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Analysis of influencing parameters on calibration of piston-operated pipettes with air cushions

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Foreword

DKD Guidelines are recommendations with regard to technical issues arising in connection with the practical work of accredited calibration laboratories. In the DKD Guidelines, procedures are described which may serve the accredited calibration laboratories as a model for the determination of internal procedures and regulations. DKD guidelines may become essential components of the quality management manuals of calibration laboratories. Through the application of the Guidelines, the state of the art in the respective technical field will be transposed into laboratory practice. The aim of this is a harmonization of the procedures and a more efficient way of working in the calibration laboratories.

The DKD Guidelines should not impede the further development of calibration procedures and processes. Deviations from the Guidelines as well as new procedures are allowed if there are technical reasons to support this action.

This expert report has been approved by the DKD Managing Board and was drawn up by the following authors:

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REPORT
Accompanying DKD-guideline DKD-R 8-1
„Calibration of piston-operated pipettes with air cushions"

Analysis of influencing parameters on calibration of piston-operated pipettes with air cushions

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Abstract

For illustrating and supplementing Guideline DKD–R 8-1the effect of the major influencing quantities on measurement uncertainty of piston-operated pipettes with air cushion is explained.
The thermodynamic description of the pipetting process enables to identify three system- related influencing factors: 1) temperature differences between test liquid, air and pipette, 2) external air moisture and 3) air pressure.
Air pressure dependence and the respective sensitivity coefficient are described by formulae.
Empirically determined values are given for the sensitivity coefficients in 1) and 2).

Furthermore instrumental and system-related effects such as angle of inclination, waiting period, pipetting frequency, immersion depth, operational force, reproducibility of piston stroke, counter hysteresis and heat transfer of hand warming are examined. The respective influences are assessed quantitatively.
Additional contributions are the standard deviation of the repeatability and the uncertainty due to gravimetry. A model equation for measurement uncertainty calculation is developed for the identified influencing quantities.

1. Functional principle of piston-operated pipettes with air cushion

Piston-operated pipettes with air cushion are constructed according to ISO 8655-part 2 and therein are addressed separately from positive-displacement piston-operated pipettes.

With an air cushion approximating zero, the positive-displacement piston-operated pipette can be seen as a marginal case.

The system-influencing parameters addressed below (chapter 2) impact the air cushion both directly and indirectly. Therefore these phenomena do not exist with positive-displacement piston-operated pipettes, as such the calculations of measurement uncertainty for pipettes with air cushion differ from positive-displacement pipettes in essential areas.

Annotation:
The expression "piston-operated pipette with air cushion" is replaced by „pipettes" in the following report.

1.1 Functional Description

The piston, wiper-sealed to the shaft, is first put in the lower position (1st stop). The pipette tip is immersed into the test liquid and begins to take up test liquid while the piston slides upwards until reaching its upper position.
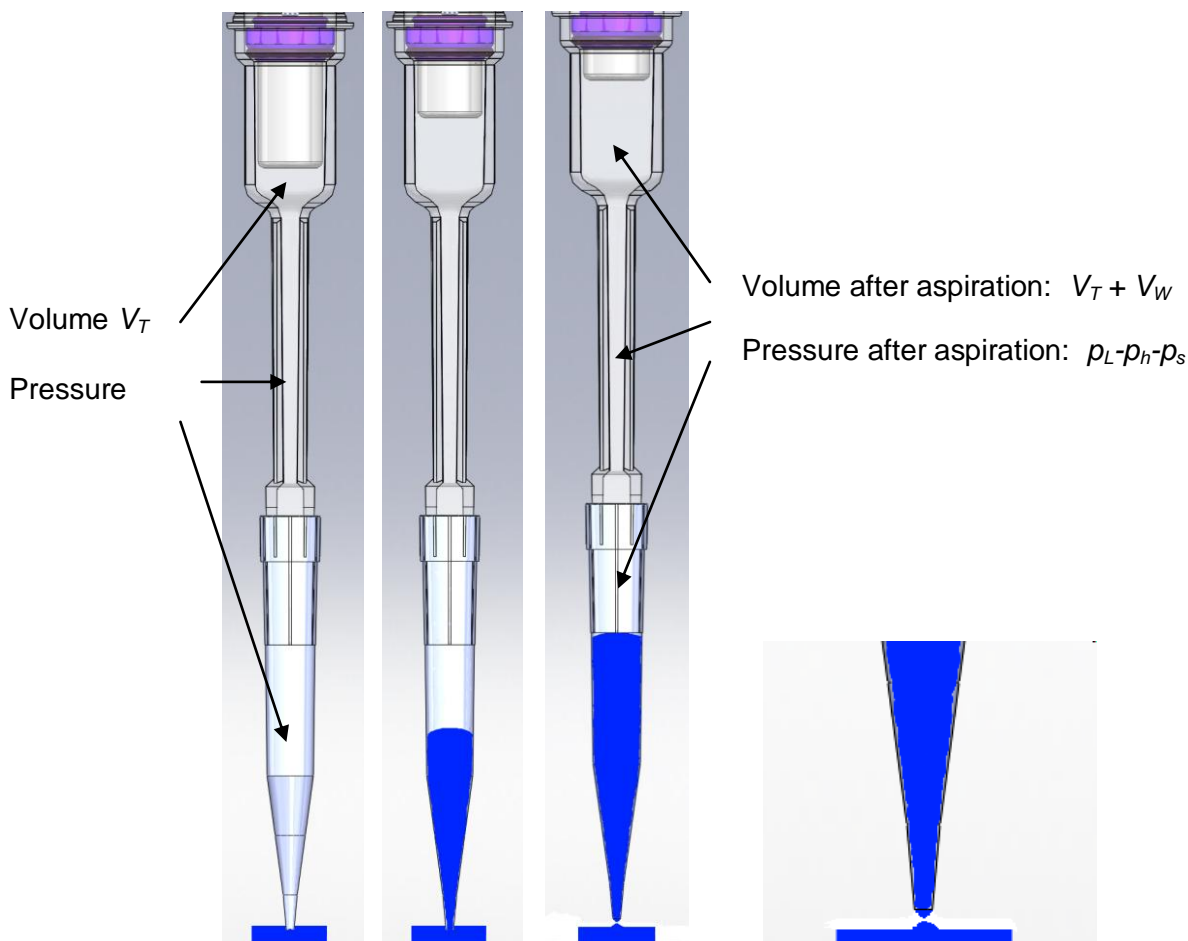

Volume V_T

Pressure

Volume after aspiration: $V_T + V_W$

Pressure after aspiration: p_L-p_h-p_s

Figure 1: Aspiration of liquid in the pipette

After aspirating the test liquid and observing a waiting period of 1 sec for small and medium-sized pipettes and about 3 sec for larger-sized pipettes (as seen in table 2, chapter 4.4), the pipette tip has to be removed from test liquid slowly without touching the tube inner wall and without jolting. The liquid surface has to be planar where the tip emerges.

