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Influences on Volume in Piston-operated Air-displacement Pipettes

Author: Rainer Feldmann, BRAND GMBH + CO KG
Wertheim, Germany
Dr. Karl Heinz Lochner, Fraunhofer Insitute for Silicate Research
Bronnbach Branch Office, Wertheim, Germany

Abstract

Air-displacement pipettes are easy-to-use instruments for small-volume liquid transfer, but they are prone to volume errors resulting from handling, ambient conditions and liquid properties.

Analytical thermodynamic approaches were used to describe and quantify influencing factors affecting volume. Results were compared with measurements. Effects related to temperature difference between liquid and instrument by far prevail over the dependence on common temperature level of liquid, instrument and environment. An equation based on Fick's laws was developed to calculate volatilization effects in the captive air volume explaining the dependence on ambient moisture, pipetting pace, waiting period and unconditioned starting. Furthermore dependence on barometric pressure, angle of inclination, liquid density, immersion depth and surface energy in tips was calculated and/or measured. The review allows a comprehensive insight into the size of volume errors in calibration and use. These effects are typically much higher than those resulting from gravimetric measurement.

Introduction

The concept of a high-precision air-displacement pipette with disposable tips was patented for Schnitger [1]. In the seventies several publications described effects on pipette accuracy and coefficient of variation. Joyce and Tyler [2] investigated hand-warming effect. Ellis [3] measured and correctly explained volume dependence on temperature difference between water and instrument.

Zeman and Mathewson [4] assumed volatilization in the tip to be a possible reason for errors.

Curtis [5] presented a photometric method in the early nineties. In 1995 a method for calibrating low volume pipettes using an evaporation trap was described by Michel, Sommer and Spieweck [6].

In 1992 a research project named AIF 9152 was launched by „Forschungsgemeinschaft Technik und Glas" in Wertheim/Germany by K. H. Lochner published in 1995 [7] (complete report) and in 1996 [8] (major results). This comprehensive project identified the major influencing factors on volume. In a number of cases the influences could be quantified. This project is referenced in many parts of this review.

The investigations of Michel et al. and Lochner et al. gave rise to a revision of the German DIN 12650 standard, resulting in the international ISO 8655 standard for pipette calibration.

From the late nineties on, the number of accredited laboratories in this field grew worldwide, and the call for inter-laboratory comparisons arose.

In Germany details of a bilateral comparison were published [9], culminating in developing the guideline DKD-R-8 1 [10] and DKD expert reports [11], [12] and [13] to calculate measurement uncertainty in DAkkS accredited laboratories. Important work was also done by E. Batista et al. [14]. Basic knowledge on air-displacement pipettes is also found in K. Ewald's booklet [15].

The present work now specifies influences precisely by using mathematical terms. In particular, the challenging phenomena of evaporation in the captive air volume and influence of temperature differences will be treated using mathematical modeling. Calculated results will be compared to actual measurements, where possible. Timing of axial and radial heat transfer in the captive air volume, liquid and tip will be calculated. The factors contributing to dependence on common temperature level between water, instrument and ambient air are quantitatively assessed and compared to experiment, as well as influence of surface tension of the liquid and surface energy of the pipette tips.

Functional principle of piston operated air-displacement pipettes

Piston operated air-displacement pipettes are constructed according to ISO 8655 part 2. The piston is first put in its lower position (1st stop, Fig. 1). The pipette tip is immersed into the test liquid whose surface has to be planar. Any surface curvature influences the aspirated volume. The piston slides upwards to aspirate liquid until reaching its upper stop. After aspirating liquid and observing a waiting period [12], the pipette tip is removed from the test liquid slowly without touching the tube inner wall and without jolting. The volume is defined when detaching completely from the liquid source.

Only evaporation on the liquid's surface around the tip orifice and residues in the tip could result in a decrease in the dispensed relative to the aspirated volume.

Thermodynamic approach

Events in the captive air volume can be approximately described as isothermal change of state of ideal gas.

Immediately before immersion, barometric pressure p_a exists in the captive air volume V_D (Fig. 1). After aspiration the pressure in the system is reduced by the hydrostatic pressure of the liquid column p_h and the capillary pressure of meniscus p_S. Due to this pressure change the captive air volume V_D is enlarged by the piston stroke volume V_{Str} and reduced by the volume V of the aspirated liquid. V_{Str} is always a little larger than V. The difference between V_{Str} and V is denoted by ΔV_W. Following equation results:

$$p_a \cdot V_D = (p_a - p_h - p_S) \cdot (V_D + V_{Str} - V) \qquad (1)$$

Hydrostatic pressure $p_h = \rho_W \cdot g \cdot h$ is placed in Eq. (1):

$$p_a \cdot V_D = (p_a - \rho_W \cdot g \cdot h - p_S) \cdot (V_D + V_{Str} - V) \qquad (2)$$

ρ_W: liquid density, g: gravitational acceleration. Eq. (2) enables the calculation of effects resulting from changes in barometric pressure, capillary pressure, angle of inclination, changes in liquid density and gravity. Eq. (2) resolved to actual pipette volume V is:

$$V = V_{Str} - V_D \cdot \frac{\rho_W \cdot g \cdot h + p_S}{p_a - \rho_W \cdot g \cdot h - p_S} = V_{Str} - \Delta V_W \qquad (3)$$

To use this term for calculation, test liquid lift h in the tip and volume of captive air volume V_D have to be measured.

Physical operations during use of an air-displacement pipette can be mathematically included by using additional summands ΔV_{EV} and ΔV_T to contribute for evaporation effects and temperature effects in the captive air volume, respectively:

$$V = V_{Str} - \Delta V_W - \Delta V_{EV} - \Delta V_T \qquad (4)$$

Equations were derived also in [7], [8] completed by p_S.

