Gravimetric determination of pipette errors

In chemical measurements (for instance in titrimetric analysis) it is very important to precisely measure amount of liquid, the measurement is performed with use of various measuring vessels. Presently laboratories use all kinds of glass measuring vessels like: beakers, flasks, etc., for measurement of bigger volumes of liquid, and in case of small volumes glass pipettes. Glass pipettes have been used for very precise measurements since the 70's of 20^{th} century.

In 1964 during XII Measuring Conference, a measuring value of a "liter" from outside SI set has been accepted for official use. A liter [l] officially stands for a cubic decimeter [dm3].

In analytical chemistry, it is much more convenient to express volume in liters, and such is used in various documents, norms and publications.

Development of science results in development in metrology of various sizes.

Today one can observe even more precise and accurate measuring instruments. It is not different in case of measuring liquids with use of pipettes, commonly known as *„liquid handling"*. Presently, laboratory operators use modern pipettes, which can dose volumes like tenth of microliter [ul].

Volume Measuring Metrology - pipettes

An important issue relating to all kinds of measuring instruments applicable particularly in laboratories is proper supervision over these instruments. Supervision includes calibration and checking procedures of measuring instruments. Pipettes, like other measuring instruments, have norms and regulations describing their parameters. Presently, an international standard referring to pipettes is EN ISO 8655-2:2003 *Piston-operated volumetric apparatus Part 2: Piston pipettes* (in content of ISO 8655-2). The norm describes design requirements which are valid for pipettes manufacturers, and also maximal permissible errors which are constitute for norms to the manufacturers, and can also be used as acceptance criteria for pipettes operators.

In case of piston pipettes, one distinguishes two kinds of errors: systematic error *es* and random error *CV*.

In order to determine above mentioned errors for piston pipettes, laboratories use weighing method, which process and means of error calculations are described in norm EN ISO 8655-6:2003 *Piston-operated volumetric apparatus – Part 6: Gravimetric methods for the determination of measurement error* (in content of ISO 8655-6).

Gravimetric method for determination of measurement error specifies the amount of liquid that is purged from piston pipette to a specific vessel, with application of an analytical balance, and conversion of obtained measuring result in mass unit into volumetric unit, which is a microliter or milliliter, as specified above. A physical relationship described by a formula:

$$V = \frac{m}{\rho} \tag{1}$$

Where:
V – stands for volume
m – stands for mass
Z – stands for density

Gives a result, in which volume depends on mass and density of a substance. In order to determine volume of a liquid used for checking of a piston pipette, one should use a formula:

$$V_i = m_i \cdot Z \tag{2}$$

Where:
Vi – stands for volume value
mi – stands for mass obtained as result of weighing purged liquid
Z – stands for corrective coefficient depending on temperature and air pressure in [□l/mg]

Mass is read directly from measuring instrument, i.e. analytical balance, and Z coefficient is determined from a formula:

$$Z = \frac{1}{\rho_w} \times \frac{1 - \frac{\rho_a}{\rho_b}}{1 - \frac{\rho_a}{\rho_w}} = \frac{1}{\rho_b} \times \frac{\rho_b - \rho_a}{\rho_w - \rho_a} \tag{3}$$

where:
ρ*w* – stands for water density
ρ*a* – stands for air density
ρ*b* – stands for density of reference mass that has been used for calibration of the balance (according to R111 OIML stainless steel density ρ*b* is approximately 8000 kg/m3) and it is determined for each temperature and pressure value in Attachment A norm ISO 8655-6.