The volume is defined when the liquid column is detached from the liquid source. Only evaporation at the test liquid's surface around the pipette tip orifice and incomplete dispensing of the test liquid could result in a decrease of dispensed volume. Residual test liquid in tips always has to be assessed as artifact when using water as the calibration test liquid. This is also true for droplets at the tip's external surface. Occurrence of these artifacts requires a change of pipette tip and/ or use of fresh water.

1.2. Thermodynamic Approach

Aspiration of liquid is a dynamic procedure. Events in air cushion (without disturbing influences) can be approximately described as isothermal change of state of ideal gas. In reality a polytropic (ranging between isothermal and adiabatic) change of state of real gases can be observed. This approximation describing the contributions to measurement uncertainty can undoubtedly be justified.

The following is an examination of the process from the tip's immersion into, and subsequent emersion out of the liquid.

Immediately before immersion, barometric pressure p_L exists in the air cushion V_T. Disruption of the test liquid column at emersion pressure in the air cushion is reduced by the hydrostatic pressure of the test liquid column p_W and the internal pressure of meniscus p_S. Internal pressure is directed downwards when polyproylene-tips and water are used for testing.

The air cushion V_T is enlarged by partial vacuum by V_W. V describes the actual volume of test liquid in the tip. So it is less than the piston lift volume V_{Hub} by V_W. That means $V= (V_{Hub}-V_W)$ resp. $V_W= (V_{Hub}-V)$.

$$p_L \cdot V_T = (p_L - p_h - p_S) \cdot (V_T + V_{Hub} - V).$$ (1)

Hydrostatic water pressure p_h is calculated as a product of test liquid density ρ_W, gravitational acceleration g and test liquid lift in the tip h_W:

$$p_h = \rho_W \cdot g \cdot h_W.$$

Symbols placed in (1):

$$p_L \cdot V_T = (p_L - \rho_W \cdot g \cdot h_W - p_S) \cdot (V_T + V_{Hub} - V).$$ (2)

Formula (2) enables the calculation of effects resulting from changes in barometric pressure (see chapter 2.3.), angle of inclination (4.1.) during aspiration, and changes

in test liquid density and gravity, etc..Therefore equation (2) has to be resolved to actual pipette volume V, which leads to:

$$V = V_{Hub} - V_T \cdot \frac{\rho_W \cdot g \cdot h_W + p_S}{p_L - \rho_W \cdot g \cdot h_W - p_S} = V_{Hub} - V_W .$$ (3)

To use this term for calculation, test liquid lift h_W in the tip and volume of air cushion have to be measured. Formula (3) shows, that piston volume V_{Hub} is slightly higher than the actual pipette volume V.
The difference V_W equals air cushion expansion, created by hydrostatic and internal pressure of the test liquid.

A number of important interferences can be included by using additional summands. Having that term ([2] and [3], slightly modified), physical operations during use of a pipette with air cushion can be shown mathematically:

$$V = V_{Hub} - V_T \cdot \frac{\rho_W \cdot g \cdot h_W + p_S}{p_L - \rho_W \cdot g \cdot h_W - p_S} + A_{fl} \cdot p_d \cdot (1 - S) \cdot k \cdot t_{AnsW} + V_{eff} \cdot \frac{\Delta T}{T} .$$ (4)

1) Piston lift volume   2) Expansion of air cushion   3) Evaporation inside tip   4) Temperature effects

A_{fl}	Contact area of test liquid versus air cushion
p_d	Vapor pressure of test liquid
S	Vapor saturation of air cushion (% rel. humidity / 100%)
t_{AnsW}	Aspiration time plus waiting period
k	Evaporation factor (Volume per area, vapor pressure and time)
V_{eff}	Effective part of air cushion
$\Delta T/T$	Relative temperature change in the air cushion

It can be noticed that in the third and fourth summand V_T does not appear directly. The third summand describes effects of evaporation into the air cushion during aspiration. Evaporated test liquid increases the air cushion's volume V_T and displaces test liquid downwards out of the tip (chapter 2.2.). When using water as the test liquid one nanoliter evaporated water becomes 1300 nanoliters of vapor (under normal conditions), that means 1300 times more volume.

