula (7)].

B.5.1.2 Results

B.5.1.2.1 Standard uncertainty of the ALHS mean volume

The standard uncertainty of the mean delivered volume is calculated according to Formula (10):

$$u(\bar{V}) = 0{,}741 \text{ µl}$$

NOTE See Table B.2 for the source of the 0,741 µl value.

B.5.1.2.2 Expanded uncertainty of the ALHS mean volume

The expanded uncertainty of the measurement is calculated by multiplying the standard uncertainty of the measurement by the coverage factor k, according to Formula (11). In this example, a coverage factor of (k = 2) is used, but that value can vary (see Clause 11).

$$U(\bar{V}) = 0{,}741 \text{ µl} \times 2{,}0 = 1{,}5 \text{ µl}$$

B.5.1.2.3 Expression of the measurement result

The overall result of the measurement, including the expanded uncertainty of measurement, can be expressed according to Formula (12):

V_M = 199,2 µl ± 1,5 µl (k = 2)

B.5.1.3 General remarks

The numerical values of the quantity estimation, standard uncertainty, and sensitivity coefficients used in this example apply only to this example and are not applicable to other uncertainty calculations. Other numerical values will result in different uncertainty budgets.

B.5.2 Example of testing a single channel using gravimetric regression

B.5.2.1 Measurement conditions

The measurement conditions for this example are as follows:

— 24-fold measurement of a single channel dispensing device delivering approximately 54 nl of test liquid (water);

— a seven-place microbalance meeting the minimum requirements of ISO 23783-2:2022, Table 3;

— thermometer and other measuring instruments meeting the requirements of ISO 23783-2:2022, Table E.1;

— mean volume: $\bar{V}$ = 53,74 nl;

— random error of measurement (standard deviation, n = 24): s_r = 0,762 nl;

— standard deviation of the mean $s_r\left(\bar{V}\right) = s_r / \sqrt{24}$ = 0,156 nl;

— the systematic error of measurement is not calculated as no selected volume is set for this liquid delivery device.

The determination of the uncertainty for these conditions is given in Tables B.3 and B.4. Contributions from the optional uncertainty components described in 7.5 and 7.6 are not included in this example.

Table B.3 — Measuring system standard uncertainty of the regression example

Uncertainty component	Unit	Symbol	Estimation	Distribution	Standard uncertainty	Sensitivity coefficient	Uncertainty contribution nl	Degrees of freedom	Percent contribution %
Weighing (before) [a]	mg	m_{before}	305,56	normal	$1{,}09\times10^{-5}$	1 004	included in weight	30	
Weighing (after) [a]	mg	m_{after}	305,61	normal	$2{,}06\times10^{-5}$	1 004	included in weight	30	
Weighing (measured) [b]	mg	$m_{measured}$	0,05	normal	$2{,}33\times10^{-5}$	1 004	included in weight	46	
Weight [c]	mg	m	0,05	normal	$4{,}70\times10^{-5}$	1 004	$4{,}72\times10^{-2}$	754	99,93
Water temperature	°C	t_W	23,70	rectangular	$8{,}70\times10^{-2}$	omitted	included in water density	∞	
Weights density	g/ml	ρ_B	7,95	rectangular	$1{,}40\times10^{-2}$	$9{,}75\times10^{-4}$	$1{,}37\times10^{-5}$	∞	0
Water density	g/ml	ρ_W	0,997 4	rectangular	$2{,}23\times10^{-5}$	$5{,}42\times10^{1}$	$1{,}21\times10^{-3}$	∞	0,07
Air density	g/ml	ρ_A	0,001 14	rectangular	$2{,}74\times10^{-7}$	$4{,}74\times10^{-1}$	$1{,}30\times10^{-5}$	∞	0
Measuring system standard uncertainty	**nl**	$\boldsymbol{u_{MS}}$					$\mathbf{4{,}72\times10^{-2}}$	**755**	**100**

[a] This is the uncertainty of the regression confidence interval (k = 1) and omits other sources of balance uncertainty.

[b] Weighing (measured) is a combination of the two preceding regression confidence intervals, each at k = 1.

[c] Weight includes contributions from weighing (measured), balance drift and balance resolution before and after. It does not include other sources of balance calibration uncertainty listed in EURAMET cg-18[4] or ASTM E898[9].