According to requirements of ISO 8655-6, the measuring procedure anticipates performance of 10 series of measurements from each tested volume and calculation of average volume according to formula (2) and calculation of average value according to a relationship:

$$\bar{V} = \frac{1}{10} \times \sum_{i=1}^{10} V_i \tag{4}$$

In case where the temperature in a weighing room is different from the temperature described in norm ISO 8655-2, and set as 20ºC, and corrective factor of thermal expansion of a device (pipette) *Y* is specified, than formula (2) can be replaced with below one:

$$V_l = m_l \cdot Z \cdot Y \tag{5}$$

Corrective factor *Y* is determined by a formula:

$$Y = 1 - \alpha_c (t_d - t_{d20}) \tag{6}$$

Where:

αc – stands for a volumetric expansion coefficient expressed in w ºC-1
td – stands for temperature expressed in ºC
*td*20 – stands for constant temperature 20ºC
Y coefficient value equals approximate to a unit.

Error characteristics of piston pipettes

As has been mentioned above, norm ISO 8655-6 determines characteristic errors for piston pipettes.

Systematic error

Systematic error, (also known as pipette accuracy error), according to International Vocabulary of Basic and General Terms of Metrology (VIM), is a difference between average and infinite quantity of measurements of the same measured value, performed in repeatable conditions, and real measured value.

Norm EN ISO 8655-1:2003 *Piston-operated volumetric apparatus – Part 1: Terminology, general requirements and user recommendations,* gives definitions for this error with reference to piston pipettes, and describes it as a difference between volumetric measurement value and nominal or selected value of tested pipette.

On analysis of both methods, it occurs that both are correct.

Systematic error is expressed in volumetric units, microliters [□l] and percents [%]. It is marked with *es* symbol and calculated with a formula:

$$e_s = \bar{V} - V_s \tag{7}$$

If the error is expressed in microliters, and

$$e_s = 100 \cdot (\bar{V} - V_s) / V_s \tag{8}$$

If the error is expressed in percents

Where:

Vs – stands for value of tested volume.

In case of pipettes with constant volume, tested volume Vs is also the nominal volume V0 and can be replaced by it in formula (8).

Random error

Random error (also known as pipette repeatability error), according to the definition from International Vocabulary of Basic and General Terms of Metrology (VIM) is a difference between measurement result and average from infinite quantity of measurements of the same measured value, performed in repeatable conditions (the difference between measurement error, i.e. the difference between measuring result and real value of measured value, minus systematic error). It should be stressed here, that it is possible to perform only finite quantity of measurements, thus it is only possible to estimate the value of random error.
For pipettes, norm EN ISO 8655-1:2003 *Piston-operated volumetric apparatus – Part 1: Terminology, general requirements and user recommendations* defines the error as dispersion between of measuring result of volume around average value of volume.
On analysis of both methods, it occurs that both are correct, if assumed that in both cases the measure is defined as standard deviation.

Random error is expressed in volumetric units: microliters [□l] and percents [%]. Random error is marked with *CV* symbol. The measure of random error is standard deviation calculated by a formula

$$s_r = \sqrt{\frac{\sum_{i=1}^{n}(V_i - \bar{V})^2}{n-1}} \tag{9}$$

Where:
n – stands for quantity of repetitions, in this case 10 series of repetitions.

Random error can also be expressed in percents, and in such case, it is expressed by a formula:

$$CV = 100 \cdot \frac{s_r}{\bar{V}} \tag{10}$$

Gravimetric method of measuring process

In order to correctly go through procedure of gravimetric calibration of a piston pipette, it is necessary to have adequate measuring equipment, for which metrological requirements are defined by in norm ISO 8655-6.

Analytical balance

The most important measuring instrument for process of piston pipettes calibration is an analytical balance, which is selected with consideration of pipette volume. Norm ISO 8655-6 precisely defines the requirements for applicable balances:

Tested volume *V*	Reading unit *d* mg	Repeatability and linearity mg	Standard measurement uncertainty mg
1 ul *V* < 10 ul	0,001	0,002	0,002
10 ul < *V* < 100 ul	0,01	0,02	0,02
100 ul < *V* < 1000 ul	0,1	0,2	0,2
1 ml < *V* < 10 ml	0,1	0,2	0,2
10 ml < *V* < 200 ml	1	2	2

Chart 1: Minimal requirements for balances (according to ISO 8655-5)

In case, where standard measurement uncertainty of liquid weighing process is determined (for instance from balance calibration certificate), than it should be used as an acceptance criterion instead of repeatability and linearity parameters. It should be assumed here, that standard deviation is not bigger than two or threefold of reading unit *d*.