The fourth summand describes effects of temperature changes in the air cushion during aspiration. Parts of this air cushion V_{eff} undergo a relative temperature change $\Delta T/T$, which leads to a volume change and consequently a change of test liquid volume (chapter 2.1.).
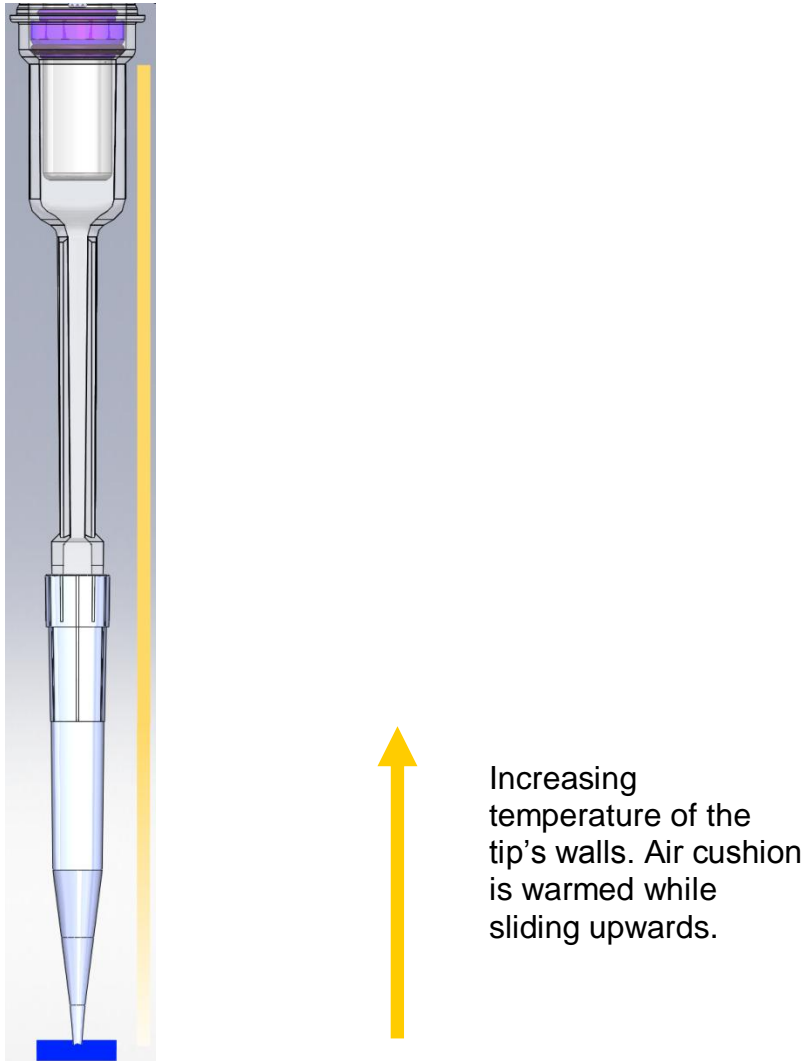
Some of these variables (S, k, V_{eff}, T) are not known exactly, and can only be calculated by approximation. So these effects have to be determined experimentally.

2. System Effects

Effects resulting from that thermodynamic approach are referred to as system effects, such as: temperature differences, humidity and pre-moistening of the air cushion, as well as barometric pressure.

2.1. Temperature Differences

Figure 2: Pipetting of cold liquid

Experiments show that the absolute temperature level has only small effects on volume (see chapter 2.4.). Whereas large effects resulting from temperature differences on volume can be observed.
If air temperature diverges from a common temperature of pipette and liquid, the effect still is relatively small. However the effect increases up to 3-fold, when the pipette's temperature diverges from a common temperature of water and air. An analogical effect – and more easily proven via experimentation – can be observed if water's temperature differs from the common temperature of air and pipette (q.v. [2]).

The latter case can be explained in detail as follows (see fig. 2):
In the case of a test liquid being colder than the common temperature of pipette and air, the tip cools down in the test liquid-contacting area during pipetting or when the

air cushion is pre-moistened.

To aspirate liquid, first the piston is put in the lower position, followed by immersion of the tip. The temperature of air-caught in the already cooled down part of the pipette tip- starts to decrease. While filling the tip with test liquid, the cooled-down air is shifted into the upper part of the pipette tip, this upper part maintaining its first temperature, rewarming the air again. Even a minimum increase of air temperature leads to a remarkable change in air cushion volume. Accordingly the actual pipette volume is decreased by the amount of air cushion expansion.
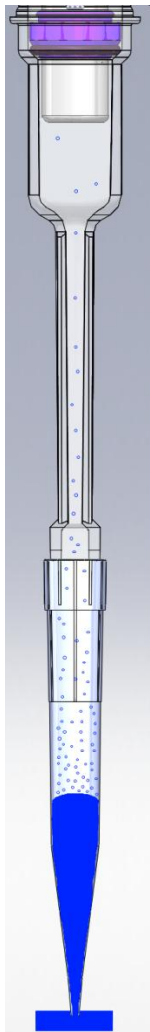
Annotation: According to [2], working with tips not pre-rinsed in the circumstances described above results in a volume increase at first aspiration, because air cushion contracts when contacting cooler test liquid. Thus aspiration of higher volumes of test liquid occurs.

Measurements have shown that pipettes with nominal volumes of about 1000µl and 100µl have sensitivity coefficients c_{TDif} of approx. 0.22% change of accuracy due to 1 K temperature difference between water and pipette/air. For pipettes with a nominal volume of 10µl, 0.19% per 1 K applies. Other nominal volumes have different coefficients, but the deviation is small.

Additionally, exchange of heat from the operator's hand can lead to temperature differences on the inside of a pipette, always attended by a decrease of volume. The extent depends on design, temperature, heat transfer and size of human hand. Effects of hand warming typically show measurement tendencies toward lower values and / or a decrease of nominal volume compared to aliquots (partial volumes). Occurrence of the hand warming effect has to be considered in measurement uncertainty.

Temperature effects behave approximately proportional to the aspirated volume in aliquot ranges. The smaller the volume of air that is moved inside the pipet's shaft, the smaller the effect on volume.

2.2. Air moisture, Pre-moistening of the air cushion

During aspiration, liquid evaporates at the test liquid's surface into the air cushion.

Tiny evaporated amounts of liquid result in enormous volume changes (see chapter 1.2.).

The originating vapor displaces test liquid during the aspiration and the waiting period and causes a volume decrease.

The higher the vapor saturation in the air cushion, the smaller the amount of liquid that evaporates during aspiration.

After delivery of test liquid, the piston slides back to its start position, ready to start another pipetting process. Sliding back, external air gets into the pipette and mingles with air of the cushion. So relative humidity in the air cushion is strongly dependent on the moisture of the ambient air, which means: high ambient air moisture results in higher volumes and vice versa.

