EURAMET PROJECT 1295

Guide on the Calibration, Operation and Handling of Micropipettes

Final REPORT

Pilot: Hellenic Institute of Metrology (NQIS/EIM), Greece
Dr. Zoe Metaxiotou, Head of Flow & Volume Laboratory

Participants:

- IPQ, CMI, BOM, FORCE, (EURAMET)
- BRAND GMBH + CO KG (Germany)
- ARTEL (USA)
- ZMK & ANALYTIK GmbH
- EXHM, General Chemical State Laboratory (Greece)
- *GILSON**
- *Mettler-Toledo**

** Late participants*

January 2019

Contents

1 Introduction

1.1 The trigger

The experience gained from several bilateral and multilateral EURAMET intercomparison projects during the last decade has shown that the calibration and handling of micropipettes is a delicate work that requires a substantial amount of experience on a technical level in order to achieve optimized and reliable results. In addition, it is recognized among the national metrology institutes, members of EURAMET that the influence of several experimental / environmental factors is not quite clear nor adequately or exhaustively described in the existing international documents usually referred to as the standards for the calibration procedure. Moreover, although research has been conducted in this field, mainly driven by the major manufacturers of micropipettes, it is not clear whether the outcomes of these investigations have been widely disseminated in the metrological community and have reached the level of end users in a comprehensive and systematic manner that affects the way these instruments are operated on a daily routine work basis in laboratories.

On the other hand, the reliable measurement of small volumes in the micro- or nano- scale is of major importance for analytical laboratories in the medical / pharmaceutical sector and gains increased attention during the past few years.

2 Project description

2.1 The Scope

Based on the aforementioned facts, the Volume Subgroup of the TC-F committee of EURAMET during its annual meeting in 2014 suggested a new EURAMET research project which would be focused on the development of a guide entitled: "*Guide on the Calibration, Operation and Handling of Micropipettes*". This guide aims to be a comprehensive and reliable instruction manual containing the accumulated scientific and metrological experience in this field, as well as recent research developments covering the most significant experimental aspects of the calibration procedure and operation of micropipettes. The guide is expected to contribute to the harmonization of the calibration procedure of pipettes which in turn will assure the reliability and comparability of measurement results both on national and international level, especially in the field of medical and pharmaceutical applications. In addition, the guide might be disseminated and adopted in the accreditation field contributing further to the harmonization of calibration practices and uncertainty estimation in the measurement of small volumes. Last but not least, the guide might provide a solid basis for the update of existing international standards.

2.2 The aim

The experienced gained by NMIs in the past decade during several EURAMET inter-comparison projects on the calibration of air cushion pipettes reveals a lack of in depth knowledge in this field which among other things is believed to be the reason for the deficit of harmonization both on calibration, uncertainty estimation and handling of pipettes by NMIs, accredited laboratories and users. An extensive literature survey was conducted on

the subject in the beginning of this project. The literature survey was particularly focused on the possible experimental factors that may affect the calibration and handling of pipettes. To this end, all available technical documents were collected and reviewed while stored in a restricted access data base which was created for this reason in the web site of EIM.
Based on the findings of the literature survey, the present research project was decided to be focused on the investigation of the influence of four parameters considered to be of major importance for the proper calibration and operation of a pipette. The parameters chosen were the type of liquid, the type of tip, the degree of experience of the operator, and the temperature of the liquid.
The current work is focused and limited to piston operated pipettes with air cushion. This term is replaced though the entire report by „pipettes“.

2.2.1 The Transfer Standard

A transfer standard (TS) package appropriate for the subject of this investigation was chosen. The pipettes in this project were selected so as to extent the volume range examined during previous EURAMET intercomparison projects. To this end, a TS package consisting of four different pipette sizes with at least two pieces in each size was used:

i. 1 - 10 µL
ii. 10 - 100 µL
iii. 100 – 1000 µL
iv. 100 µL (constant volume)

The pipettes were provided by the participants and were all of “air cushion” type from four different manufacturers (Eppendorf, BRAND, Gilson and Sartorius).

2.2.2 The experimental design scheme

Using the TS, the influence of the four parameters has been investigated on a single, double or triple level basis regarding the values of the parameters. In particular, two different investigation schemes were applied, and are presented in Table 1 and Table 2, respectively.

Table 1. Type I experimental scheme

Pipette	**(e.g. 100 – 1000 μL / S/N: ………)**	
No	Parameter	Level
1	Operator	1. Experienced (+) 2. Less experienced (-)
2	Calibration liquid	1. Water (+) 2. Other (-)
3	Temperature difference between (P-A)/L	1. No gradient (+) 2. Gradient (-)
4	Tip type	1. Manufacturer (+) 2. Other (-)

In particular, the approach of experimental scheme Type I was to investigate separately the effect of each parameter for the given levels, keeping all other parameters constant at the + level. On the contrary, the Type II experimental scheme is more extended and investigates beyond the effect of a single parameter, the simultaneous combined effect of two or three parameters taking values different than the ones corresponding to the reference conditions (+).

Table 2. Type II experimental scheme

Pipette	**Operator**	**Tip**	**Solution**	**Temperature**
Experiment	**Parameter Level**			
1	+	+	+	+
2	+	+	+	-
3	+	+	-	+
4	+	-	+	+
5	+	+	-	-
6	+	-	-	+
7	+	-	-	-
8	+	-	+	-

In real practice, the situations that can be found within Type II scheme are very common. Two typical situations are given in the following examples.

Example 1

In a hospital laboratory two trained operators of different level of skill within the frame of two similar blood tests are dispensing the same nominal volume of different liquids which are thermostated at different temperatures using the same pipette but with different tips. Would their test results be within the specifications of the methods applied?

Example 2

In a hospital laboratory an experienced operator within the frame of a blood test is dispensing the same nominal volume of two different reagents which are thermostated at different temperatures using the same pipette but with different types of tips. The analysis protocol requires the addition of the same amount of the two reagents in the test vial in order to obtain the requested result within the accuracy specifications of the method.

The workload within Type I and Type II experiments was partitioned among all participants, while the pilot laboratory had the additional responsibility for dealing with the logistics of the project, interpreting the data and issuing the intermediate and Draft A and B progress reports.

2.2.3 The calibration protocol

All participants were advised to follow the following procedure during pipette calibration.