Influences resulting from piston stroke

Pipette volume is primarily determined by stops defining the piston stroke (Fig. 2).

Measurements of stroke repeatability and reproducibility identified deviations in the micrometer range due to mechanical imperfections.

Increased force against the first stop results in slightly higher volume delivered and vice versa. Differences in volume delivery occur for different users applying different operational forces.

Collectively, a standard uncertainty at a minimum of 0.014 % of nominal volume has to be assumed to contribute for stroke dependent influences for well trained personnel. For fixed volume pipettes even a smaller amount can be justified [12]. Electronic pipettes of course are not influenced by operating forces which is undoubtedly an advantage.
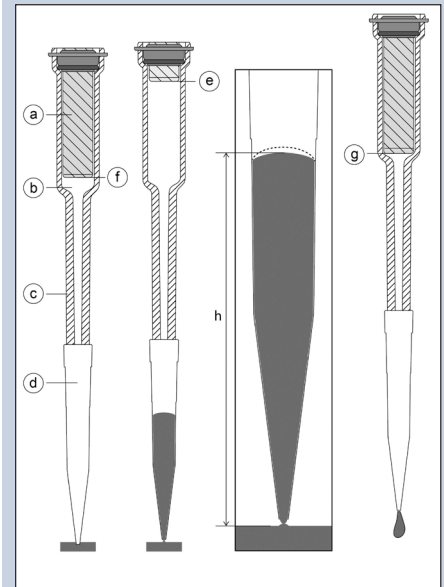
Figure 1: After immersion of pipette with tip (d) the piston (a) slides up and expands the captive air volume (b). The piston is wiper sealed to the shaft (c). The pipette takes up liquid until the piston reaches its upper stop. When lifting the pipette surface tension forms a small neck of liquid above the surface until liquid in the tip detaches completely from the recipient vessel (amplified section). h denotes the liquid lift in the tip. It is measured from the liquid surface to the meniscus vortex. Approximately the liquid lift after withdrawing the pipette can be used for h. When lifting the pipette (tip is still immersed), the meniscus is flattened (amplified section) instead of sinking while conserving its shape. (e) denotes the upper stop (rest position), (f) the 1st stop and (g) the 2nd stop (blowout position)
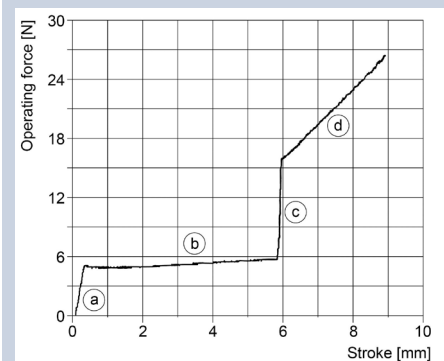
Figure 2: Typical force to path dependence of a mechanical 200-µl pipette. The rest position is denoted by (a), the pipetting stroke by (b), the 1st stop by (c) and the blow out stroke by (d). In section (c) the curve is not completely vertical causing operating force dependency. From Lochner [7] (modified)

Effects of barometric pressure, liquid density, angle of inclination and capillary pressure

Volume change resulting from the variables barometric pressure p_a, liquid density ρ_W, capillary pressure p_S, gravimetric acceleration g and liquid rise in the tip h are calculated using Eq. (3).

$$\Delta V \approx -V_D \cdot \rho_W \cdot g \cdot h \cdot \left(\frac{1}{p_{a,X2}} - \frac{1}{p_{a,X1}}\right) \tag{5}$$

The liquid rise h in the tip can approximately be regarded constant calculating the mentioned dependencies. The effect of different gravimetric accelerations g around the globe is unmeasurably small. Hydrostatic pressure and capillary pressure can be neglected against air pressure p_a in the denominator.

Eq. (5) then shows that the influence of all four mentioned factors is approximately proportional to the captive air volume V_D and the liquid rise h.

Dependence on barometric pressure

The volume dependency on the change in barometric pressure results from Eq. (5) when changing from atmospheric pressure $p_{a,X1}$ at location X1 to atmospheric pressure $p_{a,X2}$ at location X2 at constant ρ_W and small p_S

$$\Delta V \approx -V_D \cdot \rho_W \cdot g \cdot h \cdot \left(\frac{1}{p_{a,X2}} - \frac{1}{p_{a,X1}}\right) \tag{6}$$

This equation can be used to calculate the dependence on meteorological pressure changes, too.

For small volume pipettes include capillary pressure as in Eq. (5).

Adjustment for altitude in manual pipettes is correct only for a specific volume. However, electronic pipettes with two-point adjustment allow matching the whole specified volume range.

The dependence on altitude has been validated in the Bernese Alps in Switzerland by Spaelti Chr et al. [13] and also by Rodriguez G [16] on Mount Washington (NH, USA). The effect of meteorological changes in air pressure is low. It shall be considered in the uncertainty budget [10].

Influence of capillary pressure in the tip

From Eq. (5) a simple expression can be derived to approximately calculate the influence of a liquid's density:

$$\Delta V \approx -V_D \cdot \frac{g \cdot h}{p_a} \cdot \Delta \rho_W \tag{7}$$

If water is exchanged against sulphuric acid (density 1.8 g/ml) and assuming a 1000-µl pipette, the calculated volume decrease is 0.78 % at nominal volume. Yet a change in liquid entails a change in vapor pressure and surface tension, too. Therefore volume deviations different to these calculated values will be observed in reality.

