

Novel gravimetric measurement technique for quantitative volume calibration in the sub-microliter range

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Abstract

We present a novel measurement method based on the gravimetric principles adapted from the ASTM E542 and ISO 4787 standards for quantitative volume determination in the sub-microliter range. Such a method is particularly important for the calibration of non-contact micro dispensers as well as other microfluidic devices. The novel method is based on the linear regression analysis of continuously monitored gravimetric results and therefore is referred to as ‘gravimetric regression method (GRM)’. In this context, the regression analysis is necessary to compensate the mass loss due to evaporation that is significant for very small dispensing volumes. A full assessment of the measurement uncertainty of GRM is presented and results in a standard measurement uncertainty around 6 nl for dosage volumes in the range from 40 nl to 1 μ l. The GRM has been experimentally benchmarked with a dual-dye ratiometric photometric method (Artel Inc., Westbrook, ME, USA), which can provide traceability of measurement to the International System of Units (SI) through reference standards maintained by NIST. Good precision (max. CV = 2.8%) and consistency (bias around 7 nl in the volume range from 40 to 400 nl) have been observed comparing the two methods. Based on the ASTM and ISO standards on the one hand and the benchmark with the photometric method on the other hand, two different approaches for establishing traceability for the GRM are discussed.

Keywords: gravimetric measurement, micro liquid-handling calibration, micro dispenser calibration, linear regression, measurement uncertainty assessment

(Some figures may appear in colour only in the online journal)

1. Introduction

The gravimetric method is one of the most commonly used quantitative and accurate volume determination methods in research laboratories and in industry. Gravimetric volumetric calibration standards like ASTM E542 [1] and ISO 4787 [2]

have been established over 20 years to calibrate volumetric apparatus and liquid-handling devices with the capacity from 100 μ l to 2 l. Standard operation procedures (SOP), traceability and uncertainty of the gravimetric approach in calibration of liquid-handling devices have been researched and discussed many times [3–6].

The lower limit of detection of the traditional gravimetric measurement approach for volumetric calibration of liquid-

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handling systems mostly depends on the readability [7] of the primary measuring device—the balance. Modern precise laboratory micro balances like XP2U/M from Mettler Toledo Inc. (Greifensee, Switzerland) [7] provide readability down to 0.1 μg that corresponds to about 0.1 nl water under standard conditions. However, the effective measurement uncertainty can be much larger depending on the applied measurement method. The main reason for this is probably the evaporation of liquid which proceeds for water at about a rate between (2 and 7) $\text{nl s}^{-1} \text{cm}^{-2}$ under laboratory standard conditions ($T = 20^\circ\text{C}$ and $P = 1013.25 \text{ hPa}$) according to our experiments. The evaporation rate depends on humidity as well as further details of the experimental setup like the balance tray, weighing dish, the type of windshield, etc. The unavoidable loss of liquid leads to a steady drift of the weighing mass.

Usually, every balance needs a so-called settling time of a few seconds (usually 2–10 s) to reach equilibrium again after loading the measuring target. In particular, when the liquid is provided by an impinging droplet issued from a micro dispensing device, a considerable momentum is transferred to the balance weighing dish. During the settling time, the dispensed liquid volume can lose several nanoliters by evaporation, which in comparison to the measured volume is no longer negligible in the sub- μl range as is the case for larger volumes. So far, no standard procedure for measurements in the sub- μl range has been established yet that takes this effect into account. The well-known conventional gravimetric volume measurement procedure as described, e.g., in ISO 4787 and ASTM E542—which does not account for this effect—is not ideally suited for the sub- μl range, but it can be used as a guideline for adaptation of the method to smaller volumes as described in this work.

By the use of non-contact dispensing devices, liquid handling for laboratory automation purposes has accelerated its tendency toward miniaturization in recent years. Several non-contact dispensers for the sub- μl range like PipeJet (BioFluidix GmbH, Freiburg, Germany) [8], Multidrop Microplate Dispensers (Thermo Fisher Scientific Inc., Waltham, MA, USA) [9] and DeeracTM GX Series (Labcyte Inc., Sunnyvale, CA, USA) [10] present very good precision according to relative volume measurements. Such measurements in the sub- μl range are usually based on optical and photometric methods [11, 12] that are partly also routinely applied in the calibration of diagnostic laboratory automation systems. However, these methods are usually not able to provide traceable volume results and a reliable uncertainty analysis regarding all referenced input quantities in the measurement chain. The only exception until now is the commercial liquid measurement system from Artel Inc. (Westbrook, ME, USA)—the multichannel verification system (MVS) [13, 14]. This system is based on a dual-dye ratiometric photometric method which provides traceability according to the National Institute for Standards and Technology (NIST) [15] in the volume range down to 30 nl. However, this method is quite complex and limited to the use of a specific testing liquid (proprietary dye–water mixture from Artel Inc.). For liquids other than water, a separate calibration of the photometric setup is required and the traceability is lost.

In comparison to photometric measurement principles, the gravimetric measurement is largely independent of the liquid used for measurement, which is an important feature for application near characterization of dispensing devices. Only the effective density has to be known to relate the measured mass to the dispensed volume. Environmental influence factors like temperature or pressure can be considered straightforward with the same corrections as for larger volume measurements. Therefore, the gravimetric method should in principle be able to provide similar benefits to micro volume characterization.

In previous work it has been proposed to combine photometric measurements with gravimetric determination of the total mass of the well plate to determine the average accuracy [12, 16]. Although this method provides an improvement compared to single-color photometric methods, the absolute volume of a single dispense cannot be determined gravimetrically. Therefore, the direct gravimetric measurement of a liquid aliquot as recently described in [17] would be more desirable. In this publication, the authors demonstrate the basic feasibility of gravimetric liquid measurements in the sub- μl range considering evaporation, but they fall short of providing a proper uncertainty analysis and do not establish traceability of the proposed measurement approach according to international standards. In particular, the effect of non-equilibrium balance oscillations and the error of the linear regression calculation are not considered there. Both of which can be the major sources of error. In order to overcome these limitations, the gravimetric regression method (GRM) as described in the following and two different ways to establish traceability for this method are presented in this work.