The latest studies indicate that pipettes with nominal volumes of 1000 µl and 100 µl have sensitivity coefficients $c\phi$ of about 0.07% in accuracy of the aspirated volume per 10%rH change of moisture. Values of 0.1% accuracy per 10%rH a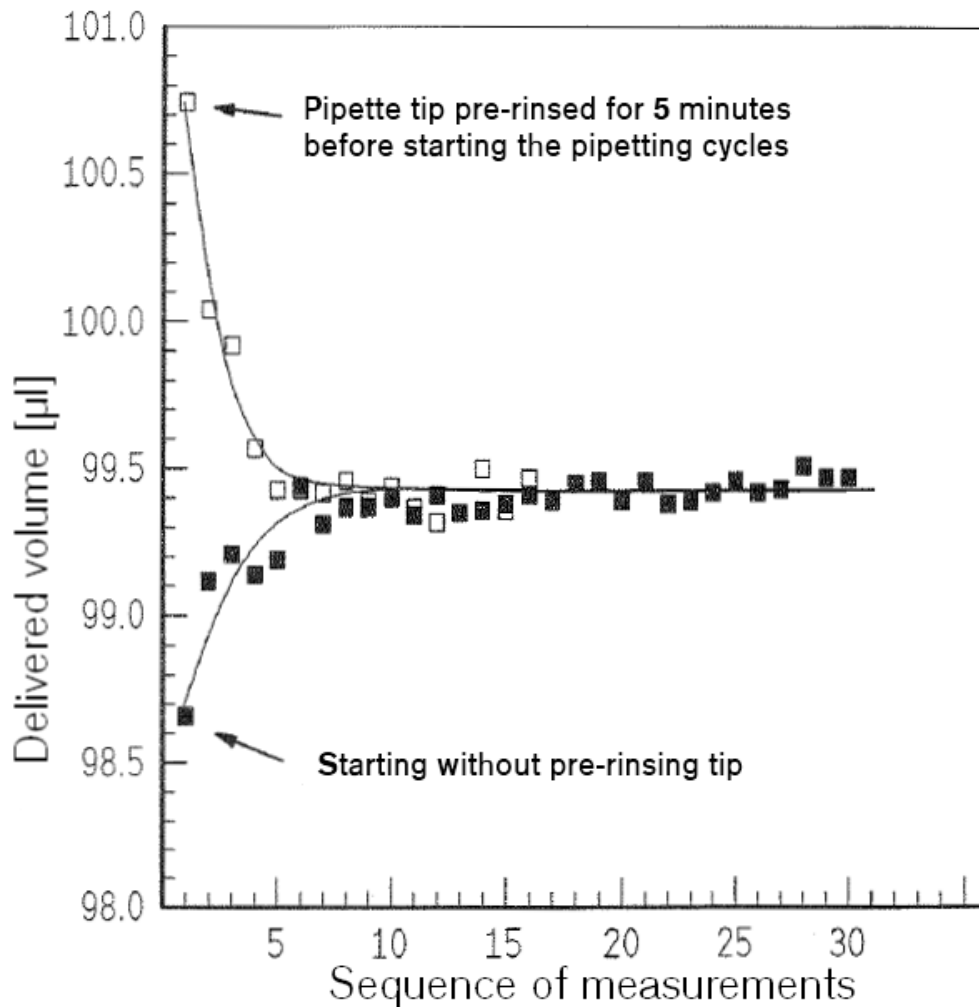pply to pipettes with nominal volumes of 10µl. Consequently a large effect for tips not pre-rinsed can be expected. First the delivered volume is considerably low and then rises during the next 4 – 5 pipetting cycles to reach a final value at thermodynamic equilibrium. Thus every new pipette tip has to be pre-rinsed 4 – 5 times before starting the pipetting process, always discarding the aspirated volume.

Figure 3: Liquid evaporating during aspiration

Annotation: The frequently-used term "prewetting" is not applicable, because there is no wetting of the tip's surface. However, humidity of the air cushion is increased during pipetting cycles.

If a pipette is stored with a filled tip for an extended period of time to reach vapor saturation in the air cushion, the dispensed volume of the following pipetting cycle is very high, decreasing rapidly during the consequent 4 – 5 cycles down to a mean volume corresponding to thermodynamic equilibrium. The drift behavior of measured values is taken from [2]:

Figure 4:

Effect of humidity on delivered volume using the example of two pipetting sequences without changing tip. With non pre-rinsed tips, the delivered volumes increase. Starting with an extremely long pre-rinsed pipette tip delivered volumes decrease down to a common equilibration value. Room temperature T: 20.2 °C, water temperature: 19.5 °C, pipette's nominal volume: 100 µl.

Annotations about changing of pipette tips are made in section 3.

2.3. Barometric Pressure

Lower barometric pressure causes higher compressibility of air cushion. To generate a partial vacuum holding the liquid column in the pipette tip, air cushion has to be expanded even more at lower external pressure. As a result, smaller volumes are aspirated.

This effect can be calculated by formula (3). The liquid's internal pressure was neglected against the hydrostatic pressure herein. The volume difference ΔV results when changing from atmospheric pressure $p_{L,X1}$ at location *X1 to* atmospheric pressure $p_{L,X2}$ at location *X2*:

$$\Delta V \approx -V_T \cdot \rho_W \cdot g \cdot h_W \cdot \left(\frac{1}{p_{L,X2} - \rho_W \cdot g \cdot h_W} - \frac{1}{p_{L,X1} - \rho_W \cdot g \cdot h_W} \right) \qquad (5)$$

Based on (5) sensitivity coefficient c_{pL} is calculated by differentiation:

$$\frac{\partial \Delta V}{\partial p_{L,X2}} \cdot dp_L = V_T \cdot \rho_W \cdot g \cdot h_W \cdot \left(\frac{1}{p_{L,X2} - \rho_W \cdot g \cdot h_W} \right)^2 \cdot dp_L \approx V_T \cdot \frac{\rho_W \cdot g \cdot h_W}{p_{L,X2}} \cdot \frac{dp_L}{p_{L,X2}} = c_{pL} \cdot dp_L \quad (6)$$

Because hydrostatic pressure is a small fraction of atmospheric pressure, it could be neglected in the nominator of the equation.
From this it can be seen that uncertainty is given as a product of three terms: air cushion volume, ratio of hydrostatic pressure and atmospheric pressure, and rel. pressure change.

As an example a pipette with nominal volume of 1000µl (liquid column of 50 mm) at an atmospheric pressure of 1008 hPa and an air cushion of 2700µl shows a sensitivity coefficient c_{pL} of 0.014 µl/hPa at its nominal volume.
For a pipette with nominal volume of 100µl, assuming typical design data, a sensitivity coefficient of c_{pL}=0.0012 µl/hPa applies, whereas for a pipette with nominal volume 10µl c_{pL}=0.0003 µl/hPa is correct.

Being dependent on atmospheric pressure means calibration location, relative to sea level, influences results, which can be recognized directly in term (6), for atmospheric pressure changes significantly with altitude. For more detailed information see [6]. Test liquid lift in the tip h_W was considered to be approximately unrelated to atmospheric pressure, respectively altitude, in formula (6). Dependency is far too small and therefore is negligible for purposes of uncertainty estimation.