In order to maintain measuring coherence, a balance used for calibration of pipettes should have valid calibration certificate at the time of measuring procedure.

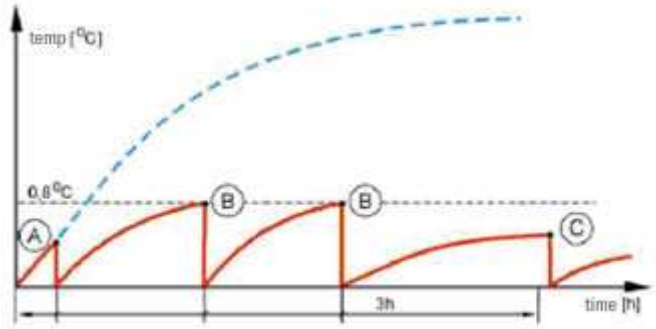
Before starting the procedure of pipette calibration, a balance designed for pipettes calibration should be properly prepared according to manufacturer recommendations (in most cases it refers to user manual recommendations on balance installation at a workstation). Depending on balance design, the adjustment to present operation conditions in a weighing room (like adjustment to gravitiational acceleration force, and errors sourcing from its change and ambient conditions) is performed:

- With properly selected standard mass (standard mass designed for balance calibration); class and nominal weight of the standard mass are determined by balance manufacturer,
- With use of automatic internal calibration system by build-in reference mass, which is an integral part of a balance.

The process of balance adjustment is comparing mass of standard / reference mass (internal or external) with mass that is saved in balance memory. As result of this comparison, balance accuracy is corrected. In case of balances equipped with automatic internal calibration system, it is possible to select three kinds of calibration:

- Start-up calibration, which initiates automatically on plugging a balance to mains; start-up calibration automatically compensates the errors sourcing from differences in gravitational acceleration force of the manufacturer site and user site,
- Temperature calibration, which is initiated automatically if a balance sensor detects temperature change inside balance mechanism which exceeds temperature thresholds set in service menu of a balance,

- Time calibration (with consideration of elapsing time from last calibration), which is initiated automatically after amount of time declared in service menu of a balance.



A – start-up calibration
B – temperature calibration
C – time calibration
Broken line stands for balance indication error if calibration process is not initiated.

Fig. 2: Balance indication drift in relation to temperature changes, for instance during self heating of a balance.

From the point of liquid handling, and process of pipettes calibration, it is important to notice, that temperature and time calibration may have negative influence on measuring process of such small increments of mass. This may also be caused by temperature limits which are mentioned in norm ISO 8655-6.
Below table presents values of temperature sensitivity drift calculated into error values:

Balance model	Maximal capacity - *Max*	Reading unit - *d*	Division of balance *Max/d*	Sensitivity drift	Temperature error for balance Max capacity	Temperature error for $\Delta m = 100g$
	[g]	[mg]	[units]	Ppm/ºC	[mg]	[mg]
MXA 31	31	0,001	31.000.000	2	6,200	0,000068
XA 60/220/X	60	0,01	6.000.000	2	4,80	0,000033

Chart 2: balance temperature errors

Analyzing possible temperature drifts as specified by the manufacturers in ppm/ºC (*parts per million*), and bearing in mind that they refer to temperature range between 18ºC and 30ºC, it can be concluded, that calibration of a balance is needed in order to compensate its temperature errors. As has been mentioned above, balances are equipped with system of automatic internal calibration dependant on time and temperature. If balances are used in verifiable conditions, i.e. they are controlled by legal metrology, system of automatic internal calibration has to be active, with no access to settings modification by an operator. In case of balances that are used for pipettes calibration, calibration process initiation can be an obstacle in procedure realization. As observed from results in chart 3, temperature errors for very small increments of mass (e.g. use of pipettes) are so small that they do

not affect measuring accuracy. It is worth mentioning here, that test procedure for a single volume of a pipette is taken within short period of time.
Having regard to above, it is convenient that balances used for pipettes checking have possibility of adjusting parameters of automatic internal time and temperature calibration. Thus, the best solution for pipette checking is application of balances that are not controlled by legal metrology. It is the only way to allow the operator to adjust balance parameters relating to calibration features.