2. Description of the GRM

In order to determine the volume of a liquid aliquot in the sub- μl range based on the gravimetrically measured mass change caused by this liquid volume on a high precision balance, similar equations as provided by the ASTM E542 and ISO 4787 standards are used. The formulae and the basic measurement procedure are discussed in other publications for large volume calibrations in detail [1–6]. For the purpose of this work, the equation for determination of the liquid volume V_{20} based on a gravimetrically measured mass m is considered as follows:

$$V(T) = m \cdot \frac{1}{\rho_w(T) - \rho_a} \cdot \left(1 - \frac{\rho_a}{\rho_c}\right), \quad (1)$$

$$V_{20} = V(T) \cdot [1 - \gamma(T - 20)], \quad (2)$$

where $V(T)$ is the total volume of measured liquid contained in the weighing dish at measurement temperature T . The temperature of measured liquid is supposed to be the same as air temperature during measurement. V_{20} is the volume at reference temperature 20°C and m is the mass of the liquid contained in the weighing dish on the balance. ρ_w is the density of the measured liquid at measurement temperature T . ρ_a is the density of air, approximately to be $0.0012 \text{ mg } \mu\text{l}^{-1}$. ρ_c is the density of the weights used to calibrate the balance

($8.006 \times 10^3 \text{ mg } \mu\text{l}^{-1}$ according to the product certificate of the balance used in this work). γ is the thermal coefficient of expansion of the measured liquid.

The measured primary quantity is the total mass m on the weighing dish of the balance, which is conventionally weighed by the used balance. The mass change caused by the addition of a liquid volume ΔV is denoted by Δm . The mass of the additional liquid is supposed to be the difference of mass before (m_{before}) and after (m_{after}) the dispensing event as given in equation (3):

$$\Delta m = m_{\text{after}} - m_{\text{before}}. \quad (3)$$

The mass values m_{before} and m_{after} are of course subject to a measurement error δm resulting from the error of the weighing process especially from the error of the used gravimetric balance.

Simply speaking the mass of the dispensed liquid volume is mainly determined by the difference of the mass before and after the dispensing event. Usually, the average values of a series of weighing results that are acquired before the dispensing event and after a settling time of a few seconds are considered as appropriate measurement results for m_{before} and m_{after} . However, due to evaporation, the mass of the liquid loaded on the weighing dish of the balance changes over time. After the settling time, the mass detected is no longer corresponding exactly to the mass directly after the dispensing event. It is therefore not admissible to consider simple average values of balance readouts for determining m_{before} and m_{after} . One possible method for overcoming this problem is the linear regression method described in [17]. In this method, the value determined by a linear regression based on several balance readouts acquired before and after the dispensing event is regarded as an appropriate measure for the mass before and after the dispensing event. However, as liquid keeps evaporating from the weighing dish, the balance never reaches equilibrium during the measurement, and thus, the balance reading does not exactly correspond to the real mass value. It is therefore questionable to consider the balance readouts under such non-equilibrium conditions as mass equivalents to determine the values m_{before} and m_{after} without any further consideration of the validity of the results. Therefore, the uncertainty of the linear regression analysis has to be taken into account as an indicator of the validity and reliability of the balance readings.

In order to establish an uncertainty analysis for the GRM, the gravimetric measurement procedure is considered as follows. A weighing dish filled with evaporating liquid measured repeatedly n times results in a series of decreasing balance readouts I_i at time t_i . The i th weighing readout can then be supposed to yield the mass m_i of the weighing dish filled with liquid and an independent error ε_i which represents a random measurement error. Assuming that the evaporation rate of the testing liquid is constant under ideal laboratory conditions with constant temperature, humidity and air pressure, m_i is supposed to have a linear relation to time t_i . And the balance readings I_i can be expressed as follows:

$$I_i = m_i + \varepsilon_i = a - bt_i + \varepsilon_i. \quad (4)$$

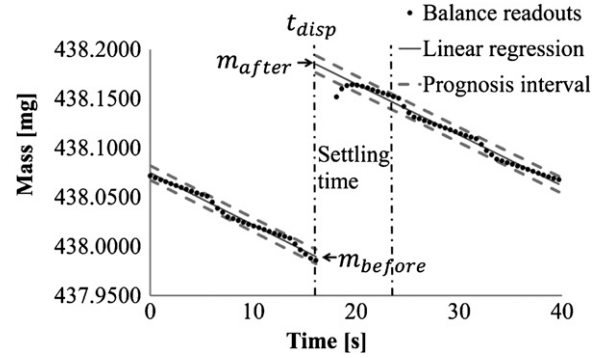


Figure 1. Plot of a typical measurement: balance readouts (dots) before and after dispensing are displayed as a function of time. Readouts acquired between the two vertical lines are not used for calculation (settling time of the balance).