1. Set the volume
2. Attach a new tip and rinse five (5) times in order to establish a humid environment in the dead volume of the micropipette.
3. Change tip - rinse once
4. Take the first 3 out of 10 repeats with this tip.
5. For the next 4 out of the remaining 7 repeats use a new tip, pre-rinsed one time.
6. Change tip - rinse once - take the last 3 repeats
7. In each repeat apply approximately 3 mm immersion depth and 1 sec waiting time
8. Set a new volume
9. Repeat steps 3-8 two more times

3 Literature review

3.1 Existing theoretical and experimental investigations

A vast amount of publications, most of which are in the form of “Application Notes” or “Best practice notes” from major pipette manufacturers, exists in the available literature [1-10]. However, there is a rather limited amount of scientific publications that deal with targeted theoretical and experimental investigations on the influence of various parameters on the performance of air cushion pipettes with respect to their accuracy, repeatability and compliance with the specifications set by manufacturers or the relevant standards [11-20]. In particular, detailed investigations on the influence of system-related parameters, such as temperature difference between liquid-pipette-air, relative humidity and air pressure, as well as parameters related to pipette handling, such as inclination angle, waiting time, immersion depth, operational force, hand warming, etc., can be found in Feldmann and

Lochner [11], while other studies more or less extensive on the effect of temperature disequilibrium, operator experience, hand warming, tip pre-rinsing, tip immersion depth, plunger speed and pressure, liquid density can be found elsewhere [12-15].
The influence of altitude on the dispensed volume for both fixed and variable volume air cushion pipettes of various sizes has been investigated by Spälti et al. [16] and Rodrigues [17], while comparative evaluations of the influence of tip type from different tip manufacturers can be found in various application notes issued by major manufacturers of pipettes [18].

3.2 Basic theoretical approach

The aspiration of a liquid using an air cushion pipette (Fig. 1) can be approximately described as a process of isothermal change of state of an ideal gas [11]. This approximation is justified and adequate in order to describe the possible experimental influence factors that affect the volume delivered by the pipette and can be mathematically expressed by equation (1).

$$P_a \times V_o = (P_a - P_h - P_s) \times (V_o + V_{str} - V) \qquad (1)$$

Where:

P_a : air pressure
V_o : captive air volume
P_h : hydrostatic pressure
P_s : capillary pressure
V_{str} : stroke volume piston
V : aspirated liquid volume

While hydrostatic pressure, P_h, due to the liquid column is given by equation (2),

$$P_h = \rho_w \times g \times h \qquad (2)$$

Where:

ρ_w : density of liquid (water)
g : acceleration of gravity
h : liquid lift inside in tip

Rearranging (1) in combination with (2) and solving with respect to the aspirated liquid volume, V, we get the delivered liquid volume in equation (3), which also accounts for effects related to evaporation, temperature differences and handling.

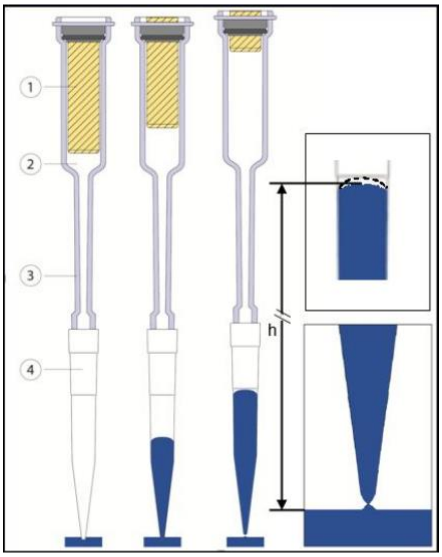


Fig. 1 Aspiration of a liquid using and air cushion pipette: (1) piston, (2) captive air, (3) shaft, (4) tip, while h denotes liquid lift in the tip (*by courtesy of BRAND GMBH +CO KG*).

$$V = V_{str} - V_o \times \frac{(\rho \times g \times h) + P_s}{P_a - (\rho \times g \times h) - P_s} - V_{ev} \pm V_{Tdiff} \pm V_{handling} \quad (3)$$

Where:

V_{ev} : volume decrease due to evaporation into the air cushion during aspiration
V_{Tdiff} : effect due to temperature change in the air cushion during aspiration
$V_{handling}$: volume change due to various handling effects

Evaporated liquid inside the tip escapes in the air cushion space, which expands and displaces test liquid downwards out of the tip, resulting always in a decrease of the delivered volume. On the other hand, parts of the air cushion volume, V_{eff}, are subject to a relative temperature change ΔT/T which leads to a volume change in the air cushion and consequently to a change in the delivered volume.
Finally, handling effects include factors such as the angle of inclination during aspiration, waiting time after aspiration during which tip is still immersed in the test liquid, time between subsequent aspirations, immersion depth of the tip inside the test liquid, operational force on the piston, counter hysteresis, and heat transfer due to hand warming.

The terms of equation (3) referring to the physical operations of evaporation and temperature change can further be expanded, as shown in equation (4) [11].

$$V = V_{str} - V_o \times \frac{(\rho \times g \times h) + P_s}{P_a - (\rho \times g \times h) - P_s} + \left[A_{fl} \times P_d \times (1 - S) \times k \times t_{AnsW}\right] \pm \left[V \times m \times \frac{\Delta T}{T}\right] -/ + \left[A_{fl} \times b \times \frac{\Delta T}{T}\right] \pm V_{handling} \quad (4)$$

Where:

A_{fl} : contact area between liquid and air cushion
P_d : vapor pressure of liquid
S : vapor saturation of air cushion in % rH
t_{AnsW} : aspiration time plus waiting time

k : evaporation factor (volume per area, vapor pressure and time)
V :aspirated volume
m: factor accounting for radial heat conduction. m = 0.3 …. 0,9, depending on pipette size b: factor accounting for axial heat conduction (volume per area, time dependent on tAnsW).
$\Delta T/T$: relative temperature change in the air cushion

Since some of the parameters in these terms are not exactly known (e.g. S, k, m, n), their effect must be determined experimentally.
The above theoretical analysis, expressed by equation (4), constitutes a model which could be used to establish the influence of the so called system effects, such as temperature differences, humidity conditions prevailing within the air cushion, and barometric pressure in the delivered volume.