Ambient conditions for pipette calibration

Measurements relating to pipette calibration process should be performed in a weighing room with stable ambient conditions, and free from breeze of air. According to regulations, weighing room temperature should be maintained at constant level which does not exceed $\pm 0{,}5$℃ change during measuring process. Ordered temperature range should be set between 15℃ and 30℃, and relative humidity should be maintained at level of approximately 50%.
In order to run the process of pipette calibration properly, the pipette, tips and distilled water should be stabilized for temperature in the weighing room. Norm requirements specify temperature stabilization period for at least 2 hours before weighing process. Pipette and distilled water temperature should equal to weighing room temperature.
Procedure of pipette calibration requires continuous monitoring of weighing room ambient conditions, with special attention paid to air temperature and air pressure. These two are necessary for calculations. Additionally, it is necessary to know humidity and distilled water temperature.
According to requirements from norm ISO 8655-6 measuring instruments should be compatible with norm requirements referring to standard uncertainty:
- thermometer, with standard uncertainty lower or equaling 0,2 ℃;
- hygrometer, with standard uncertainty lower of equaling 10 %;
- barometer, with standard uncertainty lower or equaling 0,5 kPa.

All above uncertainty parameters are specified for expansion coefficient $k = 1$.
Distilled water used for pipette calibration should be compatible with requirements of class 3 or should be prepared according to specification of international norm ISO 3696 *Water for laboratory use – Specification and test methods*.

Evaporation

An important aspects referring to pipette calibration process is liquid evaporation during weighing procedure.
Evaporation process is a natural physical phenomenon, in which liquid changes its state of aggregation to gas, which in this case is water vapour. In most cases this process takes place on the surface of liquid. Evaporation speed depends on temperature, humidity and partial pressure, which is a pressure which is created by a selected component of gas mixture, if this component would occupy the same area of vapour over liquid in a specific temperature. If vapour pressure is equal to saturated

vapour in a specific temperature, than evaporation process does not exist. This status is also expressed as equilibrium between evaporation and condensation. In case of weighing distilled water. Evaporation process is observed in any case. Norm ISO 8655-6 requires that evaporation process is taken into consideration during calculations and that possible measures are provided in order to decrease the risk possible measuring errors. In case of volumes below 50 □l the norm requires application of a weighing vessel with cover or use of other methods for compensation of this unfavourable phenomenon. The norm also requires that a series of measurements of a single volume should as short as possible (recommended period of time is approximately 60 seconds), as quick and repeatable operation positively influences the error size caused by evaporation process.

Process of pipette calibration is highly affected by stabilization time of weighing process, which is related to real measurement time. Long weighing time is influenced by ambient conditions, like breeze of air, vibrations, temperature drifts, etc. Most of balances that are dedicated to pipette calibration, provide the operator access to adjustment of parameters corresponding to weighing process optimization with present ambient conditions.

Each balance that leaves manufacturer site is adjusted to certain ambient conditions. Generally it is assumed, that such conditions are compatible with manufacturer requirements on temperature range, humidity or possible disturbances. As practice shows, such assumptions are not always correct. For most of electronic balances, process of adjusting them to ambient conditions requires:

- selection of different setting for signal filtering processes /stronger or weaker filter setting /
- selection of different criteria for determination of stable measurement.