Assume that each ε_i obeys an independent distribution with variance of σ_i^2 ; then, with the method of least squares, a and b can be estimated based on a weighing series as follows [17]:

$$a = \frac{\sum_{i=1}^n \frac{t_i^2}{\sigma_i^2} \cdot \sum_{i=1}^n \frac{I_i}{\sigma_i^2} - \sum_{i=1}^n \frac{t_i}{\sigma_i^2} \cdot \sum_{i=1}^n \frac{t_i I_i}{\sigma_i^2}}{\sum_{i=1}^n \frac{1}{\sigma_i^2} \cdot \sum_{i=1}^n \frac{t_i^2}{\sigma_i^2} - \left(\sum_{i=1}^n \frac{t_i}{\sigma_i^2}\right)^2};$$

$$b = \frac{\sum_{i=1}^n \frac{1}{\sigma_i^2} \cdot \sum_{i=1}^n \frac{t_i I_i}{\sigma_i^2} - \sum_{i=1}^n \frac{t_i}{\sigma_i^2} \cdot \sum_{i=1}^n \frac{I_i}{\sigma_i^2}}{\sum_{i=1}^n \frac{1}{\sigma_i^2} \cdot \sum_{i=1}^n \frac{t_i^2}{\sigma_i^2} - \left(\sum_{i=1}^n \frac{t_i}{\sigma_i^2}\right)^2}. \quad (5)$$

Here, n is the total number of weighings and σ_i^2 is the variance of ε_i .

Under certain conditions—explained in more detail below—it can be assumed that $\sigma_i^2 = \sigma^2 = \text{const}$, and the following simplified equations can be applied to calculate a and b :

$$a = \frac{\sum_{i=1}^n t_i^2 \cdot \sum_{i=1}^n I_i - \sum_{i=1}^n t_i \cdot \sum_{i=1}^n t_i I_i}{n \cdot \sum_{i=1}^n t_i^2 - \left(\sum_{i=1}^n t_i\right)^2};$$

$$b = \frac{n \cdot \sum_{i=1}^n t_i I_i - \sum_{i=1}^n t_i \cdot \sum_{i=1}^n I_i}{n \cdot \sum_{i=1}^n t_i^2 - \left(\sum_{i=1}^n t_i\right)^2}. \quad (6)$$

If there were no evaporation—as is the case for solid objects—then the balance readouts I_i would oscillate around the mean mass value with decreasing amplitude after loading of the weighing dish until the balance mechanism reached equilibrium again. After a settling time, I_i can then be supposed to be equal to m_i , which means that the expectation value of ε_i is zero. However, in the case of liquid, the load is continuously changing and the balance never reaches equilibrium. As a consequence, the mass readouts I_i are continuously oscillating around the actual equilibrium value with is constantly decreasing, as shown in figure 1. It has to be pointed out that these non-decaying periodic oscillations are not caused by the droplet impact onto the weighing dish, but appear as a consequence of the (electro-) mechanical properties of the balance mechanism and do not decay until all the liquid on the weighing dish has completely evaporated.

With constant evaporation rate b and constant time intervals $t_i - t_{i-1}$ between the readouts, the change of mass due to evaporation with respect to the previous measurement $m_i - m_{i-1}$ can be supposed to be the same at each time t_i . Therefore, the approximation of σ_i^2 by a constant value is justified and the regression line provides a reasonable estimate of the continuously decreasing mass value. With equation (6) it is easy to obtain the linear regression parameters ($a_{\text{before}}, b_{\text{before}}$) and ($a_{\text{after}}, b_{\text{after}}$) independently, based on two series of balance readouts before and after dispensing. Using the parameters ($a_{\text{before}}, b_{\text{before}}$) and ($a_{\text{after}}, b_{\text{after}}$), two values $m_{\text{LR, before}}(t_{\text{disp}})$ and $m_{\text{LR, after}}(t_{\text{disp}})$ can be readily calculated, which are the projected mass values at the time of dispensing t_{disp} :

$$\begin{aligned} m_{\text{LR, after}}(t_{\text{disp}}) &= a_{\text{after}} + b_{\text{after}} \cdot t_{\text{disp}}, \\ m_{\text{LR, before}}(t_{\text{disp}}) &= a_{\text{before}} + b_{\text{before}} \cdot t_{\text{disp}}. \end{aligned} \quad (7)$$

Here, t_{disp} indicates the moment when the dispense of the liquid volume is triggered, which is—except for an offset of a few milliseconds—the point in time when the liquid just impinges on the weighing dish.

Denoting the values estimated by the linear regression by subscripts ‘LR’, Δm is supposed to be the difference of these two linear regression values at time t_{disp} :

$$\Delta m = m_{\text{LR, after}}(t_{\text{disp}}) - m_{\text{LR, before}}(t_{\text{disp}}). \quad (8)$$

The analysis of the regression coefficients before and after dispense enables one to verify the validity of the measurement result Δm obtained this way. If, e.g., b_{before} and b_{after} deviate significantly from each other, this indicates unsteady and therefore unreliable measurement conditions. Otherwise, the evaporation rates would be constant, and b_{before} and b_{after} would be the same. Such unreliable conditions might be caused by environmental effects like vibrations of the balance due to wind gust or mechanical disturbance. Therefore, the comparison of the regression parameters b_{before} and b_{after} provides an important means to assess the quality and reliability of the measurement, by verification of the constancy of the evaporation rate.

For the experiments reported in this work the measurement conditions have been established that consistent regression parameters before and after dispensing with less than 5% variation could be obtained. The data acquisition time before and after the measurement has been set to 16 s corresponding in our case to 32 data points to obtain a sufficiently detailed statistic on the regression analysis and the uncertainty estimation. Further details of the experimental setup that were applied to reduce statistical errors are given in section 4.

3. Uncertainty estimation of the GRM

As explained before, the balance never reaches equilibrium during measurement due to the mass change caused by evaporation. Although the linear regression method accounts for the evaporation, it also induces an error into the calculation of the measurement result Δm due to the back projection

of the mass value to the point t_{disp} in time. Since both $m_{\text{LR, before}}(t_{\text{disp}})$ and $m_{\text{LR, after}}(t_{\text{disp}})$ are time-projected values, it is mandatory to consider the uncertainties coming from the prognostic calculation which are denoted as $u(m_{\text{LR, before}}(t_{\text{disp}}))$ and $u(m_{\text{LR, after}}(t_{\text{disp}}))$. Considering $u(m_{\text{LR, before}}(t_{\text{disp}}))$ and $u(m_{\text{LR, after}}(t_{\text{disp}}))$ correctly makes a major difference in the uncertainty analysis of the measurement result when compared to the method described in [17].