3.3 Previous experimental studies & results

As shown by equation 4, the delivered liquid volume with an air cushion pipette depends on a series of parameters, some of which are not known exactly. At the same time the number of process variables is significant and a straightforward dependence study is far from realistic, since there might be a coupling between two or more process parameters to some degree.
Up to now and to our knowledge the existing experimental studies have been focused on the investigation of the influence of some of the parameters mentioned above using a single variable investigation type of design [11-18]. Quantitative as well as qualitative estimations of the effect of various parameters have been experimentally established and the main results are briefly presented in the following paragraphs.

3.3.1 Temperature difference effect

It has been experimentally established [19] that it is the temperature difference rather than the absolute temperature level that has an effect in the liquid volume delivered. In particular, the effect increases when the pipette temperature differs from the common temperature of liquid-air or even more when liquid temperature differs from the common temperature of pipette-air. In the case of a liquid which is colder than the pipette-air system, the tip cools down when immersed in the liquid. The temperature of air in the air cushion 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. During aspiration, as the air is travelling up again into the upper warmer part of the pipette tip, it warms up again and expands leading to a volume increase in the air cushion, which in turn causes a volume decrease in the dispensed volume. In the case of a liquid which is warmer than the pipette-air system, the situation is reversed. The case has been explained and quantified in terms of accuracy change in the aforementioned work using the ideal gas law.

$$\frac{\Delta V}{V} = \frac{\Delta T}{T} = \frac{\Delta T}{293.2K}, \ \frac{1K}{293.2K} \approx 0.34\% \qquad (5).$$

This implies a 0.34% volume change for a temperature difference of 1 K at 20 ^{o}C. Usually, the thermal state of the air cushion as described above is also affected by the heat transfer from the liquid surface to the air cushion (axial heat transfer) which counter acts to some degree the previous effect, the overall change being lower than the one predicted by equation 5.

3.3.2 Air humidity effect

The humidity in the air cushion directly affects the evaporation from the liquid surface in the tip into the air cushion. The higher the vapor saturation in the air cushion, the smaller the evaporation from the liquid to the gas phase which leads to higher delivered volume of liquid. In addition, the ambient air moisture content directly affects the relative humidity of the air cushion, the two parameters being directly proportional as a coarse approximation to each other. In particular, for 1000 µL and 100 µL pipettes it has been established [19] that the relative humidity sensitivity changes by approximately 0.07% for 10% rH change in moisture content. This sensitivity coefficient was found to be slightly higher (0.10%/10%rH) for 10 µL pipettes. This effect is directly linked to the influence of pre-rinsing the tips. Consequently, tips that are used without pre-rinsing have lower moisture content and this leads to lower delivered volumes due to considerable evaporation from the liquid phase to the air cushion, which in turn expands and suppresses the liquid column out of the tip. If the same tip is used in the subsequent cycles, it will deliver higher volumes reaching finally an equilibrium value [19].

3.3.3 Barometric pressure effect

According to equation (3), the delivered volume is expected to decrease when barometric pressure is lower, the decrease being proportional to the nominal volume of the pipette. Indicatively, for a 1000 µL pipette at ambient atmospheric conditions of 1008 mbar and air cushion of 2700 µL the pressure sensitivity coefficient is 0.014 µL/mbar, while for a 100 µL and 10 µL the corresponding coefficients are 0.0012 and 0.0003 µL/mbar, respectively [11,16]. The barometric pressure effect is due to the compressibility of air. At lower barometric pressures the density of air inside the cushion is lower, meaning that in order to compensate for the weight of the liquid column inside the tip, the air cushion must expand more, leading to lower aspirated volume.

3.3.4 Absolute temperature effect

As it has already been mentioned, it is the temperature difference rather than the absolute temperature level of the system air-pipette-liquid that has an effect on the liquid volume delivered. No experimental results pointing to a specific dependence exist to our knowledge, although it is known that several parameters of those involved in equation (3) are temperature-dependent. Mutually cancelling or complex effects, as well as unknown factors make it difficult to isolate a dominant absolute temperature dependence.

3.3.5 Exchange of tip

Exchange of tip during calibration and use of micropipettes is an established practice in accordance to ISO 8655/6. This practice is considered as more representative of the conditions of use of micropipettes in typical laboratory conditions. However, as mentioned in § 3.3.2, the exchange of tip and its use with or without pre-rinsing is directly affecting the humidity and heat transfer conditions prevailing within the air cushion, while having at the same time consequences in ergonomics, cost effectiveness and uncertainty in measurement.

3.3.6 Handling effects

It has been established [19-20] that several handling aspects have an influence on the delivered volume. Such handling aspects include among other inclination angle of pipette, tip immersion depth, waiting time between subsequent aspirations, waiting time before withdrawing the tip from the liquid vial after aspiration, force applied on the piston, hysteresis and hand warming effects. The effect of those handling parameters can vary from small to significant depending on the size of pipette and the volume measured.

4 Data interpretation – Results

During the course of this project a vast amount of experimental data was collected by the participants. The data were summarized, tabulated and interpreted in order to reveal the outcomes of the experimental investigation. To this end certain criteria were adopted in order to reach solid conclusions about the significance of the influence of the experimental parameters investigated on the volume delivered by air cushion pipettes of various sizes.

4.1 Evaluation criteria

As a reference point for the current investigation the specifications limits posed by EN ISO 8655-2:2002 as shown in Figures 2, 3 and 4 for small (1 – 10 µL), medium (10 – 100 µL) and large size (100 – 1000 µL) pipettes, respectively, are given below.