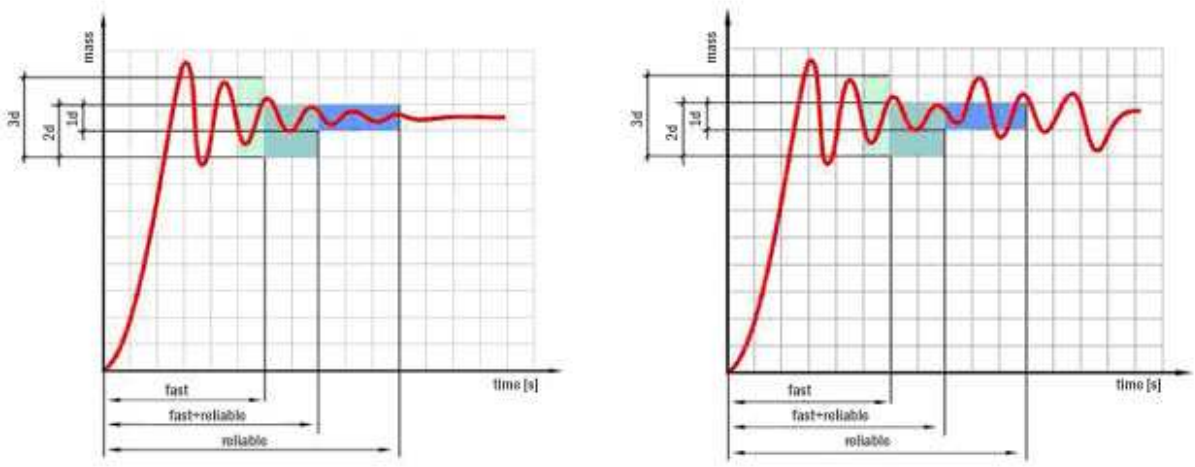


Fig.2. Graph indicating stabilization of weighing process

Due to practical application, filter settings are described as fast, average, slow and very slow. Criteria for determination of weighing result stability are presented as: fast, fast + reliable and reliable. It is possible to form a general rule: **the bigger the disturbances, the higher filter settings**

The consequence of such filter settings is lengthening of weighing process, but in some cases this is the only possibility for obtaining correct weighing result. However, the best option is to eliminate the source of disturbances in process of pipette calibration.

Measuring retraceability in gravimetric pipette calibration

One of the most important aspects related to measurements is maintaining measuring retraceabiility.

Retraceability, is a feature of measuring process or reference mass according to which, it is possible to bind it with its references, in most cases with national or international standard masses. The binding is continuous, and element of this binding has its uncertainty determined.

Maintaining measuring retraceability is a condition for uniqueness of measurements, due to which it is possible to compare each measurement result to another one.