For determining the uncertainty $u(m_{\text{LR, before}}(t_{\text{disp}}))$ and $u(m_{\text{LR, after}}(t_{\text{disp}}))$ stemming from the regression analysis, the prognosis interval ($m_{\text{LR}}(t) \pm D_{\text{prog}}(t)$) has been applied. This interval covers the expectation value under a probability of $(1 - \alpha)$ at time t and the half-width of the prognosis interval $D_{\text{prog}}(t)$ at time t is calculated as follows [18, 19]:

$$D_{\text{prog}}(t) = t_{n-2, 1-\frac{\alpha}{2}} \cdot s \cdot \sqrt{1 + \frac{1}{n} + \frac{(t - \bar{t})^2}{\sum_{i=1}^n (t_i - \bar{t})^2}}, \quad (9)$$

where

$$s = \sqrt{\frac{1}{n-2} \sum_{i=1}^n (m_i - m_{\text{LR}, i})^2},$$

$$\bar{t} = \frac{1}{n} \sum_{i=1}^n t_i.$$

Here, s is the deviation of the linear regression residuals for least squares calculation, \bar{t} is the average time of n measurement points, $m_{\text{LR}, i}$ is the resulting mass determined from the linear regression at time t_i , n is the total number of weightings and $t_{n-2, 1-\frac{\alpha}{2}}$ is the t -distribution (Student distribution) for $(n-2)$ degrees of freedom with a probability of $(1 - \frac{\alpha}{2})$ [18, 19]. Finally, α is the probability of error (taken as 5% for all calculations presented here).

In the case of $\alpha = 0.05$, the prognosis interval ($m_{\text{LR}}(t_{\text{disp}}) \pm D_{\text{prog}}(t_{\text{disp}})$) covers with 95% probability the value of the measured mass at time t_{disp} , corresponding to the expanded uncertainty in the ‘Guide to the expression of uncertainty in measurement’ (GUM) [20] with $k = 2$. Here, k denotes the k th α -quantile of the Gaussian normal distribution. Thus, the uncertainties $u(m_{\text{LR, before}}(t_{\text{disp}}))$ and $u(m_{\text{LR, after}}(t_{\text{disp}}))$ can be expressed as follows:

$$\begin{aligned} u(m_{\text{LR, before}}(t_{\text{disp}})) &= \frac{1}{2} D_{\text{prog, before}}(t_{\text{disp}}), \\ u(m_{\text{LR, after}}(t_{\text{disp}})) &= \frac{1}{2} D_{\text{prog, after}}(t_{\text{disp}}). \end{aligned} \quad (10)$$

Based on this uncertainty assessment, the combined standard uncertainty $u(V_{20})$ of the measured volume V_{20} , calculated according to equations (1) and (2) using the mass difference determined from the regression, can be expressed according to EURAMET cg-19 [6] and the method described in the GUM [20] as follows:

Table 1. Uncertainty calculation of GRM for a typical measurement (the same measurement data as shown in figure 1).

Input quantity (*)	Unit	Value	Standard uncertainty u^*	Sensitivity coefficient
Δm	μg	199.0	5.9842	1.0030
δm	μg	0	3.05	1.0030
ρ_w	$\mu\text{g nl}^{-1}$	0.996 6465	1.52E-06	-200.5053
ρ_a	$\mu\text{g nl}^{-1}$	0.0012	2.89E-07	172.3952
ρ_c	$\mu\text{g nl}^{-1}$	8.006	0.01	0.0037
γ	$^{\circ}\text{C}^{-1}$	0.000 207	2.89E-07	-1339.1259
T	$^{\circ}\text{C}$	26.7	0.05	-0.0414
V_{20}	nl	199.6		
$u(V_{20})$	nl	6.7		
$U(V_{20}), k=2$	nl	13.5		

$$\begin{aligned}
u(V_{20})^2 = & \left(\frac{\partial V_{20}}{\partial m} \right)^2 u^2(\Delta m) + \left(\frac{\partial V_{20}}{\partial m} \right)^2 u^2(\delta m) \\
& + \left(\frac{\partial V_{20}}{\partial \rho_w} \right)^2 u^2(\rho_w) + \left(\frac{\partial V_{20}}{\partial \rho_a} \right)^2 u^2(\rho_a) \\
& + \left(\frac{\partial V_{20}}{\partial \rho_c} \right)^2 u^2(\rho_c) + \left(\frac{\partial V_{20}}{\partial \gamma} \right)^2 u^2(\gamma) \\
& + \left(\frac{\partial V_{20}}{\partial T} \right)^2 u^2(T), \quad (11)
\end{aligned}$$

where

$$u^2(\Delta m) = u^2(m_{\text{LR,after}}(t_{\text{disp}})) + u^2(m_{\text{LR,before}}(t_{\text{disp}})). \quad (12)$$

The value of $u(\delta m)$ in equation (11) is given by the uncertainty obtained from the Swiss Calibration Service (SCS) for the Mettler-Toledo XP2U/M balance used for the presented experiments (calibration identification number 140538). The SCS calibration is based on OIML R111-1:2004 [21]. According to the calibration certificate, the expanded uncertainty ($k = 2$) of weighing conventional mass (stainless steel with the density of $7950 \pm 140 \text{ kg m}^{-3}$) of 100 mg is $2.6 \mu\text{g}$ and of 2 g is $6.1 \mu\text{g}$. This uncertainty value can be assumed as the uncertainty $u(\delta m)$ of a normal weighing process including the error of repeatability, linearity, eccentric loading and sensitivity offset of the gravimetric balance. The value $u(\Delta m)$ stems from the uncertainty analysis of the linear regression calculation presented before. It is calculated based on the balance readouts according to equation (12). The uncertainty of the other factors in equation (11) concerns the volume conversion from mass as well as temperature corrections, which are calculated in a similar way as performed by the well-known standard procedures for macroscopic volume measurements [1, 6].