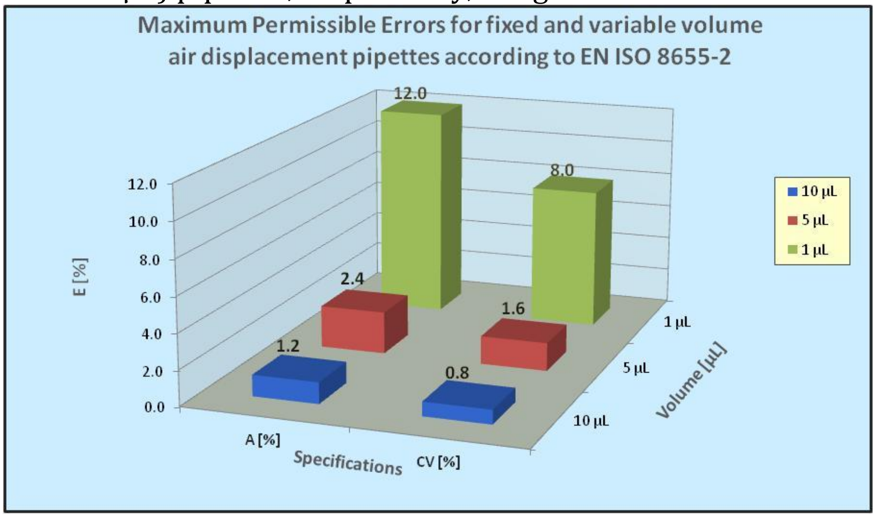


Figure 2. EN ISO 8655-2 specifications for 1 – 10 µL variable volume pipettes

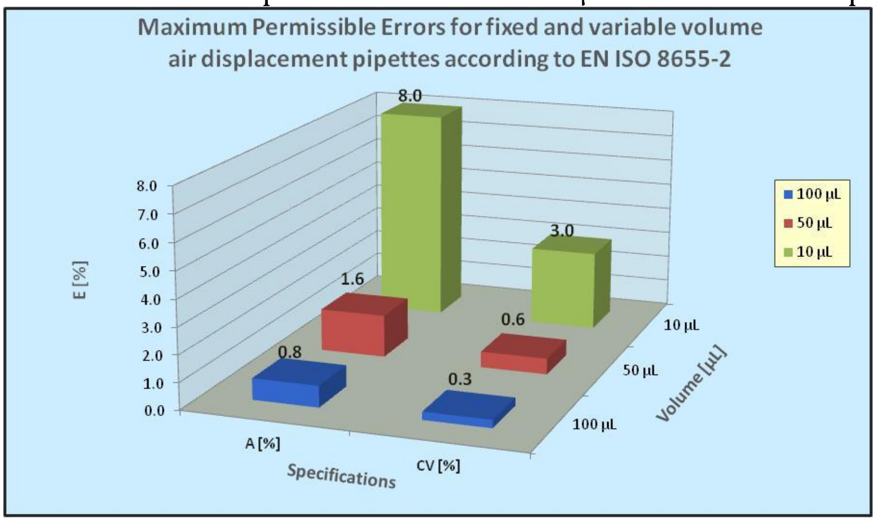


Figure 3. EN ISO 8655-2 specifications for 10 – 100 µL variable volume pipettes

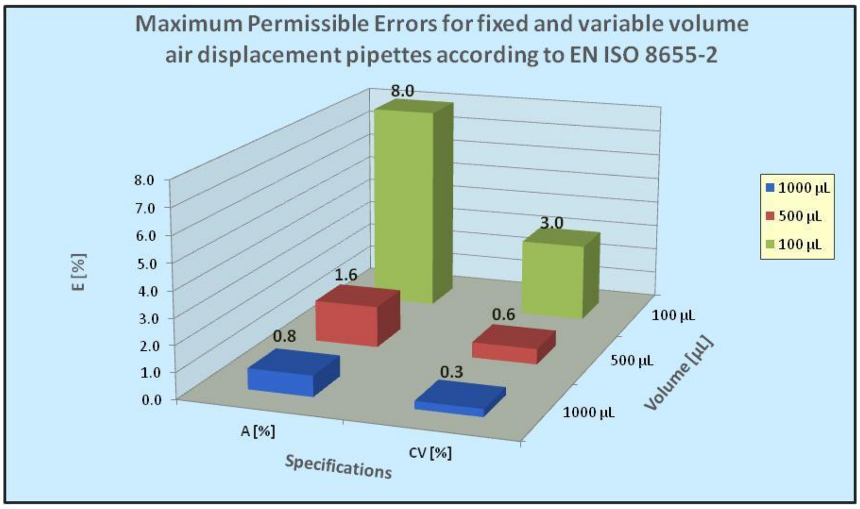


Figure 4. EN ISO 8655-2 specifications for 100 – 1000 µL variable volume pipettes

The evaluation, however, of the obtained results in the present investigation was done on a ***qualitative*** and a ***quantitative*** basis according to the criteria given in Figure 5.