Angle of inclination

If a pipette is inclined during aspiration by an angle ε to the vertical line, a higher volume is obtained. Only the projection of hydrostatic pressure to the vertical line is effective. The expression

$$\Delta V \approx V_D \cdot \frac{g \cdot \rho_W}{p_a} \cdot (h - h \cdot \cos \varepsilon) \tag{8}$$

applies in this case. Using a 1000-µl pipette at an inclination of 45 ° to the vertical line increases volume by 0.28 % at nominal volume, a value which corresponds well to the 0.27 % measured by K. H. Lochner et al. [8].

Nominal volume [µl]			10 ml	1000	100	20
	Altitude [m]	Air pressure [hPa]	Deviation [%] from nominal volume			
(1)	3500	658	-1.2	-0.53	-0.74	-1.56
(2)	2300	766	-0.74	-0.31	-0.44	-0.93
(3)	518	953	-0.15	-0.06	-0.09	-0.18
(4)	0	1013	0.00	0.00	0.00	0.00

Table 2: Calculated volume deviation depending on altitude assuming typical pipette design data using Eq. (6). (1) Jungfraujoch (Switzerland), (2) Mexico City, (3) Munich, (4) sea level

Influence of capillary pressure in the tip

Contact angle θ between liquid and tip leads to a capillary pressure p_s. Assuming spherical shape, $p_s = \frac{4 \cdot \sigma \cdot \cos\theta}{d}$ results.

σ is the surface tension of the liquid, and d the tip's internal diameter. Only in small tips, a spherical surface is developed. Tips of several millimeters in diameter show a profile flattened by gravity (Fig. 3).

The capillary pressure influence could be checked using pipette tips with reduced surface energy, showing a significantly higher contact angle to polypropylene (measured values 115 ° versus 104 °). From Eq. (5) a simple equation results to describe the capillary effect on volume:

$$\Delta V \approx -V_D \cdot \frac{\Delta p_S}{p_a} \qquad (9)$$

The effect is small for pipettes with nominal volume greater than 100 µl. 1000-µl pipettes show a maximal volume reduction of 0.1 % at nominal volume, while 1 µl pipettes show a volume reduction of 1% or more. Calculation complies well with measurements. Due to these volume deviations reduced surface energy on tips seems to be disadvantageous, but in fact these tips avoid residues in the tips, a characteristic which entails much more beneficial effects for many liquids.

Temporal development of vapor concentration in the captive air volume

This chapter discusses the summand ΔV_{EV} in equation (4). The comprehensive calculations use Fick's first law:

$$j = -D \cdot \frac{\partial c}{\partial z} \qquad (10)$$

and Fick's second law (here in one dimensional form):

$$\frac{\partial c}{\partial t} = D \cdot \frac{\partial^2 c}{\partial z^2} \qquad (11)$$

j is the diffusional flux in mol/(m²s), c is the vapor concentration in mol/m³. The captive air volume is modeled as a cylinder of volume V_D and cross section equal to the liquid surface with diameter d (Fig. 4), z runs along the tip's central axis, t is the time elapsed, D is the diffusion constant, L is the cylinder height.

This differential equation can be solved applying the Laplace transform method. For finite L, the solution is (see [17], page 41):

$$c(z,t) = c_0 + (c_S - c_0) \cdot \left[1 - \frac{4}{\pi} \cdot \sum_{n=1}^{\infty} \frac{(-1)^{n-1}}{2 \cdot n - 1} \cdot \cos\left(\frac{2 \cdot n - 1}{2} \cdot \pi \cdot \frac{z-L}{L} \right) \cdot e^{-\left(\frac{2 \cdot n - 1}{2} \cdot \pi \right)^2 \cdot \frac{D \cdot t}{L^2}} \right]$$

$$(12)$$

This solution is also exact for long time intervals but doesn't converge well at very short time intervals.

It can be seen well that even after 1 second, most of the released vapor is still located not more than 12 mm above the liquid surface (Fig. 4). Delivery and next filling of course will mix up moist and dry parts of the captive air volume.

It should be kept in mind that of course also convection forced by the movement of the liquid surface plays a role. However, close to the surface parallel flow lines exist (piston flow). Thus, in the region close to the liquid surface diffusion can be assumed to develop undisturbed.
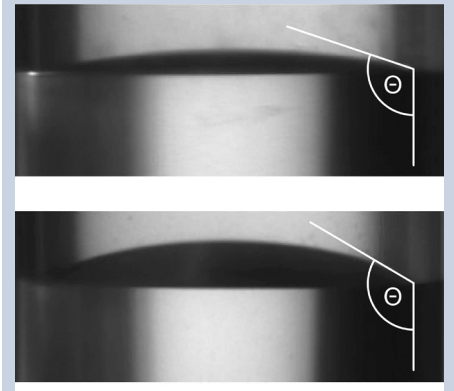
Figure 3: Shape of liquid surface in the pipette tip after aspiration and pipette lift-off from liquid. Upper: Pure polypropylene tip. Volume 1000 µl. Lower: BRAND® PP ULR tip showing a higher bulge
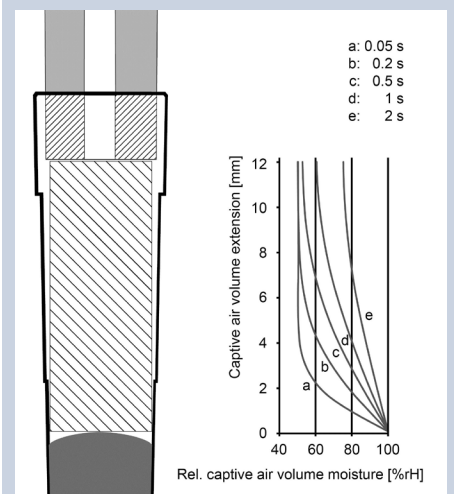
Figure 4: Calculated temporal development of captive air volume moisture concentration in a 1000 µl pipette tip during 0.05 s to 2 s after first contact between air and water. Relative humidity in the captive air volume prior to water contact: 50 %. After 2 s the concentration reaches about half of the possible moisture saturation. The hatched cylinder represents the mathematical model of the captive air volume