The values of the individual uncertainty components and the finally resulting measurement uncertainty for a typical measurement according to the proposed method based on the measurement results shown in figure 1 are given in table 1. The total standard measurement uncertainty for measuring a liquid volume of about 200 nl results in $u(V_{20}) = 6.7 \text{ nl}$. In contrast to $u(\delta m)$, which represents the uncertainty of a normal weighing process of a non-evaporating mass, the uncertainty $u(\Delta m)$ takes the regression calculation and the balance oscillation into account. Therefore, both of these values have to be considered for calculating the resulting uncertainty $u(V_{20})$ of the final volumetric result V_{20} . Since $u(\Delta m)$ has considerable size,

even larger than $u(\delta m)$ for the presented case, it is evident how important it is to consider the error from the regression analysis and the prognostic calculation correctly, in order to avoid underestimation of the uncertainty of the final measurement result.

To enable the direct comparison with the Artel MVS—for which the uncertainty is provided by the manufacturer for the 95% confidence interval (i.e. expanded uncertainty, $k = 2$)—the uncertainty for the GRM will also be discussed in terms of the expanded uncertainty which can be calculated from the standard uncertainty $u(V_{20})$ by multiplication with $k = 2$: $U(V_{20}) = 2 \cdot u(V_{20})$.

4. Experimental section

The primary measurement device of the gravimetric liquid measurement system used in this study is a high precision laboratory micro balance type XP2U/M from Mettler Toledo, Switzerland. A micro weighing dish (ID \times H 13 mm \times 3.5 mm, VWR Order no 611-1356) filled with about 200 μl of the same testing liquid that is provided to the dispensing device (i.e. deionized water or Artel's proprietary dye-water mixture) is placed at the center of the weighing pan and is covered by an aluminum draft shield (ID \times H 80 mm \times 24 mm) with a circular opening (\emptyset 6 mm) on the top. The testing liquids involved in this experiment are deionized water with water quality of 18 M Ω cm (Quality 1 in ISO 3696) [22, 23] and Artel sample solution (Artel's proprietary dye-water mixture) of type C (for volumes from 500 nl to 2.5 μl in Artel standard 384-well plates, density $1.0059 \mu\text{g nl}^{-1}$ at room temperature according to manufacturer), type D (for volumes from 150 nl to 500 nl in Artel standard 384-well plates, density $1.0020 \mu\text{g nl}^{-1}$ at room temperature according to manufacturer) or type E (for volumes from 30 to 150 nl in Artel standard 384-well plates, density $1.0077 \mu\text{g nl}^{-1}$ at room temperature according to manufacturer). For temperature correction to reference temperature 20°C , we used the same thermal coefficient of expansion for all types of liquids. The nozzle of the dispensing device to be characterized is put directly in closest proximity over the opening of the draft shield. The balance as well as the dispensing device are placed on a vibration isolated granite table (Johann Fischer Aschaffenburg DIN876-126 hardstone surface plate and Newport VH3660W-OPT vibration isolated workstation) to reduce mechanical vibrations and a large

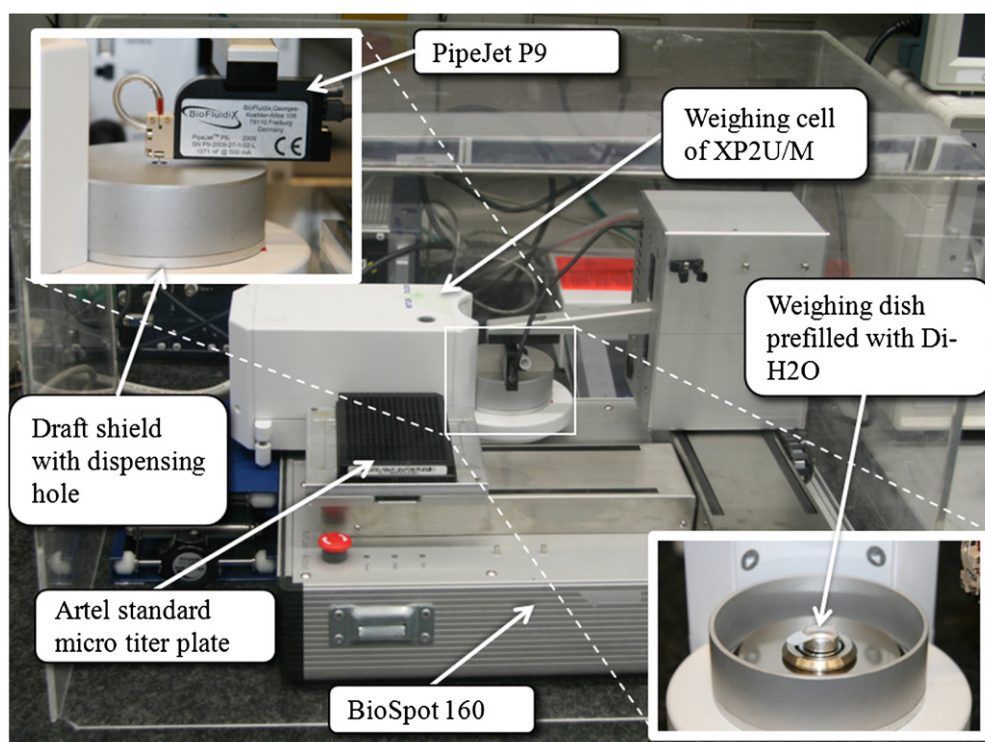


Figure 2. Experimental setup for gravimetric measurement as well as for micro plate preparation for subsequent photometric evaluation (equipment of Artel-MVS not shown). Di-H₂O: laboratory deionized water.

wind shield cover made of transparent PMMA is placed over the whole measurement setup to isolate it from surrounding air convection as shown in figure 2. The acquisition of the balance readouts, the evaporation compensation according to the proposed linear regression method and the calculation of uncertainty of GRM as well as triggering the dispenser were automatically executed by a self-programmed vb.net software program.