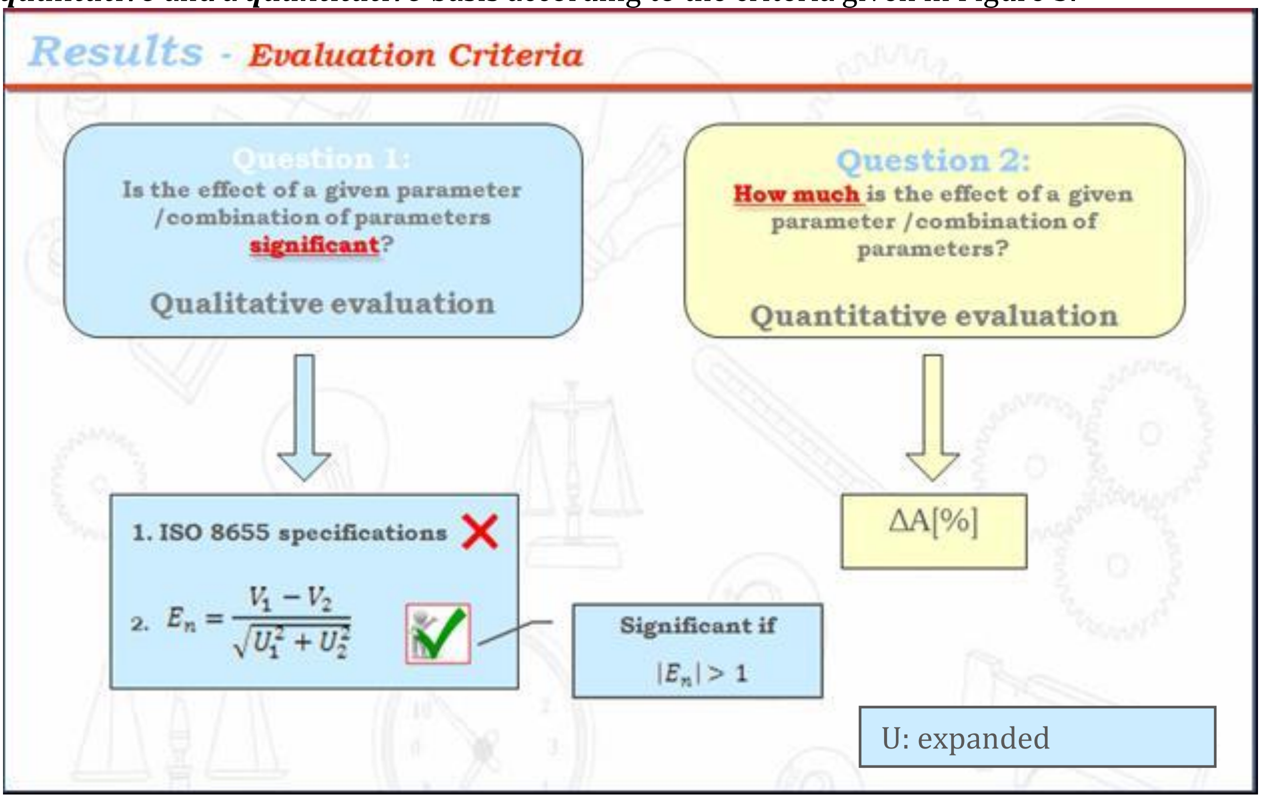


Figure 5. Evaluation criteria for the interpretation of the results

The ISO 8655 specifications were not chosen to be the criterion for the evaluation because they are considered to be too high and modest for this type of investigation.

Applying this type of evaluation the results obtained during the course of this project are presented graphically in the next paragraphs categorized in two main groups:

- Type I investigation (single parameter)
- Type II investigation
  - ✓ Single parameter (Experiments 2-4)

- ✓ Multiple parameters (Experiments 5-8)
- ✓ Experiment 1: reference value

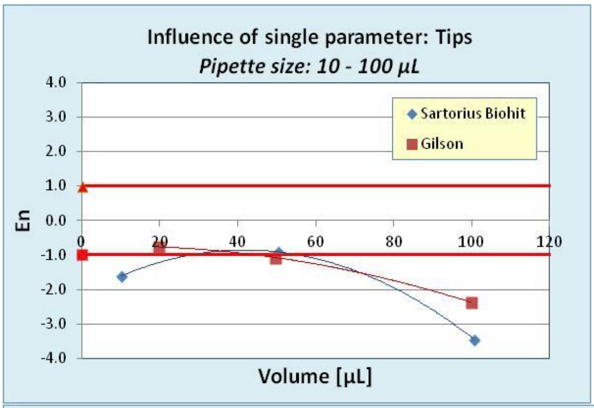
For each group the results are evaluated both on a qualitative and on a quantitative level whenever feasible.

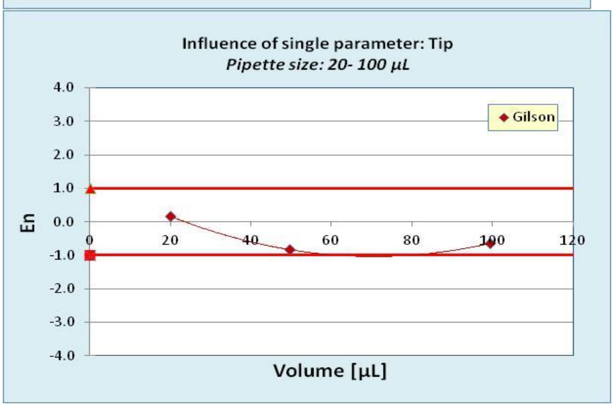
4.2 Type I investigation

The result of the evaluation of the Type I investigation is depicted in the following diagrams for the single parameters: tip type, operator, solution type and water temperature, respectively, for different pipette sizes and different manufacturers. The red lines in all diagrams represent the criterion, En, limits.

4.2.1 Influence of tip

A tip of different type may indicate differences in shape, material and quality which in turn affect several important parameters such as the size of air cushion, raising height of liquid inside the tip, fit of tip on the cone, tip orifice imperfection, water retention and leachables. All of the above can have an influence on the accuracy and reproducibility of the pipette, while they can also affect the way the pipette behaves in tip change or in autoclaving [18]. In the present investigation good quality tips of origin other than the manufacturer were used, but there was no information available about specific differences with respect to the above geometrical, physicochemical and constructional parameters. Having this background in mind, it is not possible to attribute the observed behavior to a certain feature of the tips. It becomes, however, obvious that the type of the tip can have an influence on the delivered volume.





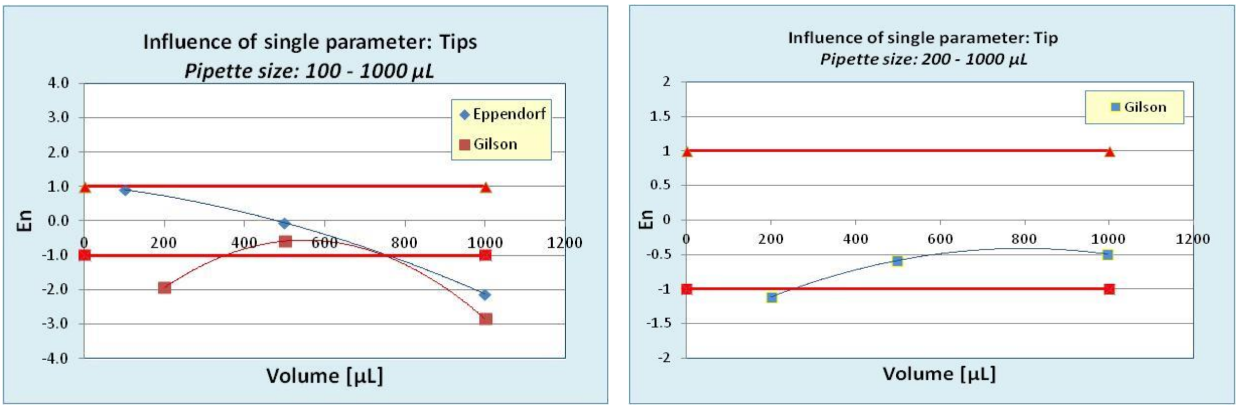


Figure 5. Influence of tip type in medium and large size pipettes

4.2.2 Influence of operator

Operator skill refers to the consistency that an operator executes a specific pipette calibration. Ideally an operator's skill should be demonstrated through documented quantitative evidence with respect to his ability to perform the calibration of a tested good quality pipette with the required degree of precision and accuracy. The impact of the operator's skill and experience on the calibration and use of a pipette is unquestionable. However, this impact is not easily correlated to the degree of expertise of the operator. In the present study results were obtained with an experienced and a less experienced operator (Figure 6). However, no documentation regarding any type of quantitative evaluation of the operator's skills was available. The obtained effect as qualified by En score is not easily interpreted and should, therefore, be considered just indicative of the potential effect of lack of operator's experience because any other correlations with the size of the delivered volume cannot be determined.