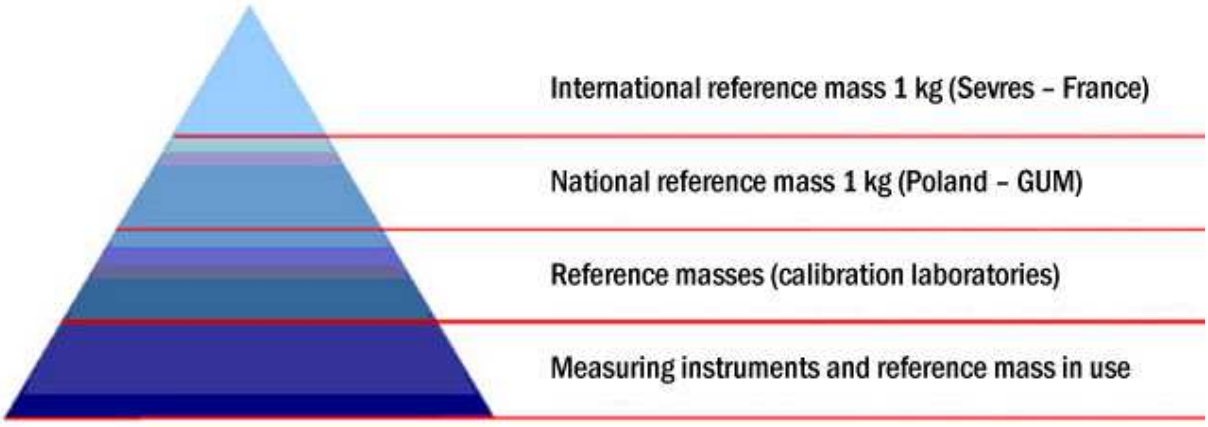


Fig.3. Retraceability schema

International Standard mass 1 kg BIMP – France 100)	National Standard mass 1 kg (Central Office of Measures)	Reference mass Calibration Laboratory, RADWAG	Analytical balance (RADWAG MXA 21/P)	Piston pipette (RADWAG) RP-PV10-

Fig. 4. Piston pipette retraceability visualization

From the point of view of measuring instruments and reference masses operators, the best option for maintaining their retraceability is calibration of these instruments and masses by accredited laboratories, and checking them according to internal timetable for calibration and checking. The internal control should be performed with use of selected instruments and reference masses.

Gravimetric method measuring uncertainty

Measuring uncertainty is connected to testing and/or measuring procedures valid in laboratories. According to the natural rights, there are no accurate measurements, it is only possible to calculate the range in which obtained result is positioned. The size of this range depends on accepted trust level. Another issue refers to thorough analysis of each component of uncertainty. If an uncertainty budget analysis is performed incorrectly, it may lead to a situation where accepted result is incorrect, or correct result may be dropped off as incorrect.

Uncertainty of measurement is recently under thorough analysis of testing and research laboratories and scientific offices.

According to International Vocabulary of Basic and General Terms of Metrology, uncertainty of measurement is a parameter related to measuring result, characterized by dispersion of indications, which can be with justification assigned to specific measured value.

Such is the instance in case of standard deviation and its multiplication – standard deviation from a series of measurements is also uncertainty of measurements.

Uncertainty of measurements is divided into two kinds, depending on parameters source. One distinguishes between Type A and Type B uncertainty of measurement.

Method A of standard uncertainty calculation, is realized through static analysis of observed series.

For uncertainty Type A normal distribution is accepted, which graphically is presented as Gausses curve.

Uncertainty type B is determined by a scientific analysis based on all accessible information on changeability of initial value. Those data are: based on previously performed measurements, operator's experience, characteristic features of measured materials and measuring devices. Uncertainty type B utilizes data from manufacturer's product specification, uncertainty reference data, handbook and manual content, all accessible publications and other resources. Another important source of data comes from calibration certificates of measuring instruments, reference masses and sizes from physical objects or any other certificates.

With use of above mentioned analytical balance, it is possible to determine components of uncertainty type B, which are:

- reading unit *d*,
- repeatability, which is determined by standard deviation set earlier by an operator or during calibration process,
- balance indication error, specified in calibration certificate,
- uncertainty while determining an indication error.

With a more thorough analysis, it is possible to find more parameters, but they may have no effect on a measurement, depending on its accuracy.

Complex uncertainty

Complex uncertainty – in simple words – is a connection of uncertainty type A and type B. the most common is the complex uncertainty, there are, however, some cases, where complete uncertainty analysis is based on the type B.

Extended uncertainty

Extended uncertainty is a value describing the range of values surrounding the measuring result, which, as expected, can cover a large part of values distribution, which are commonly assigned to measured value.
According to Guide to Expression of Uncertainty in Measurements, letter *u* has been assigned to match uncertainty, and expression of extended uncertainty is realized by capital letter *U*. Graphic presentation of measurement uncertainty is shown on below chart:

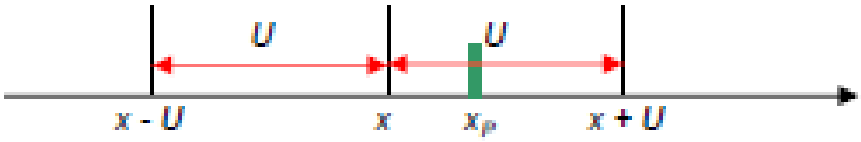


Fig.5 graphic interpretation of uncertainty of measurement

where:
x – measurement result
xP – measurand

As result of value measurement xP, value x has been obtained. As seen above, the result of measurement is not equal to measurand - there are no ideal measurement in the environment. One can only discuss the range in which the measurand is positioned. Depending on the accuracy of a measuring process and the uncertainty related to it, the range can have bigger or smaller scope. Scope size depends for instance on applied measuring device, ambient conditions, operator, and also proper analysis of measuring uncertainty.
An extension ratio *k* is a numerically expressed ratio, used as a multiplier of standard complex uncertainty, determined to set extended uncertainty.

The extended uncertainty is expressed by a below relationship:

$$U = k \cdot u(x) \tag{13}$$

Where: U – extended uncertainty
k – extension ratio
u(x) – complex uncertainty
In case of calibration of piston pipettes, it is possible to distinguish between two sources of uncertainty:
- uncertainty related to measuring instrument (pipette)

- uncertainly related to measuring method (gravimetric method).

According to Guide to Expression of Uncertainty in Measurements, both sources of uncertainty have to be analyzed and taken into account during estimating of complex uncertainty while calibration process.

Norm ISO 8655-6 mentions a statement, that uncertainty level resulting from a series of gravimetric measurements, with use of measuring equipment is low if compared with results from measuring process. It is assumed here, that instruments utilized for measuring procedure (balance, barometer, thermometer, etc.) are used according to specification from this norm. Thus, it is a hint for the operators to neglect uncertainty level in measurements, and concentrate only on systematic and random measuring errors resulting from a series of ten repetitions of tested volume measurement for each instrument.
It should be stressed here, that systematic measuring error does not influence the uncertainty evaluation of gravimetric method. It is a measuring result which includes consideration of random error, that is characteristic to volume measurement.
With above specified assumptions, norm ISO 8655-6 provides a simplified formula for uncertainty calculation, which may be used for evaluating measuring uncertainty for piston pipettes calibration with 95 % of trust level:

$$u = |e_s| + 2 \cdot s_r \tag{14}$$

A successful evaluation of uncertainty depends mostly on thorough and correct analysis of complete measuring process. It is important to evaluate measuring uncertainty according to its accuracy, as in some cases some of uncertainty components may have influence on the measuring result. For this reason, in case of very small volumes, or more thorough analysis (i.e. obtaining more data on uncertainty budget), formula (12) is not applicable. A detailed analysis of gravimetric method uncertainty evaluation is described in ISO/TR 20461 document.

Sources of errors in process of pipettes checking

Analysis of norm ISO 8655-6 requirements, and tests results provided by RADWAG in section 2, specify, that measurements performed during calibration process of piston pipettes (and other kinds of measurements) are affected by potential occurrence of measuring errors. Research performed by RADWAG Measuring Laboratory was to define the main sources of potential risk and defining its influence on measuring result. Sources of errors have been observed in:

- incompatible balance used for test procedure,
- incorrect weighing vessel,
- liquid evaporation process during weighing procedure,
- incorrect ambient conditions in a weighing room or lack of their continuous monitoring,
- incorrect workstation (vibrations, breeze of air),

- incorrect liquid used for calibration of piston pipettes,
- calculation errors,
- incorrectly selected pipette tips (other than recommended by a manufacturer),
- inappropriate physiological condition of an operator (e.g. too low or too high body temperature during test procedure),
- non-ergonomic workstation.

Most of presented error sources have been analyzed in the above article. Conclusion from performed tests on inappropriate or incorrect ambient conditions in a weighing room, lack of their continuous monitoring, vibrations and breeze of air which influence balance operation, incorrect calculation and workstation ergonomics have been discussed in section 3 of this article. It is RADWAG research and development department response for the problem of piston pipettes calibration.