In order to investigate the performance of the proposed gravimetric volume measurement procedure and experimental setup, a dispensing device with high repeatability and reproducibility is required. Therefore, a PipeJet P9 dispenser (BioFluidix GmbH, Freiburg, Germany) with 500 μm dispensing pipe was selected. This dispenser is known to operate with very high reproducibility (CV: coefficient of variation $<1\%$) at single droplet volumes in the range from 10 to 60 nl. The PipeJet P9 dispenser was mounted on a liquid-handling robot (BioSpot, BioFluidix GmbH, Freiburg, Germany) in such a way that liquid could be dispensed onto the micro balance or into an Artel standard 384-well plate. Thus, the identical setup was used for gravimetric measurements as well as to fill liquid into a micro well plate for executing the certified measurement method with the Artel MVS system.

For a first benchmark between the Artel MVS method and the proposed gravimetric method, the balance weighing dish and the PipeJet P9 dispenser were loaded with Artel sample solution (proprietary dye–water mixture from Artel Inc.). Artel sample solution of type C, D or E was used for the different volume ranges as described in the operating instructions. First, the PipeJet was automatically moved over the micro balance to execute 21 times the gravimetric measurement as described

above. Directly after the gravimetric measurement the PipeJet was automatically moved to fill the same volume into 21 wells of a certified Artel 384-well micro plate (that had been prepared before, according to the method described in detail in [13, 14]) for subsequent spectrophotometric measurement. The first dispense of a measurement series was always used as priming shot and subsequently excluded from evaluation. Thus, the number of results reduces to $n = 20$ per experiment in both cases. Such a priming shot is important to reduce the effect of liquid evaporation from the nozzle after a certain undetermined idle time of the dispenser that can lead to a drift of the droplet volume or dye concentration which causes systematic errors.

After one of these measurement cycles, the PipeJet moved back to the balance and started the next measurement cycle with a different dispensing volume. Different dispensing volumes were generated by adding multiple droplets (1, 2, 4, etc) to the balance weighing dish. In total, eight different test volumes from about 40 nl (i.e. one droplet) to about 1 μl (i.e. 25 droplets) were measured, whereas the PipeJet dispenser was always operated by the same parameters (down-stroke length = 25 μm , up-stroke velocity = 1 $\mu\text{m ms}^{-1}$, hold time = 10 μs , down-stroke velocity = 100 $\mu\text{m ms}^{-1}$ and shot delay = 50 ms) to produce an individual droplet. Details of the PipeJet working principle and the meaning of the individual control parameters are described in [8].

After the completion of three consecutive experiments with three different volumes, the automatic execution of the experiment was stopped and the filled micro titer plate was processed according to the Artel MVS method. This interruption was necessary because the accuracy of the Artel

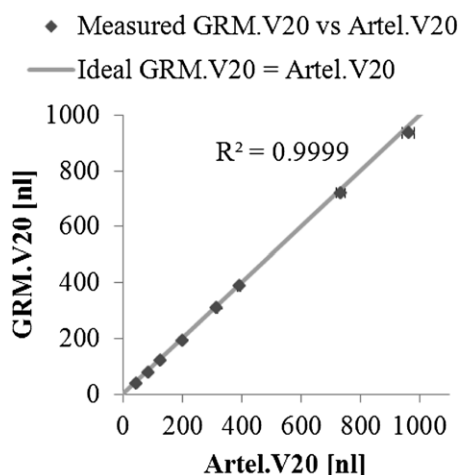


Figure 3. Benchmark results of the presented gravimetric measurement method compared to the Artel-MVS method. Standard deviation of 20 measurements is shown as error bars in the *x*- and the *y*-direction. Environmental conditions during the measurements: temperature = 29.4 °C, relative humidity = 51%, air pressure = 975 hPa. GRM: gravimetric regression method.

method is also influenced by evaporation effects, such that prepared well plates should not be stored for an extended period of time before reading. Furthermore, liquids C, D or E had to be supplied to the dispenser when the corresponding volume range was addressed. After reading of the microliter plate results with the Artel MVS reader, a new plate was prepared and the experiments have been continued as described above.

After the benchmark experiment with the Artel sample solution of type C, D or E, the gravimetric measurement was carried out once more with the same experimental setup but using deionized water. The same dispensing parameters, as well as measurement configuration was used like before, except of the fact that dispensing was performed onto the balance only and no Artel MVS measurements were made this time. This second experiment was carried out in order to determine whether the measurement results obtained gravimetrically with deionized water and the Artel solution

of type C, D or E are the same. The time interval between these two experimental series with the same hardware and dispensing parameters was about 12 h with an ambient temperature difference of about 3 °C.