Via (6), correction of measured volumes to standard pressure (e.g. 1013 hPa, at sea level) could be achieved in the calibration certificate. But there are several reasons to suggest a correction for altitude not be applied:

Even correction is accounted by a defined uncertainty, leading to higher measurement uncertainty.
Furthermore, most users' laboratories are located in similar or comparable altitude to their calibration laboratory, impairing comparability when altitude correction has been implemented.
In the end, altitude correction would be a disadvantage for all laboratory sites not being located on sea level.

Meteorological fluctuation of atmospheric pressure should be considered, too, because contribution to measurement uncertainty isn't large. Not considering this contribution would mean generating a calibration certificate only valid for one pressure and would impede mutual comparability of different certificates.

Computational correction of altitude should be made by user and not be included in the calibration certificate, e.g. for interlaboratory comparisons.

Electronic pipettes with an option for altitude correction should be corrected to the altitude of the calibration lab. The set correction value during calibration shall be stated on the calibration certificate.

If no specific data on liquid lift and air cushion exist, typically for pipettes that are customary in the market the following maximal values for air cushion size and liquid lift in the pipette tip can be assumed to calculate measurement uncertainty contribution associated with the meteorological air pressure fluctuation:

Nominal volume /μl	Liquid lift in the tip /mm	Air cushion /μl
>5 000 - 10 000	150	20000
> 1 000 - 5 000	130	10000
> 300 - 1 000	55	3000
>100 - 300	45	1000
> 10 - 100	30	500
1 - 10	19	100

Table 1: Maximal values for liquid lift and air cushion

For these are typical maximal values, a check for the individual instrument shall be done whether the use of the stated values is justified or not.

The volume change which results from calibrations referring to different altitudes and respective air pressures, cannot be calculated exactly enough using these maximal values. The values in table 1 should be used to calculate the maximal contribution to measurement uncertainty due to changes of air pressure.

2.4. Absolute Temperature Level

If temperatures of pipette and water are equilibrated to ambient air temperature, an effect on measurement results at different absolute temperature levels have not been proven yet by experimentation.

Thermal expansion of piston and the distance between piston stops lead to volume increase. Due to different pipette types, materials and designs it is not possible to give a general value to this increase.
In case of a polymer piston combined to a polymer piston stroke mechanism typically a cubic expansion coefficient for the volume displaced by the piston of approximately $150 \cdot 10^{-6} K^{-1}$ is received (combining expansion of piston cross section and increasing distance between piston stops). That equates to a volume change of 0.15% per 10 K temperature change.

Changing temperature level also changes the volume of air cushion V_T, because of thermal expansion of pipette tip and shaft. At higher temperatures the increased air cushion shows a higher compressibility and a decrease of the aspirated volume is entailed.
However at higher temperatures, test liquid lift in the tip declines due to a higher internal tip volume, causing a rise of aspirated volume.

Additionally the test liquid's density gets smaller at higher temperatures, also leading to higher volume.

The effect of temperature on vapor pressure and on diffusion constant of the test liquid is complex to determine quantitatively. The diffusion constant describes the speed of vapor propagation in the air cushion. Higher vapor pressure usually effects a volume decrease (alcohols, [2]) at higher temperature, because during aspiration time and waiting period more test liquid is evaporating.
Currently a general proposition as to which effect dominates cannot be made because of uncertain quantification of vapor pressure and diffusion constant effects.

3. Exchange of Pipette Tip during measurement

In accordance to ISO 8655, part 6, exchange of the pipette tip is required after each pipetting cycle. Each newly mounted tip has to be pre-rinsed once before use.
This was accounted by the fact that a disposable pipette tip normally is exchanged by the user. Additionally, after longer pipetting sequences, droplets could appear on tip's surface, forcing a change.

Exchange of pipette tips always entails a disturbance of moisture level inside the pipette. Without a change, a pipette system is more stable. High quality pipette tips do not show any residuals of test liquid even after a series of pipetting cycles. Having a sequence of 10 pipetting cycles it's not necessary to change them.

Working without a pipette tip replacement has major advantages regarding ergonomics and reduction of measurement uncertainties. Also applying tip exchange after each pipetting cycle does not represent the common procedure in laboratory daily routine. Hence, there is good reason to calibrate without change of tips.

First, the tip has to be pre-rinsed by aspiration and dispensing test liquid 4-5 times, and then the calibration process can be started. As soon as residues, droplets or other disturbances occur in the tip, a change becomes necessary and the new tip shall be prepared for calibration by 4-5 cycles of pre-rinsing.

4. Effects by handling and instrument

4.1. Angle of Inclination

If a pipette is not handled vertically during aspiration, a higher volume is pipetted, because hydrostatic pressure of a liquid column is proportional to the cosine of the angle of inclination. The maximum modification off vertical line should be 10°.

4.2. Waiting Period

Calibration process -according to ISO 8655 defines a waiting period of 1 - 2 seconds. During the time after aspiration the pipetting system shall not be moved from the test liquid. Small-sized pipettes need 1 second, pipettes with nominal volumes above 1000μl need a longer waiting period of about 3 seconds to make sure aspiration is completed. This deviates from ISO 8655. At longer waiting periods evaporation into the air cushion causes volume decrease. Therefore the waiting period must be observed in a most exact way.
Deviation of defined waiting period increases the value of uncertainty. A deviation of 0.5 seconds can be defined to be realistic.

4.3. Pipetting Frequency

Beside unintended fluctuation in waiting periods, time between delivery and subsequent aspiration of test liquid can vary. If a delay occurs internal higher humidity will dissipate outside and decrease in the air cushion. Decreased internal humidity leads to volume decrease in the subsequent pipetting cycle.

4.4. Immersion Depth

The volume finally remaining in the pipet tip is defined in the moment of the disruption of the liquid column (chapter 1.1.). At that time the tip's orifice is located even a bit above the liquid surface. Therefore immersion depth doesn't influence the volume directly, however, effects exist that have an indirect impact. The resulting effect remains significantly below 0.02% in accuracy per millimeter of immersion depth deviation. Partial volumes and small pipette sizes can show remarkably higher values.

Depending on the nominal volume the defined immersion depths must be observed.