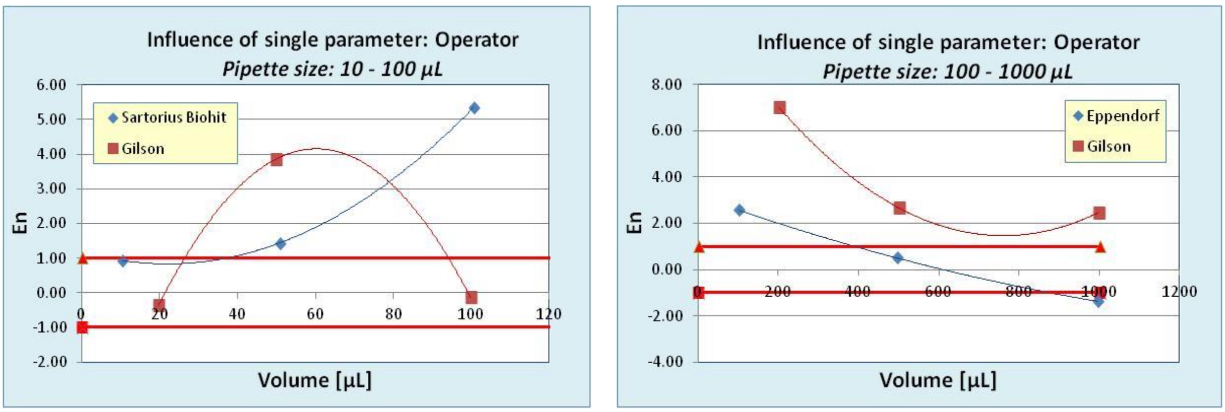


Figure 6. Influence of operator in medium and large size micropipettes

4.2.3 Influence of solution

A calibration liquid other than water can have properties (density, viscosity, surface tension, vapor pressure, etc.) that might affect the delivered volume as equation (4) implies due to its interference with the captive air. In the present study two different types of solutions were chosen to be tested.

a. *Glycerol – H_2O mixture (Solution B)*
b. *DMSO – H_2O mixtures (concentrations 10%, 30%)*

It must be emphasized that the two types of solutions chosen to be tested as calibration liquids other than water were not reference solutions of any kind. They were characterized with respect just to their density by RUDOLF and IPQ (a) and EXHM (b). Furthermore, the DMSO-H_2O mixtures (DMSO: dimethylsulphoxide) were chosen for their unique characteristics (highly polar, water miscible organic liquid, excellent solvent for organic and inorganic substances, dipolar aprotic solvent with high melting point (18.5 °C), viscosity similar to the environment of living cells, low toxicity level) and wide applications (biochemistry, biology, genetics, structural ^{1}H-NMR investigations, DNA investigations with PCR, cryopreservation, etc.) in the medical analytical laboratory field.
The densities of these two types of liquids are given in Table 3.

Table 3. Density of calibration liquids other that water

Liquid Description	Density at 20 °C [kg/L]	Density at 17 °C [kg/L]	Density at 23 °C [kg/L]	Characterized by
Glycerol+50% CMC TRITON X-100 (Solution B)	0.998212	-	-	Rudolf Research Analytical
Glycerol+50% CMC TRITON X-100 (Solution B)	0.998221	0.998794	0.997554	IPQ
DMSO 10%	1.011176	-	-	EXHM
DMSO 30%	1.040785	-	-	EXHM

As shown in Table 3 the density of Solution B does not differ from water density at 20 °C while the other two solutions have densities higher than water's.
From the diagrams in Figure 7 it is shown that in small (1-10 µL) and large (100-1000 µL) size pipettes there is an effect of Solution B in the delivered volume which is demonstrated by over delivering and under delivering in small and large volumes, respectively. In the medium size pipettes the obtained results seems to be inconclusive for this specific solution.
The influence of DMSO solutions of varying concentrations is shown in Figure 8 for small size variable volume pipettes of three different manufacturers, while the influence of the same solutions on the volume delivered by 100 µL fixed volume pipettes of the same manufacturer is given in Figure 9.