5. Results and discussion

The benchmark results of the gravimetric measurements and the photometric Artel MVS measurements are presented in figures 3 and 4. The repeatability given by the coefficient of variation (CV) is below 3% for both measurement methods in the whole considered measurement range, where the CV is defined as the ratio of the standard deviation to the mean measured volume. Furthermore, both measurement methods provide greatly consistent results. The small deviations between the results of the two different methods are best discussed in terms of the so-called bias which can be expressed in terms of the absolute bias (nl) and relative bias (%) defined as follows:

$$\begin{aligned} \text{Bias [nl]} &= \text{GRM.V20} - \text{Artel.V20}, \\ \text{Bias [\%]} &= \frac{\text{GRM.V20} - \text{Artel.V20}}{\text{Artel.V20}} \cdot 100\%. \end{aligned} \tag{13}$$

Here, GRM.V20 is the measured mean volume at the reference temperature 20 °C determined with the presented GRM approach and Artel.V20 is the measured mean volume at reference temperature 20 °C using the Artel MVS with temperature correction according to equation (2).

The bias represents the difference between the mean volume measured by the GRM approach and the reference value given by the Artel MVS. In the lower volume range from 40 to 400 nl, the absolute bias is almost constant around -7 nl. For larger volumes, the absolute bias becomes somewhat larger and reaches up to -27 nl. Given the measurement uncertainty of the gravimetric method and the Artel method [24] as shown in figure 4, all measured values have to be considered to be consistent, since the bias is always smaller than the corresponding measurement uncertainty.

In view of the proven consistency of both methods, one could argue that traceability of the gravimetric method could be established by calibration of the gravimetric results

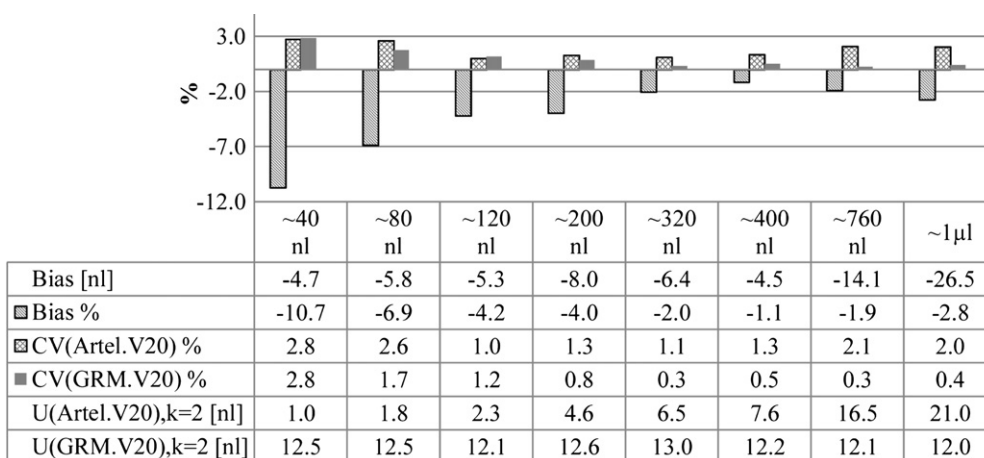


Figure 4. Bias and CV of the presented gravimetric measurement method compared to the Artel-MVS method. Environmental conditions during the measurements: temperature = 29.4 °C, relative humidity = 51%, air pressure = 975 hPa. GRM: gravimetric regression method.

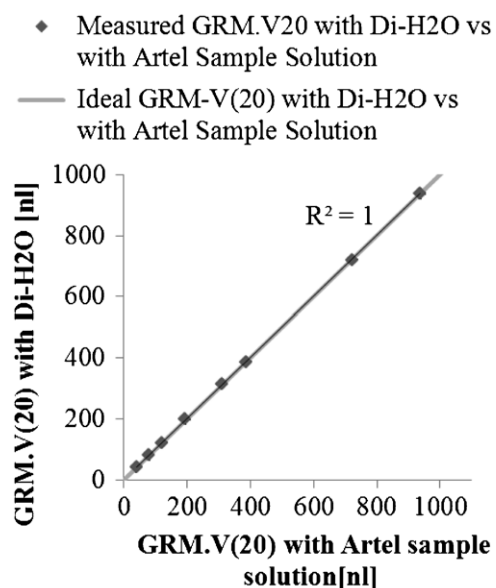


Figure 5. Comparison of gravimetric measurements carried out with Di-H2O and Artel sample solution of type C, D or E. Standard deviation of 20 measurements is shown as error bars in the x- and the y-direction. Environmental conditions for measurement with Artel sample solution: temperature = 29.4 °C, relative humidity = 51%, air pressure = 975 hPa. Environmental conditions for measurement with Di-H2O: temperature = 26.7 °C, relative humidity = 52%, air pressure = 975 hPa. GRM: gravimetric regression method. Di-H2O: laboratory deionized water.

through the benchmark with the traceable Artel measurements. This calibration line would be indeed given by figure 3. On the other hand, traceability is better established by relating the gravimetric volume measurement results directly to the gravimetric mass change acquired by the balance analogue to ASTM E542 and ISO 4787. However, ASTM E542 and ISO 4787 are not strictly valid for the small volumes considered here—in particular due to the discussed significant evaporation effect—and a corresponding standard for the sub- μ l range is not yet available. Furthermore, the gravimetric results have been obtained by using Artel sample solutions C, D, E and

not with deionized water as requested in ASTM E542 and ISO 4787.

Assuming though that the ASTM E542 and ISO 4787 could be extended to the microscopic volume range by the described GRM method, only the consistency of using Artel solution compared to deionized water for gravimetric measurement has to be shown to provide direct traceability of the gravimetric method. In order to verify whether the usage of the Artel sample solution of type C, D, or E gives the same gravimetric measurement results compared to dispensing deionized water, the second experimental series was performed as described above using deionized water only. As shown in figures 5 and 6, the same results have been obtained by the gravimetric method using either the Artel solution of type C, D, or E or deionized water in the whole volume range. The volumes obtained for either liquid are consistent within a few nl only (cf figure 6). This indicates that first the PipeJet dispenser delivers the same volumes with either liquid and, second, the gravimetric results are not influenced by the choice of either liquid. Both of which could be expected due to the very similar density and evaporation behavior of both liquids. Therefore, it seems admissible to use the measured data obtained for deionized water as well as for the Artel solution of type C, D, or E to establish traceability for the proposed method according to ASTM E542 and ISO 4787 (provided that the standard can be considered as extendable to small volumes down to 40 nl when the described GRM approach is used). Thus, traceability for the GRM could be established consistently by two different, independent approaches.