Volume region	Immersion depth / mm	Waiting period / s
0,1 μl – 1 μl	1 – 2	1
> 1 μl – 100 μl	2 – 3	1
> 100μl – 1000 μl	2 – 4	1
> 1000 μl	3 – 6	3

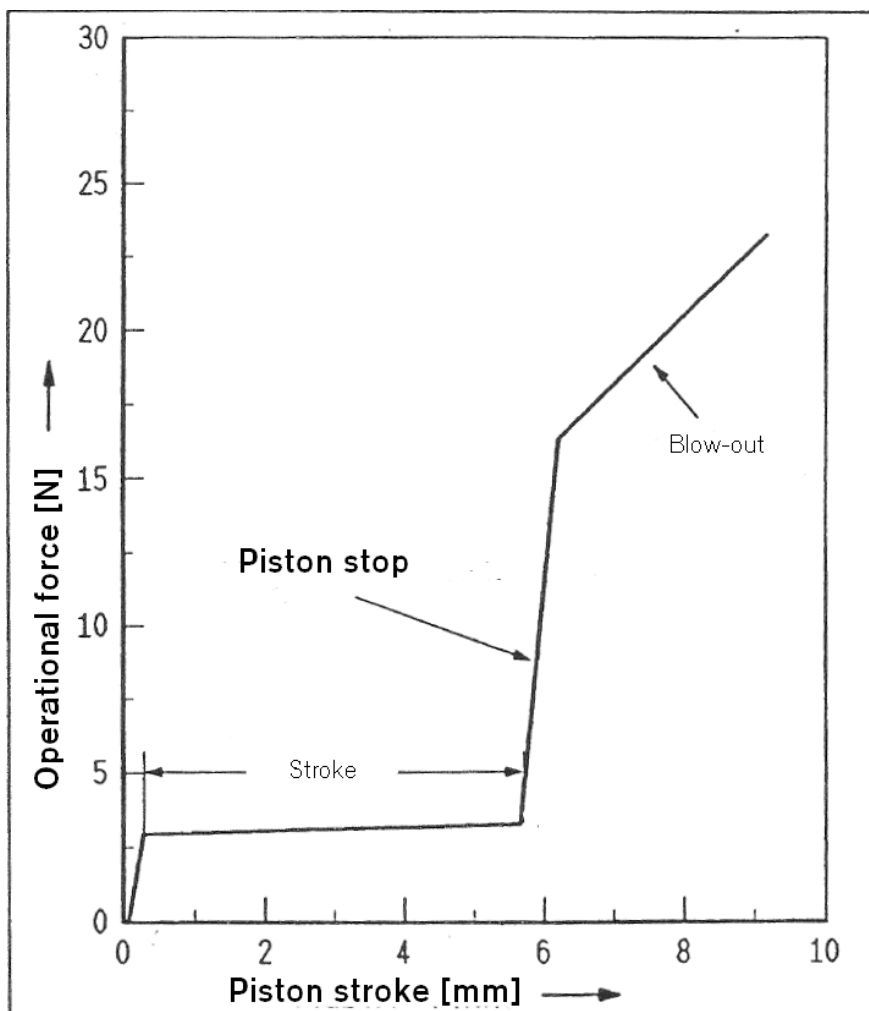**Table 2:** Immersion depth and waiting period

4.5. Operational Force

A user's operational force to achieve the first stop is subject to fluctuations. Depending on pipette design this results in greater or smaller change of piston stroke.
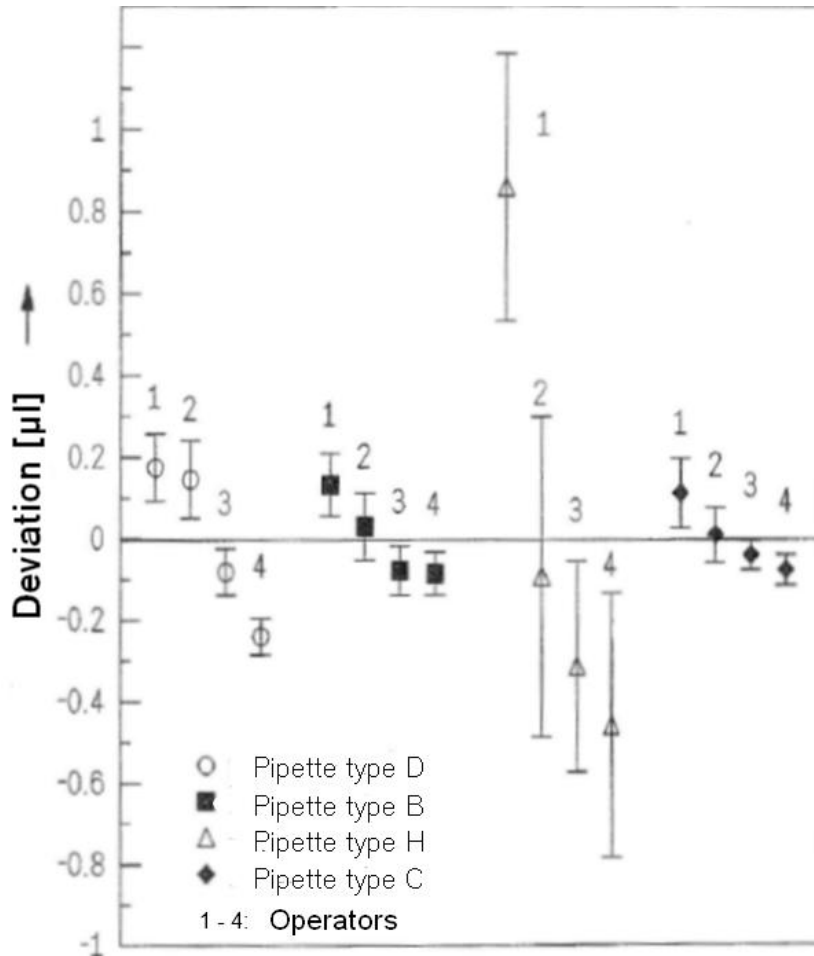
Differences occur for different users (see [2] and [3]), as well as for the same user pipetting at completely different times. Even within 10 individual measurements fluctuation of operational force exists, influencing the standard deviation of random error (chapter 5).