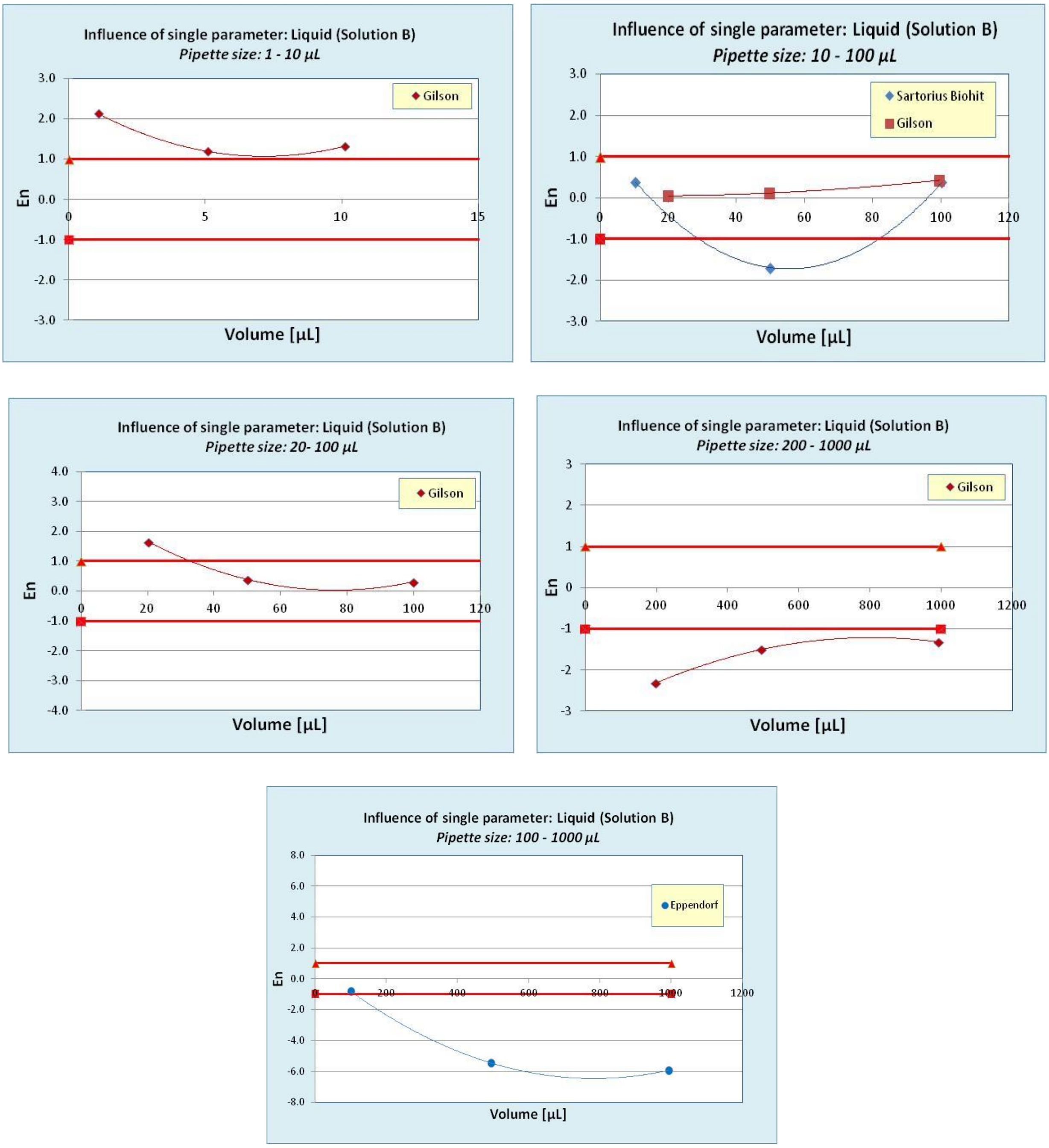


Figure 7. Influence of liquid type (Solution B) in small (1-10 µL), medium (10-100 µL/20-100 µL) and large size (100-1000 µL/ 200-1000 µL) pipettes

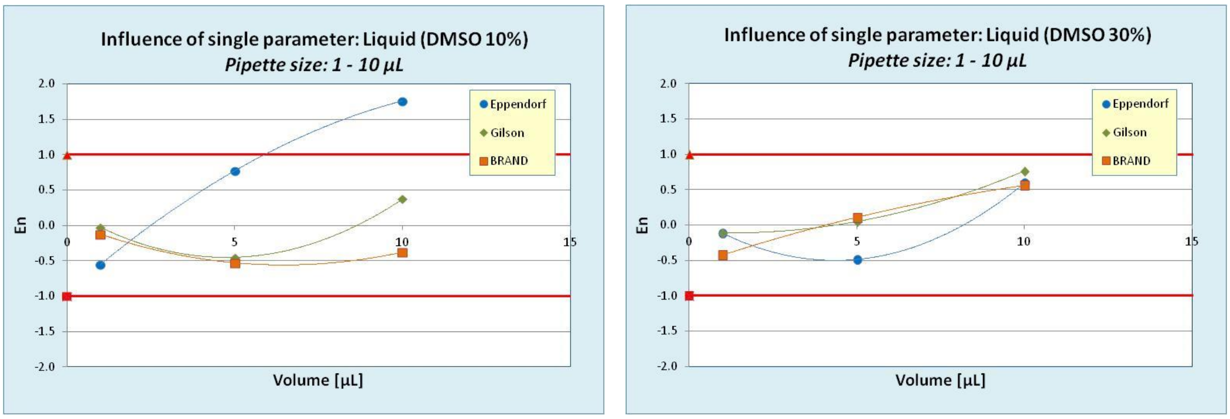


Figure 8. Influence of liquid type (10% & 30% DMSO solutions) in small pipettes of three different manufacturers

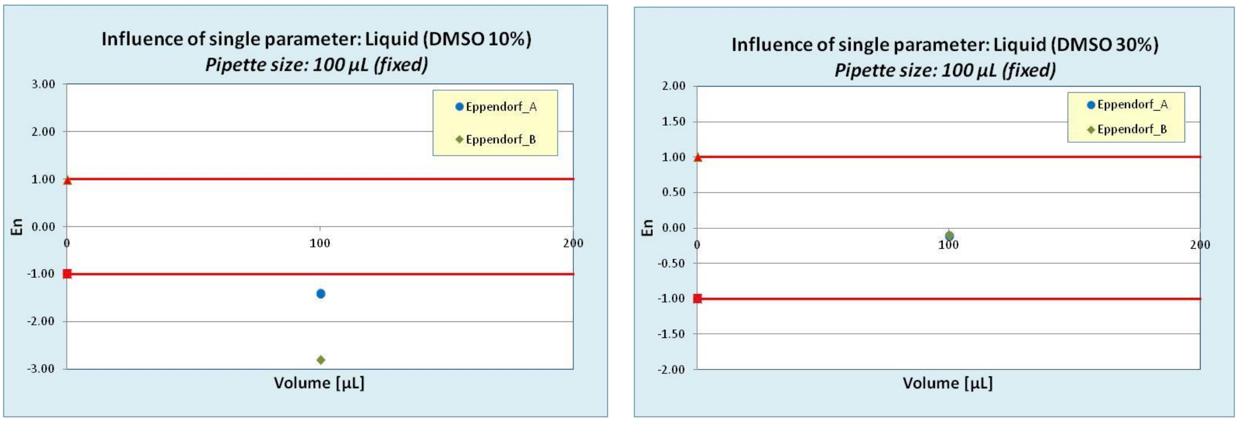


Figure 9. Influence of liquid type (10% & 30% DMSO solutions) in fixed volume 100 µL pipettes of the same manufacturer

As shown in Figure 8, small variable volume pipettes, with the exception of one manufacturer (Eppendorf) at nominal volume, are not affected by the calibration liquid in neither of the two DMSO concentrations used.