Evaporated volume

Multiplying the diffusional flux j in Eq.(10) by the liquid's surface area and integrating the expression over a period of time t_e, the evaporated number of particles ν (in mol) results:

$$\nu = -D \cdot A_{fl} \cdot \int_0^{te} \frac{\partial c}{\partial z} \cdot dt$$

The ideal gas law $V = \nu \cdot \frac{R_m \cdot T}{p_a}$ relates the vapor volume V_{EV} to ν. p_a is the air pressure, R_m the molar gas constant and T the temperature in K.

$$V_{EV} = \nu \cdot \frac{R_m \cdot T}{p_a} = -D \cdot A_{fl} \cdot \frac{R_m \cdot T}{p_a} \cdot \int_0^{te} \frac{\partial c(z=0)}{\partial z} \cdot dt$$

In standard pipetting mode, t_e equals the aspiration time plus waiting time: $t_A + t_{WT}$.

The concentration can be expressed using again the ideal gas law. Introducing the relative air moisture φ and the liquid's vapour pressure p_d the expression

$$V_{EV} = A_{fl} \cdot \frac{p_d}{p_a} \cdot L \cdot (1 - \varphi) \cdot \left(1 - \frac{8}{\pi^2} \sum_{n=1}^{\infty} \frac{e^{-\left(\frac{2 \cdot n - 1}{2}\pi\right)^2 \frac{D \cdot t_e}{L^2}}}{(2 \cdot n - 1)^2} \right) \quad (13)$$

The following fig. 5 shows the results for the evaporated volume in a 1000 µl tip. Moisture effects mainly depend on the liquid surface. Zero captive air volume of course suppresses evaporation completely.

Dependence of volume on contact time between liquid and captive air volume

The created vapor volume V_{EV} increases the captive air volume during aspiration and waiting time. It is worth noticing that from one nanoliter of water about 1300 nanoliter of vapor volume results by evaporation. This makes moisture effects large.

Increasing contact time between water and captive air volume will reduce the aspirated volume while the tip is still immersed. In [7] the dependence of volume delivery on immersion time was measured (Fig. 6).

The results were compared with values for V_{EV} calculated using Eq. (13) (subtracted from 20 µl) assuming typical design parameters of a 20-µl pipette. Even this simple model leads to good correspondence between calculation and measurement. Only the values at 95 % are non complying possibly due to experimental issues (measurement of moisture). This example clearly proves that moistening of the captive air volume determines the volume in the tip and not a "wetting" effect on the tip's walls. Water cannot wet polypropylene. Tip filling and delivery does not leave any detectable film on the surface. If so, water and/or tip quality are off-grade.

It is obvious that pipetting volatile liquids leads to poor accuracy and even dripping. This deleterious effect can be remedied e. g., by rinsing the tip several times to reach more vapor saturation in the captive air volume.
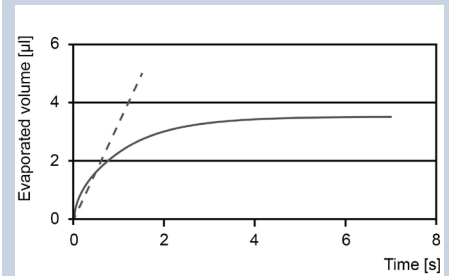
Figure 5: Evaporated volume on the liquid surface of a 1000-µl tip, filled to nominal volume. The initial moisture is 50 %. The cylindrical model was used for the captive air volume neglecting the shaft bore. Dashed line: linear model, full line: use of Eq. (13) showing a convex curvature and reaching a saturation end point. The time scale shows the sum of aspiration time and waiting period
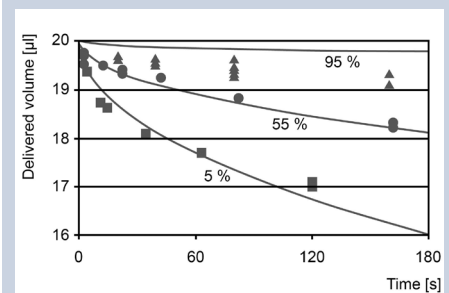

Figure 6: Dependence of delivered volume on immersion time in a 20-µl pipette. Delivered volume was measured applying three different settings of ambient moisture (5 %, 55 %, 95 %). A new, not preconditioned, tip was used for each weighing. The full lines represent calculated values. Each curve reaches a saturation endpoint. From Lochner [7] (modified)

Initial effects in sequential pipetting without tip change

Fig. 7 shows that settling to stable values takes up to 5 times of pre-rinsing the tip to achieve equilibrium, a fact which has entered into many guidelines. Pipettes with high ratio captive air volume / selected volume can require a higher cycle number.