Precision of stroke stops with regard to force dependency is specific to pipettes (see [2] and [3]). It has been experienced that well-trained employees reach more even results (lower standard deviation of random error) than untrained employees.

The following graph (taken of [2]) shows typical force-to-path dependence of pipettes.

Figure 5: Typical force-to-path dependence of a 200μl pipette

Figure 6 taken of [2]:
Volume deviation caused by the variation of operational force from person to person. Results of 4 operators using 4 pipette types of 200 µl nominal volume are shown including the respective scattering range. The operational forces effected by the operators decreased in the sequence from operator 1 to operator 4.

Electronic pipettes are not influenced by operational forces.

Collectively standard uncertainty at a minimum of 0.014% of nominal volume has to be assumed.

4.6. Reproducibility of Piston Stroke

Beyond operational force, the piston stroke possesses a mechanically caused uncertainty. Reproducibility of 0.02% nominal volume can be assumed as standard uncertainty of the accuracy.
Even electronic pipettes show mechanically caused fluctuation of reproducibility regarding piston stroke. Up to now, no values can be mentioned to document a more precise stroke control.
The value of reproducibility is not marginal. But it should be kept in mind that we talk about a stroke deviation of about 2 – 4 µm, which already can be caused by minor interferences.

4.7. Counter Hysteresis

The piston stroke depends on from which direction a value is set on the counter at variable mechanical pipettes. Mostly, effects are rather small but -using some pipette types- can increase up to higher values. Effect is eliminated by routinely setting value from only one direction, but nevertheless variable volume pipettes show a counter setting error compared to fixed pipettes.

4.8 Heat Transfer of Hand Warming

As another influencing factor on accuracy hand warming has to be considered. It is individually dependent on user and pipette type design.
At nominal volume hand warming effects are biggest due to the position of the piston inside of the pipette. To minimize the factor „hand warming", contact intensity and time of system and user should be kept as low as possible. Hand warming leads to a decrease of volume (drifting time-dependently). If hand warming effects are appearing in a calibration their extent has to be assessed and considered in uncertainty calculations.

4.9 Balances of Handling and Instrumental Effects

Summarizing influencing factors of 4.1 to 4.8 a standard measurement uncertainty of about 0.04% (for fixed pipettes) to 0.06% (for variable pipettes) referring to nominal volume is calculated.

5. Standard Deviation of the Repeatability

The random error of 10 individual measurements s, defined in [1] yields a standard uncertainty for the mean volume of $u_{Wdh} = \dfrac{s}{\sqrt{10}}$.

The standard deviation of the random error s can be estimated applying a fraction of the manufacturer's random error tolerance (CV, coefficient of variation). It has been experienced that measured CV value on calibration usually ranges between 1/6 and 1/3 of this tolerance.

$$s \approx \left(\frac{1}{6} \text{ up to } \frac{1}{3} \right) \cdot CV .$$

This resulting s is in percent and refers to the mean volume of the measurement.

Usually multichannel pipettes have higher CV tolerances, a fact which results in a higher contribution to uncertainty in comparison to single channel pipettes.

6. Contribution due to Gravimetry

The gravimetric effect to uncertainty is calculated according to ISO TR 20 461 (see [4]).
Additionally evaporation out of weighing vessel is considered. An estimation for pipettes with nominal volume of 1000 µl is 20 µg. 15 µg apply for a nominal volume of 100 µl and 10 µg for pipettes with nominal volumes of 10 µl.

The gravimetric contribution together with the evaporation loss results in an uncertainty of about u_{grav}= 0.0035% in volume ranges of 1000µl. This represents only a small fraction of influences mentioned in chapters 2 and 4 and therefore is non-dominant.

7. Model Equation for Measurement Uncertainty Calculation

To calculate volume V_{20} referring to the reference temperature T_{M20} the following model equation is developed (7):

$$V_{20} = \frac{(W_1 - W_0)}{\rho_W - \rho_L} \cdot \left(1 - \frac{\rho_L}{\rho_G}\right) \cdot (1 - \gamma \cdot (T_M - T_{M20})) + \Delta V_{Verd} + \Delta V_{Wdh} + \Delta V_{TDif} + \Delta V_{\Phi} + \Delta V_{pL} + \Delta V_{Hand}$$

The first and second summand (ΔV_{Verd}: volume loss due to evaporation) result from the gravimetric procedure (chapter 6), see [4]. The further summands are based on the above mentioned effects in chapters 2, 4 and 5:

ΔV_{Wdh}: Volume error caused by the standard deviation of the repeatability
ΔV_{TDif} : Volume error caused by temperature differences
ΔV_{Φ}:   Volume error caused by air moisture
ΔV_{pL}:   Volume error caused by atmospheric pressure
ΔV_{Hand}: Volume error caused by handling and instrument effects
γ: Volume dependency γ consists of all components described in chapter 2.4.

Setting the reference temperature to the common temperature of test liquid/pipette/air, the bracketed term containing γ is eliminated.

For simplicity reasons gravimetric uncertainty described in [4] is technically summarized as ΔV_{Grav}.

$$V_{20} = V_S + \Delta V_{Grav} + \Delta V_{Wdh} + \Delta V_{TDif} + \Delta V_{\Phi} + \Delta V_{pL} + \Delta V_{Hand} \tag{8}$$

V_S is the selected volume.

8. Calculation of Measurement Uncertainty

Based on equation (8) according to rules of error propagation, measurement uncertainty for nominal volumes can be calculated.

For the squared combined standard uncertainty u^2 the following expression is valid:

$$u^2 = (V_S \cdot u_{Grav})^2 + (V_S \cdot u_{Wdh})^2 + $$
$$+ (c_{TDif} \cdot V_S \cdot u_{TDif})^2 + (c_\Phi \cdot V_S \cdot u_\phi(\Phi))^2 + (c_{PL} \cdot V_S \cdot u_{pL}(p_L))^2 + (V_S \cdot u_{Hand})^2$$

Annotation: The standard uncertainties and sensitivity coefficients were given in percentage of nominal volumes. So values always had to be multiplied by nominal volume V_S

Explanation of symbols:

u: Combined standard uncertainty
u_{Grav}: Standard uncertainty gravimetric procedure
u_{Wdh}: Standard uncertainty of mean volume
u_{TDif}: Standard uncertainty temperature difference water-pipette/air
u_Φ: Standard uncertainty relative air moisture
u_{pL}: Standard uncertainty atmospheric pressure fluctuation
u_{Hand}: Standard uncertainty handling and instrumental effects

9. Uncertainty of Aliquot Volumes (Scaling)

There are influencing parameters which are independent of volume and have an absolute value (such as: error of piston stroke), other parameters are approximately proportional to the selected volume (e. g. all temperature effects and parameters directly depending on the liquid column).
Parameters, related to humidity, are somewhere in between.