6. Conclusion

In this paper, we have presented an improved gravimetric volume measurement method referred to as gravimetric regression method (GRM) for determining liquid volumes in the sub- μ l range. The core element of this method is a linear regression for evaporation compensation applied on a large number of measured data points before and after addition of the liquid volume to the balance. In order to predict the

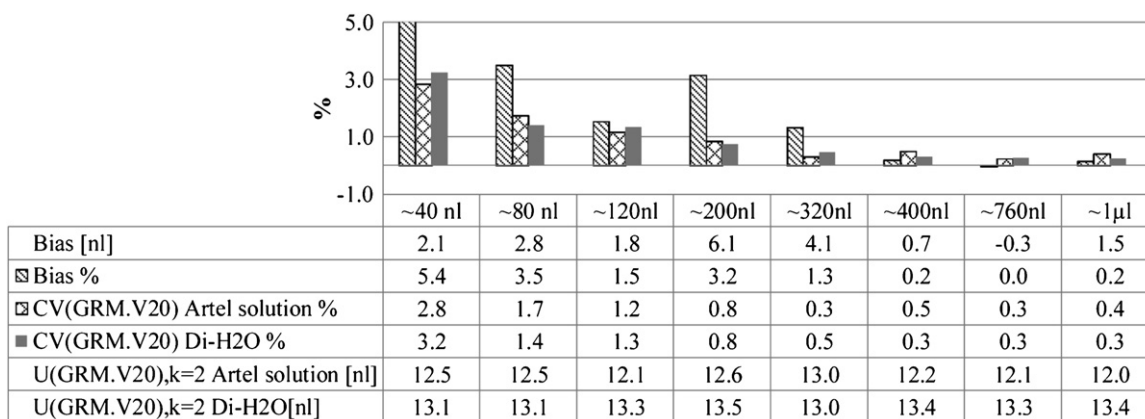


Figure 6. Bias and CV of the presented gravimetric measurement method carried out with Di-H2O and Artel sample solution. Environmental conditions for measurement with Artel sample solution: temperature = 29.4 °C, relative humidity = 51%, air pressure = 975 hPa. Environmental conditions for measurement with Di-H2O: temperature = 26.7 °C, relative humidity = 52%, air pressure = 975 hPa. GRM: gravimetric regression method. Di-H2O: laboratory deionized water.

mass difference caused by the dispensed liquid correctly, the prognostic error of the linear regression has to be considered. Therefore, a thorough uncertainty analysis has been presented for the GRM and a combined standard uncertainty $u(V_{20})$ of typically 6 nl and an expanded uncertainty $U(V_{20})$ of typically 12 nl have been determined for volumes in the range from 40 nl to 1 μ l.

According to the presented benchmark experiment, the results of the gravimetric measurements have been shown to be consistent with the Artel MVS photometric method that was used as a reference. To the knowledge of the authors, this is the first time that traceability according to international standards has been established for a volumetric measurement carried out in the sub- μ l range by means of a gravimetric method. The major advantage of such a gravimetric method compared to photometric approaches is that it can be applied in principle with any liquid which makes it more flexible and easier to use in application near measurement scenarios.

The analysis of the measurement errors revealed that the uncertainty of the new GRM is larger than the uncertainty of the photometric reference method (Artel MVS) in the lower volume range but smaller for volumes larger than 700 nl. However, since the central measuring device in the GRM, i.e. the balance, normally provides a smaller uncertainty than the spectrophotometer in the Artel MVS photometric method, the GRM may have the potential to deliver a smaller uncertainty if the measurement procedure is further improved. In this second approach, traceability to international standards is established independently of the Artel MVS photometric method, but relying on the traceability of the balance itself, analogous to the standards ASTM E 542 and ISO 4787 for large volume measurements. Although these standards are not strictly valid for the considered volume range, they still can be considered applicable for the purpose of this work in view of the fact that more suitable standards for the sub- μ l range do not exist yet. Furthermore, the experimental data obtained in this study prove that both methods to establish traceability yield the same results within the limits of uncertainty, which justifies the proposed approach for direct calibration *ex post*.

Compared to the previous work [17], this study has provided a thorough uncertainty analysis of the GRM and has established traceability according to international standards for this method. It turned out that the uncertainty caused by the use of the prognostic value of the linear regression performed on the primary balance readouts is significant and must not be underestimated. Furthermore, it has been found that the fitted value for the rate of evaporation and the magnitude of the prognosis interval can be used as criteria to determine the reliability of a measurement. If the fit values for the periods before and after addition of the volume to the balance deviate too much from each other and/or the prognosis interval becomes large, this indicates a disturbance of the measurement setup during data acquisition. If such a disturbance is considered to be too large, the corresponding measurement can be rejected. However, if the statistical measurement error caused by the disturbance is considered to be still in an acceptable range, the presented uncertainty analysis accounts for this error properly and delivers correspondingly

larger uncertainties for such disturbed measurements. Thus, in summary, the presented GRM approach is suitable to provide flexible, accurate, objective and traceable results for liquid volume measurements in the sub- μ l range. This will be very helpful for quantitative calibration of micro non-contact liquid-handling devices and other microfluidic applications in the future.

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