There is an issue comparing calculated and measured results: the span in the first weighing between saturated captive air volume and captive air volume at ambient moisture is much larger in measured values. Assumingly this behaviour can be explained by turbulence in the air.
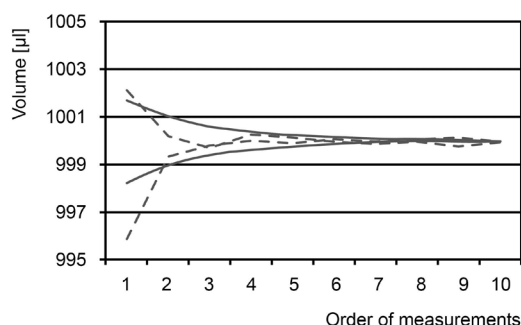
Figure 7: First 10 measurements of a 1000-µl manual pipette after tip mounting. Full lines: calculation, dashed lines: measurement. Two upper lines: captive air volume and tip were saturated before the experiment, two lower lines: measurements start with captive air volume and tip on ambient air moisture. A saturated captive air volume causes maximum volume because evaporation is zero. A tip containing ambient air moisture at first allows maximum evaporation leading to lowered values. All curves converge to the same intermediate equilibrium value

Calculation of moisture equilibrium in the captive air volume

During the pipetting cycles the captive air volume gains moisture from the liquid surface and loses moisture by the blowout stroke and re-aspiration of dry ambient air.

Calculation and experiments show that captive air volume moisture establishes in the midrange between external moisture and full saturation.

Dependence on external air moisture

Higher external moisture leads to higher equilibrium moisture in the captive air volume, allowing less evaporation during aspiration and waiting period. Therefore higher external moisture corresponds to higher volume. Additionally, the influence of initial effects and waiting period decreases with rising ambient moisture.

Aliquot volumes show lower absolute dependence in conical tips. This behaviour can be explained well because moisture effects depend on the liquid surface area which is smaller in the aliquot range.

Much higher values are reached if tips are not sufficiently pre-rinsed. Above all, if the captive air volume is much higher than the nominal volume, equilibrium moisture is reached only after many cycles of pre-rinsing, e. g. regarding a 20 µl pipette using 200 µl tips. After 2.5 s of tip immersion time and 3 times of pre-rinsing, a 2.1%–3.5 % volume change at nominal volume was measured if external moisture changed from 20 % to 80 % [7]. This value matches well the calculations assuming incomplete moistening in the captive air volume. If pipette tips are not pre-rinsed at all, dependency on moisture level is increased.

With respect to moisture effects it is essential to know the liquid meniscus cross section, the tip design and the extent of preconditioning performed.

Sometimes discrepancies between measurements of two different persons remain unfixed because one operator releases the piston after delivery while the tip is already outside the weighing vessel and moisture trap, aspirating dry ambient air, while the other, by habit, releases the piston when the pipette tip is still within the high humidity region of weighing vessel and moisture trap. The latter scenario leads to an increase in volume.

Pipette size	Measurement			Calculation		
	Aliquot of pipette volume					
	100%	50%	10%	100%	50 %	10%
10 ml	0.05	0.04	0.03	0.03	0.03	0.02
1000 µl	0.05	0.05	0.03	0.05	0.04	0.02
100 µl	0.08	0.07	0.03	0.07	0.05	0.03
10 µl	0.09	0.08	0.01	0.07	0.06	0.05

Table 2: Volume of pipettes was measured using different sizes of pipettes. Values were measured with pipettes conditioned to equilibrium moisture.The values stated show the volume deviation in percent if the external air moisture changes by 10 % rH. The values refer to percent of nominal volume! Example: According to measurements, the volume of a 1000 µl pipette at 50 % of nominal volume changes by 0.05 % of nominal volume (absolute 0.5µl) if external moisture changes by 10 %. Measurements with 10 µl pipettes using 20 µl tips showed poor reliability

Dependence on waiting period

After aspiration, a waiting period of 1 second (3 seconds for pipettes over 1000 µl) must be observed. For purposes of uncertainty calculation, the volume change must be known if the waiting period deviates from these values. Measurements and calculation for a 1000-µl pipette show a volume drop of about 0.04 % at nominal volume if the waiting period increases by 1 second because more liquid evaporates during waiting period. Measured results for other pipette sizes were inconsistent.

Dependence on common temperature level

Measurements performed by Lochner [7] and Ylätupa [18] and others showed no significant influence of common temperature level.

Effects are:

a) Longitudinal thermal expansion of the piston stop distance.

b) Thermal expansion of piston cross section. Maximum expansion occurs if using a polymer piston and stroke mechanism, resulting in a volume change up to 0.03 % per 1 K. Different designs of the pipette interior can increase or decrease this dependence.

Besides a) and b) in the captive air volume, additional effects are effective:

c) Cubic expansion of tip and shaft volume has no direct influence on measured volume, but a larger captive air volume is softer and the aspirated volume is decreased.

d) Furthermore increase of the internal tip volume is accompanied by less liquid lift causing a rise of aspirated volume and counteracting to c).

e) Liquid density decreases with increasing temperature followed by an increase in volume.

f) Increasing temperature reduces the surface tension of water.

Calculations showed negligible influence for c) to f).

g) The effect of evaporation in the captive air volume is strongly temperature dependent due to vapor pressure increasing with temperature.

Further experiments were performed on 1000-µl pipettes. The whole room including all measuring equipment was held stable at room temperature, 29 °C and 5 °C. An ambiguous temperature trend was observed depending on pipette type. No statistical significant statement could be given. But by correcting measurements for thermal expansion of piston and stroke, a small negative trend was found. Calculation using formula (13) yields 0.016 % decrease per 1 K.

For purposes of measurement uncertainty calculation it is reasonable to assume a maximum dependency of +/-0.02% volume deviation per 1 K change of common temperature level. Effects of temperature difference between liquid and pipette will always prevail.