Table B.4 — Combined standard uncertainty of the mean volume of the regression example

Uncertainty component	Unit	Symbol	Distribution	Standard uncertainty	Sensitivity coefficient	Uncertainty contribution nl	Degrees of freedom	Percent contribution %
Measuring system standard uncertainty [a]	nl	u_{MS}	normal	$4{,}72\times10^{-2}$	1	$4{,}72\times10^{-2}$	755	0,1
ALHS standard uncertainty [b]	nl	u_{ALHS}				see footnote b		
Experimental standard deviation of the mean [c,e]	nl	$s_r(\bar{V})$	normal	$1{,}56\times10^{-1}$	1	$1{,}56\times10^{-1}$	23	1
Reproducibility of the calibration [d,e]	nl	$s_d(\bar{V})$	normal	1,56	1	1,56	∞	98,9
Standard uncertainty of the mean delivered volume	**nl**	$u(\bar{V})$	**normal**	**n/a**	**n/a**	**1,57**	**>10 000**	**100**

[a] Measuring system standard uncertainty is taken from Table B.3.

[b] Included in the reproducibility of the calibration $s_d(\bar{V})$.

[c] Standard deviation of the mean is described in Clause 8. Value for repeatability of the 24 replicate measurements is supplied by IMTEK.

[d] The reproducibility is based on assumed manufacturer specifications of 5 % systematic error; see Note in 8.2 b).

[e] Standard deviation of the mean and reproducibility of the calibration are described in Clause 8. These components describe the uncertainty of the liquid delivery process, u_{LDP} [see also Formula (7)].

B.5.2.2 Results

B.5.2.2.1 Standard uncertainty of the ALHS mean volume

The standard uncertainty of the mean delivered volume is calculated according to Formula (10):

$$u(\bar{V}) = 1{,}57 \text{ nl}$$

NOTE See Table B.4 for the source of the 1,57 nl value.

B.5.2.2.2 Expanded uncertainty of the ALHS mean volume

The expanded uncertainty of the measurement is calculated by multiplying the standard uncertainty of the measurement by the coverage factor k, according to Formula (11). In this example, a coverage factor of (k = 2) is used, but that value can vary (see Clause 11).

$$U(\bar{V}) = 1{,}57 \text{ nl} \times 2{,}0 = 3{,}14 \text{ nl}$$

B.5.2.2.3 Expression of the measurement result

The overall result of the measurement, including the expanded uncertainty of measurement, is expressed according to Formula (12):

V_M = 53,74 nl ± 3,14 nl (k = 2)

B.5.2.3 General remarks

The numerical values of the quantity estimation, standard uncertainty, and sensitivity coefficients used in this example apply only to this example and are not applicable to other uncertainty calculations. Other numerical values will result in different uncertainty budgets.

Annex C
(informative)

Optical image analysis of droplets

C.1 Description of the measurement

C.1.1 General

Measurement procedures based on optical image analysis make use of calibrated optical images of the liquid/air interface formed by free liquid surfaces. Photographic images show a two-dimensional projection of the three-dimensional liquid/air interfaces that can be readily generated by a suitable digital optical setup, such as a calibrated high-speed camera or a stroboscopic imaging system working in dark-field or bright-field mode. From the acquired photographic images of the free liquid surface, the size and shape of the two-dimensional image can be extracted by appropriate image processing algorithms (e.g., as described in ISO 23783-2:2022, G.6.4). The volume of the liquid is then reconstructed from the size, position and shape of the liquid/air interface, using certain approximations and known properties of the measurement setup, for example, the optical magnification factor of the optical imaging setup and the assumption of rotational symmetry of the liquid volume and other properties are used for this purpose, as described in the corresponding methods section in ISO 23783-2 and briefly in C.1.2.

C.1.2 Optical image analysis of droplets

This procedure is only applicable to liquid volumes provided as free flying liquid droplets — for example generated by non-contact nano-dispensing technologies or inkjet printheads. The recommended volume range for applying the optical image analysis of droplets for volume measurements is 100 pl to 1 µl per droplet. Measurement of larger volumes is possible by consecutive measurements of a series of individual droplets.