A multiplicity of experiments show that the combined uncertainty at 50% of nominal volume, is 75% and at 10% of nominal volume, half of magnitude applying for nominal volume.

10. Formal Declaration of Uncertainty

To describe properly the dependence of measuring uncertainty over the whole range of pipettes' nominal volumes it is recommended to state uncertainty in percent of accuracy referring to nominal volume. A reasonable partitioning into volume ranges should be made.

The reason is that certain ranges are covered by a very similar uncertainty statement in percent. Example: In Appendix A in [5] 0.12% uncertainty is stated for a pipette with fixed volume 1000 µl. The budget for a pipette with 100 µl fixed volume would yield nearly the same value in percent.

11. Summary

Operation of piston-operated pipettes with air cushions was described as a thermodynamic system by a mathematical formula.
The following system-related influencing parameters could be analyzed:

Temperature differences of test liquid, pipette and air
- External humidity and internal moisture of the air cushion
- Barometric pressure and associated altitude

Further influencing parameters - due to user and/or instrument- are:

- Angle of inclination
- Waiting period
- Pace of pipetting cycles
- Immersion depth
- Operational force (not existent at electronic pipettes)
- Reproducibility of piston stroke
- Counter hysteresis (also not existent at electronic pipettes and fixed volume pipettes)
- Hand warming effect

In addition, the standard deviation of the mean volume due to standard deviation of repeatability has to be considered.

Measurement uncertainties, resulting from gravimetric calibration, are essentially smaller than aforementioned interferences.

For calculation of uncertainty, a model equation was developed to quantify impact of different parameters.

For handling and instrumental effects experiment-based estimates were made and an overall value was determined.

Detected uncertainties support model budgets, written in the DKD-guideline DKD-R 8-1 [5] well. The outcome of this is a necessity to define and monitor ambient conditions precisely and keep tight limits for them.

Calculations are predicated on even held tight limits of ambient conditions. So in DKD-guideline's R 8-1 model budget [5] named resulting uncertainties cannot be lower assuming regular working conditions.

12. References

[1] Volumenmessgeräte mit Hubkolben – Part 2: Kolbenhubpipetten (ISO 8655-2:2002), Volumenmessgeräte mit Hubkolben – Part 6: Gravimetrische Prüfverfahren zur Bestimmung der Messabweichung (ISO 8655-6:2002)

[2] K. H. LOCHNER: Abschlussbericht zum Forschungsvorhaben AIF 9152: „Untersuchung der Messgenauigkeit von Kolbenhubpipetten mit Luftpolster"

[3] K. H. LOCHNER, T. BALLWEG, H.-H. FAHRENKROG: „Untersuchungen zur Messgenauigkeit von Kolbenhubpipetten mit Luftpolster". J Lab Med 20 (7/8), 430-440, 1996

[4] ISO/TR 20461 „Determination of uncertainty for volume measurements using the gravimetric method" November 2000

[5] Guideline DKD – R 8-1 „Calibration of piston-operated pipettes with air cushions" including sample budgets – 12/2011

[6] C. SPÄLTI, „Influence of altitude on the dispensed volume of a piston pipette with air cushion", Expert Report DKD-E 8.3, 12/2011

13. Acknowledgements:

14. Used Symbols:

V	Actual pipette volume
V_0	Nominal volume of pipette
V_S	Selected volume of pipette
V_{20}	Volume of pipette at reference temperature 20°C
V_T	Volume of air cushion
V_W	Volume expansion of the air cushion (via water column and internal pressure)
V_{Hub}	Displaced volume by piston stroke
W_0	Balance reading tare value
W_1	Balance reading
ρ_W	Density of test liquid
ρ_G	Density of calibration weights for scales calibration (8000 kg/m³)
γ	Temperature dependence of pipetted volume
g	Gravitational acceleration
h_W	Lift of test liquid in the pipette tip
p_L	Air pressure
$p_{L,X1}$	Air pressure at location $X1$
$p_{L,X2}$	Air pressure at location $X2$
p_d	Vapor pressure of test liquid
p_h	Hydrostatic pressure of test liquid

p_S	Internal liquid pressure due to surface tension
T_M	Temperature of pipette
T_{M20}	Reference temperature of pipette (20°C)
ϕ	Relative moisture of ambient air
$\Delta T/T$	Relative temperature change in the air cushion
A_{fl}	Contact area of test liquid and air cushion
S	Degree of moisture saturation in the air cushion (% rel. moisture/ 100%)
t_{AnsW}	Aspiration time plus waiting period
k	Evaporation factor (Volume per area, vapor pressure and time)
V_{eff}	Effective part of air cushion
ΔV_{Wdh}	Volume error caused by the standard deviation of the repeatability
ΔV_{TDif}	Volume error caused by temperature differences
ΔV_{Φ}	Volume error caused by air moisture
ΔV_{pL}	Volume error caused by atmospheric pressure
ΔV_{Hand}	Volume error caused by handling and instrumental effects
ΔV_{Verd}	Volume error caused by evaporation loss
ΔV_{Grav}	Volume error due to gravimetric procedure
s	Random error
CV	Coefficient of variation
R	Accuracy (percentage deviation of actual to theoretical volume)
c_{TDif}	Sensitivity coefficient of effects due to temperature difference
c_{ϕ}	Sensitivity coefficient of effects due to air moisture
c_{pL}	Sensitivity coefficient of effects due to barometric pressure
u	Combined standard uncertainty
u_{Grav}	Standard uncertainty gravimetric procedure
u_{Wdh}	Standard uncertainty of mean volume
u_{TDif}	Standard uncertainty temperature difference water - pipette/air
$u\phi$	Standard uncertainty relative air moisture
u_{pL}	Standard uncertainty atmospheric pressure fluctuation
u_{Hand}	Standard uncertainty handling and instrumental effects