Temperature differences

Description

Using a liquid colder than pipette and environment, the tip cools down in the test liquid contacting area during pipetting or when the tip is pre rinsed (Fig. 8) and vice versa. If the captive air volume is warmed up or cooled down during aspiration, a large effect on aspirated volume results. This corresponds to a typical situation in routine lab work when a liquid is pipetted at body temperature or at refrigerator temperature, whereas pipette and air are at room temperature.

The temperature of air decreases quickly to nearly the tip wall temperature while moving down to the cold part of the pipette tip (to resume start position for aspiration). While filling the tip, the cooled down air is shifted into the upper part of the pipette tip and shaft warming up again. Even a minimum increase of air temperature leads to a remarkable increase in captive air volume during aspiration, leading to the same amount of volume decrease. The ideal gas law quantifies this volume change leading to

$$\frac{\Delta V}{V} = \frac{\Delta T}{T} = \frac{\Delta T}{293.2\ K} \approx 0.34\ \% \qquad (14)$$

volume change per $\Delta T = 1K$ temperature difference at 20 °C. However, heat transfer from the liquid surface to the captive air volume compromises the effect to a certain degree. So the real dependency is by 10%-35 % lower as measurements show. The volume deviation is proportional to the piston lift volume V_{Str} and the relative temperature change.

There is no dependence on the size of the captive air volume. Only if the captive air volume becomes smaller than the piston stroke volume are temperature effects reduced, respectively. Zero captive air volume suppresses these effects completely.

Heat transition in captive air volume and to tip

The speed of radial heat transition between tip and air can be calculated using Bessel functions [17].

Fig. 9 shows that aspiration time and waiting period are sufficient to assure a nearly complete temperature adaption of captive air volume to tip or shaft temperature except in large tips.

Concerning heat exchange between tip and water it is instructive to consider the heat capacity of tip and aspirated water (Table 3).

Heat capacity of water is always higher than that of the tip's walls. Regarding the heat capacity of air, it becomes evident that the air temperature has nearly no impact on tip temperature over short times. Complying with measurements, calculations show that a tip adapts to water temperature within few cycles of aspiration and delivery. Calculation of Nusselt number shows that assumption of heat conduction rather than convection can be justified.
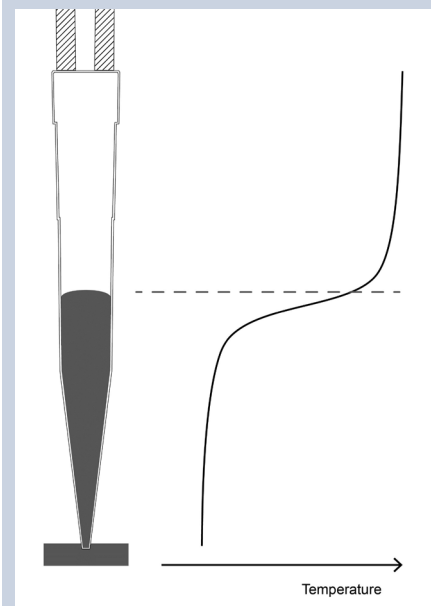
Figure 8: Pipetting of cold liquid. Previous aspiration and discharge adapts the tip's lower part to water temperature. The lateral curve shows the temperature slope from the cool lower part of the tip to its warm upper part. During aspiration the captive air volume slides from cold to warm and expands
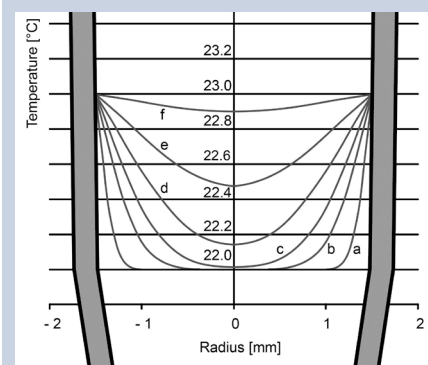
Figure 9: Calculated development of air temperature distribution in a 200 µl pipette tip if walls would immediately increase temperature. The curves show the rapid warming up of air in the tip to nearly tip temperature within 0.05 s. Legend: (a) 0.0005 s, (b) 0.002 s, (c) 0.005 s, (d) 0.01 s, (e) 0.02 s, (f) 0.05 s

Tip size	Water	Tip	Air inside tip
10 ml	42	9	0.012
1000 µl	4.2	0.9	0.0012
200 µl	0.8	0.3	0.0002
20 µl	0.08	0.05	0.00002

Table 3: Heat capacity in J/K of the aspirated water in a tip and the adjacent tip wall portion as well as the heat capacity of captive air volume equal to the aspirated water volume.

Axial temperature propagation

In many applications tips are not pre-rinsed and are discarded after each use. Unrinsed tips are not pre-cooled or preheated by previous cycles, and therefore, the tip's walls will not transfer heat to the captive air volume prior to aspiration.

However, during aspiration the captive air volume is in contact with the liquid surface and starts to adapt to water temperature. In case of low liquid temperature, the captive air volume contracts and aspirated liquid volume is increased (Fig. 10). It should be expected that both lines intersect at the room/pipette temperature of 28 °C. That they do at a lower temperature is probably due to missing moistening of the captive air volume in curve (a) lowering the volume.

Axial heat transfer on the liquid surface works as an antagonist to radial heat transfer from the preheated/pre-cooled tip surface and vice versa, whereas radial transfer prevails.