The measurement procedure is based on photographic images of liquid droplets in flight acquired by a suitable digital optical setup, such as a calibrated high-speed camera or a stroboscopic imaging system (for more detailed system requirements see ISO 23783-2:2022, Annex G). From the acquired grey-scale images of the droplet, the size and shape of the outline of the droplet is determined. The three-dimensional volume of the droplet is then reconstructed by rotating the two-dimensional projection extracted from the image around the axis defined by the flight path of the droplet. An important pre-requisite in this context is a reasonable degree of rotational symmetry of the droplet shape relative to the flight path of the droplet. This condition is strictly satisfied only for droplets in full thermodynamic equilibrium, but often reasonably well attained for small droplets generated at low Weber numbers.

C.2 Modelling the measurement

C.2.1 General

The central element of the optical image analysis method for volume measurement of droplets is the size and shape of a two-dimensional image of the droplet in flight. The shape of the droplet image is given by its outline, which takes on the shape of a perfect circle as the droplet approaches equilibrium. As the optical images are usually acquired by digital optical cameras, the shape of the droplet can be numerically determined by edge-detection or thresholding algorithms from the digital images. The results are usually represented by black and white digital images, where (by convention) all white pixels are considered to be part of the droplet image and all black pixels are considered to form part of the background. Thus, the size and shape of the droplet is recorded in a traceable way in the two-dimensional image, if the magnification factor α of the digital optical imaging system is known. With the assumption that the imaged droplet volume exhibits rotational symmetry around its flight path, the two-dimensional droplet image can be used to

reconstruct the droplet volume by rotation, approximating the droplet volume by a stack of cylindrical discs (see ISO 23783-2:2022, G.6.4 for further details).

C.2.2 Analysis of the droplets

Using the arrangement and number of white pixels which have been extracted from the processed black and white image of the droplet and the magnification factor α, the total volume of the droplet measured at temperature t can be calculated in units of µm^3 according to Formula (C.1):

$$V(t) = \sum_{j=1}^{N} \left[\pi \times \left(\frac{n_j}{2} \right)^2 \times \alpha^3 \right] + \Delta V_{\text{evap}} + \Delta V_{\text{rot}} \qquad \text{(C.1)}$$

where

$V(t)$ is the volume of the measured droplet at temperature t, in µm^3;

α is the magnification factor of the optical imaging system that is used to record the photographic droplet images in µm as given in Formula (C.2). It represents the height of one pixel.

j is the index of the row of white pixels forming the droplet image derived by image processing from the digital photographic image of the droplet;

N Is the total number of rows of white pixels forming the droplet image derived by image processing from the digital photographic image of the droplet;

n_j is the total number of white pixels in row j of the droplet image derived by image processing from the digital photographic image of the droplet;

ΔV_{evap} is the volume of test liquid that has evaporated from the droplet before the image was taken, in µm^3;

ΔV_{rot} is the volume difference between the real droplet volume and the volume of a body reconstructed from a stack of thin spherical discs of height α, which is generating the identical two-dimensional digital black and white image as the real droplet, in µm^3.

NOTE 1 Formula (C.1) is a simplification of the general Formula (G.14) in ISO 23783-2:2022 for the case that the photographic image shows exactly one single droplet (M = 1). If more than a single droplet is visible in the image, the analysis of each of the droplets can be carried out separately, and the total delivered volume determined as sum of different droplet volumes, as well as the cumulative uncertainty of the total volume can be calculated as the root sum square of the uncertainties of the individual droplets, according to Formula (5).

NOTE 2 An isotropic magnification factor α is considered to describe the magnification of the optical system. Deviations of isotropic magnification conditions are usually very small for properly designed optical lens systems and are therefore neglected.

NOTE 3 The magnification factor α can also be interpreted as scale factor mapping a real object of dimensions l (in µm) to a number of pixels on the digital image of the object: $l = \alpha \times n$. The acquired digital image is scaled to the real physical dimensions of the imaged object by the factor α.

C.3 Standard uncertainty components associated with the measuring system

C.3.1 Standard uncertainties for the analysis of droplets

The most important factor influencing the standard uncertainty of the droplet volume measurement is the magnification factor α of the optical imaging system that enters into Formula (C.1) with the power of three. Therefore, it is important to use a calibrated and precise optical imaging system for acquiring the photographic images. All uncertainties associated with the magnification factor are summarized in the standard uncertainty $u(\alpha)$.