For axial heat conduction a theoretical approach was made. The following equation could be derived (see [19], page 169), describing the volume expansion originating from axial heat conduction from the liquid surface to the captive air volume.

$$\Delta V = -2 \cdot A_{fl} \cdot \sqrt{\frac{\lambda}{c_p \cdot \rho \cdot \pi}} \cdot \frac{\Delta T}{T_a} \cdot \sqrt{t_e}$$

λ is the heat conductivity of the air, c_p its heat capacity and ϱ its density. ΔT is the initial temperature difference between water and captive air volume, and T_a the absolute captive air volume temperature in K. This volume effect depends on the surface area A_{fl} of the liquid. Comparison with measurements showed that theoretical results amount to about twice the values measured. This is plausible, since radial heat conduction compromises axial heat conduction. Table 4 shows calculation results of axial temperature propagation.

If the external liquid level exceeds the internal liquid rise (deep immersion of tip), namely selecting aliquots in low-volume tips, cooling or heating in unrinsed tips is effective from the outside during aspiration. Effects depend on pipetting pace.

Common temperature of water and air differing from pipette's temperature

This situation occurs, e. g., if using a pipette in a cold room, which has been adapted to room temperature before. This case is similar to the situation already described when liquid temperature is different from common temperature of pipette and ambient air.

Common temperature of water and pipette differing from air temperature

This situation is not a common case in a lab. If, e. g., warm air is aspirated after delivery, it will contract during the subsequent aspiration and lead to higher aspirated volume. However, after blowout the air inside the pipette has time to adapt to the pipette's temperature. In [7], the deviation of volume caused by different air temperature was measured for a 1000-μl pipette to 0.066 % of nominal volume per 1 K.
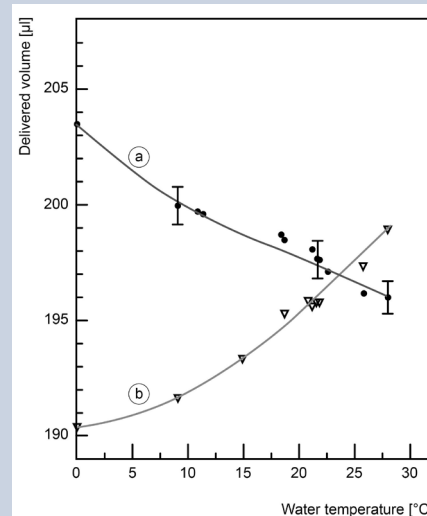
Figure 10: Dependence of delivered volume on water temperature in a 200 μl pipette in case of unrinsed (a) and pre-rinsed tip (b). Room and pipette temperature is 28 °C. At room temperature curve (b) shows a volume deviation of about 0.25 % per 1 K at nominal volume, and curve (a) of about -0.1 % per 1 K. The latter value complies with calculations in table 4. Natural convection could be a reason for non linearity. From Lochner [7] (modified)

| | Aliquot of nominal volume | | |
	100 %	50 %	10 %
1000 μl	-0.041	-0.034	-0.015
100 μl	-0.099	-0.060	-0.020

Table 4: Volume deviation in percent of the nominal volume caused by temperature difference between water and air/pipette in unrinsed tips. Values resulting from Eq. (15) were divided into half. Example: if a 1000 μl pipette aspirates 500 μl of water colder 1 K compared to the pipette in an unrinsed tip, volume increases by 0.034 % of 1000 μl (absolute 0.34 μl) according to calculation

Exchange of pipette tip during measurement

ISO 8655, part 6, demands tip change during pipetting, followed by only one pre-rinsing cycle of each new tip.

Exchange of pipette tips always entails a disturbance of moisture level inside the pipette. Without a change, a pipette system is more stable. To reduce uncertainty of measurement and also for economic reasons and fast calibration, measurements shall be taken without change of tip. See [10] as well as [20].

As soon as residues, droplets or other disturbances occur in the tip, a change of tip becomes necessary. Any new tip shall be prepared for calibration again by 35 rinse cycles to establish a new equilibrium value of moisture in the captive air volume.

Order of volume settings for calibration

Calibration shall, by convention, start at the lowest aliquot volume, then selecting the intermediate aliquot and finally the nominal volume [10, 20].

If proceeding in reverse order, measured volumes will not change if the tip is sufficiently preconditioned to reach moisture equilibrium in the captive air volume after volume change.

As a side effect of hand-warming, the order of measurements can have an influence on volume. If starting with the smallest volume, heat can take time during aliquot measurements to invade the pipette parts close to the hand and thus develops a large effect when selecting the nominal volume. Starting with the nominal volume, warming up is still observed at the beginning. The following aliquot volume does respond remarkably less to warming up.

Pipetting pace

Mainly due to moisture effects, the pace of weighings affects volume. Fig. 12 shows the effect of long waiting period, priming the pipette during calibration and aspirating moist air.

A longer waiting period leads at first to volume decrease (a) and to higher moisture in the captive air volume entailing subsequent volume increase (b). Obviously lowering the moisture by pumping dry air leads to lower volume (following c), pumping in moist air to higher volume (following d).

Immersion depth

The volume finally remaining in the pipet tip is defined in the moment of the disruption of the liquid column. At that time, the tip's orifice is located a bit above the liquid surface. Therefore, immersion depth has no direct impact on volume. However, some effects exist that impact indirectly.

If the tip is immersed deeper, the water surface reaches a higher end position after aspiration. Lifting the pipette increases the hydrostatic pressure in the tip leading to a little outflow through the tip which is still immersed. However, the meniscus of the surface does not follow this outflow at its outer margin because the contact angle shows hysteresis. Rather the meniscus is flattened in the center. This phenomenon is assumed to lead to a small dependence on immersion depth. For large pipettes the resulting effect remains significantly below 0.02 % volume deviation referred to nominal volume per millimeter of immersion depth deviation [12].

Differences between water and tip temperature are assumed to create larger effects on volume if the liquid rise in the tip is lower than the immersion depth. In this case, temperature impacts from outside the tip, changing tip and captive air volume temperature.
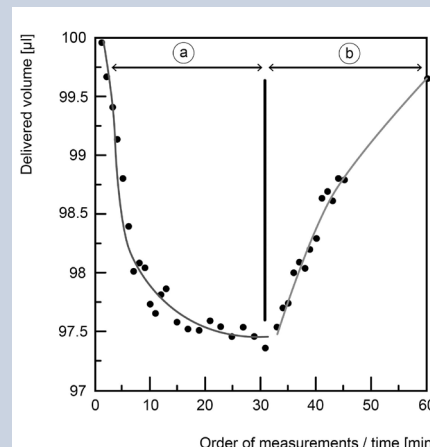
Figure 11: Influence of hand-warming on pipetted volume in a 100-μl pipette. Room temperature: 19 °C. Water temperature 17.8 °C. The instrument was held in hand without interruption and warmed up (part (a)). During the first 30 minutes, the exchange of heat from the operator's hand lead to temperature differences on the inside of the pipette, accompanied by a decrease of volume. Thereafter, insulating gloves were put on and the pipette was laid down between operations resulting in an increase of volume (part (b)). The investigated pipette represents extreme dependency. Current pipette designs usually show much lower hand-warming effects (from Lochner [7] (modified))
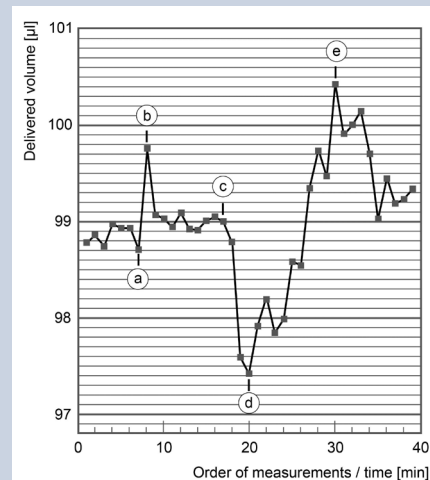
Figure 12: Influence of irregular pace and moisture disturbances during calibration in a 100-μl pipette. At point (a) the liquid was held in the tip for 30 seconds. At point (a), standardized pipetting was resumed. Repeated pumping (30 times) in dry air leads to large volume decrease (c). Following (d), air as moist as possible was aspirated. Following (e) standardized pipetting allowed volume to settle again to original values, from Lochner [7] (modified)

Handling and instrumental effects

Experiments showed that it is reasonable to assign to effects of piston stroke and counter hysteresis, angle of inclination, capillary pressure change from tip to tip, change of waiting period, dependence on common temperature level, hand-warming, pipetting pace and immersion depth a standard measurement uncertainty of about 0.04 % (for fixed pipettes) to 0.06 % (for variable pipettes) referring to nominal volume [12].

Contribution due to gravimetric calibration procedure

The gravimetric effect on uncertainty is comprehensively calculated in ISO/TR 20 461 [21]. As described in detail in [10], [12] the contribution of gravimetry to the total uncertainty of measurement is relatively low.

Conclusions

Applying captive air volume pipette design and the basic laws of thermodynamics of ideal gases and surface physics, mathematical expressions could be found to describe and quantify the basic influences on the delivered volume. Major source of error is a temperature-dependent between liquid and instrument (about 0.3 % volume deviation at nominal volume per 1 K difference), whereas dependence on common temperature level of liquid, instrument and ambient air is smaller by more than a factor 10. The latter dependency originates from tempera-ture dependent liquid vapor pressure counteracting to thermal expansion of the stroke unit.

There is also a significant impact on volume if ambient air moisture is changed.

Pre-rinsing moistens the captive air volume. The volume is much lower if tips are used without pre-rinse. Yet in this case, the dependence on temperature difference between liquid and instrument is reduced to about one-third to one-sixth. If the liquid is at body or refrigerator temperature no pre-rinsing of tips should be applied.

Liquids with high vapor pressure need intensive preconditioning to avoid a large volume error or even dripping.

It was shown that temperature difference effects behave about proportionally to the selected aliquot volume. Dependency on barometric pressure, angle of inclination and liquid density behave about proportionally to the captive air volume, whereas all moisture related effects mainly depend on the liquid surface area.

It could be demonstrated that small volume tips with low surface energy reduce the aspirated volume.

Calculating the uncertainty of measurement, these system- related effects always prevail over the gravimetric contribution acc. to [21].

Air interface pipettes are precision instruments but their limitations in application and calibration must be accepted. While observing recommendations in correct use, in practice volume errors of 2-3 % (at nominal volume) are realizable. Otherwise up to 10 % or more can be expected.

Efforts to perfect air interface pipettes are compromised by thermodynamic facts.

If precise delivery of various liquids or independence from unfavorable environmental or handling conditions is required, there is good reason to use positive displacement systems, e. g., multiple stroke dispensers.

The given calculations represent estimations sometimes based on idealized assumptions. Therefore the precision of calculated results cannot be expected to be very precise. It would be favorable to use finite element programs being able to handle two phase systems.

Nevertheless the given analytical approaches shed light on unknown phenomena by allowing a basic and simple comprehension.

Affiliations:

R. Feldmann is employed at BRAND GMBH + CO KG, Germany and K. H. Lochner is employed at Fraunhofer Insitute for Silicate Research, Bronnbach Branch Office, Wertheim, Germany.

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