Two different 100 µL fixed volume pipettes of the same manufacturer showed consistent behavior with the same DMSO solutions, but they were affected only by the 10% DMSO solution by delivering less volume compared to water. A higher density of the calibration liquid would cause larger hydrostatic pressure due to the liquid column inside the tip which in turn would cause expansion of the air cushion leading to under delivery. However, this effect is not observed with the 30% DMSO solution which has even higher density. Other possibly counter acting effects due to parameters like the affinity between DMSO solution and tip wall, surface tension, vapor pressure or capillary forces are not known, and therefore it is not easy to interpret the obtained behavior and attribute it to a certain cause.

The above results regarding the influence of tip, operator and liquid type as single parameter investigations can be interpreted mainly on a qualitative basis. On the contrary, the results presented in the next section can be evaluated on a quantitative basis as well and can also be compared to results obtained in previous similar investigations.

4.2.4 Influence of liquid temperature (T=+ 5 °C)

The influence of liquid temperature in medium and large size variable volume pipettes from three different manufacturers was investigated in experiments where the water temperature was +5 °C. The results are shown in Figure 10.

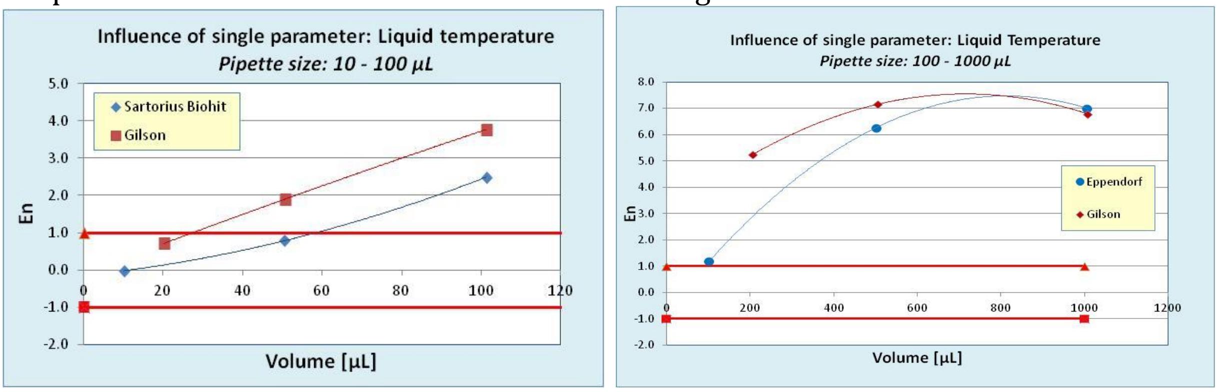


Figure 10. Influence of H_2O temperature in medium and large size pipettes (qualitative)

The quantitative effect of the water temperature in these experiments is presented in Figure 11. As shown in the corresponding graphs there is approximately an overall +1.0 to +1.5% change in accuracy for all tested pipette brands for a ΔT=-15 °C change in water temperature with respect to reference conditions (20 °C). This implies a temperature sensitivity coefficient of approximately 0.07%/K to 0.1%/K. From the two graphs it can also be observed that the delivered volume of cold water is higher compared to the one obtained at reference temperature. All the above results were obtained by the same author. The results are in contradiction with previous investigations [19] and could be explained, at least partially, by practices applied by this author which deviate from the calibration protocol with respect to pre-rinsing of the tips. An increase in the volume is expected with cold liquids only if pre-rinsing of tips was not applied during calibration.

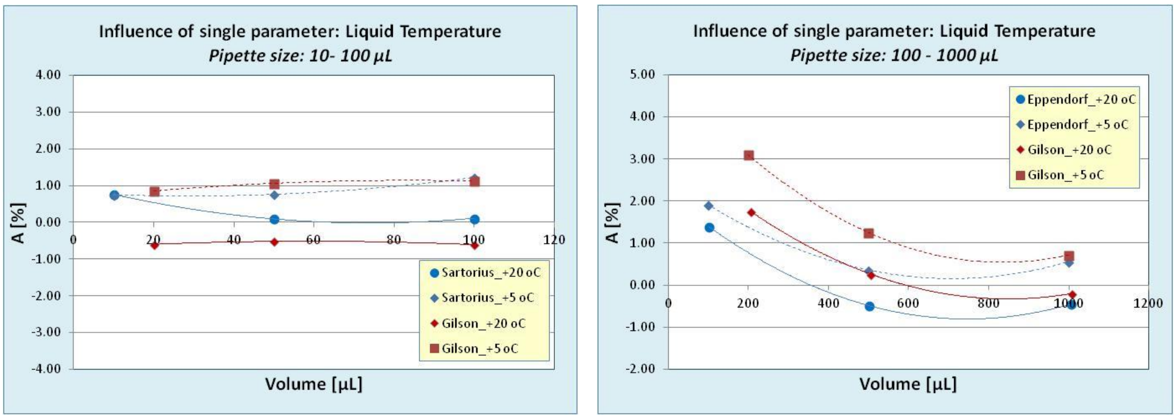


Figure 11. Influence of H_2O temperature on the accuracy of the delivered volume (quantitative)

4.3 Type II investigation

The type II investigation consisted of two distinct groups of experiments aiming at different targets.

- Group 1 investigation (performed by ARTEL)
- Group 2 investigation (performed by BRAND and ZMK)

4.3.1 Group 1 investigation

The Type II experimental scheme given in Table 2 was applied by ARTEL with the following results summarized in Table 4 below. The scheme was applied in full scale for a pipette of *medium* size 20 – 100 µL, while for pipettes of *small* size (1 – 10 µL) and *large* size (200 – 1000 µL) single parameter experiments only (#3 and #3 & #4), respectively, were performed.

Table 4. Type II experimental scheme results – Group 1

EXPERIMENTAL DESIGN SCHEME II (b) - ARTEL - GILSON Pipettes										
Pipette	1-10 µL			200-1000 µL			20-100 µL			
EXPERIMENT #	V	U	En	V	U	En	V	A [%]	U	En
1 Reference conditions	**9.9307**	**0.09**	0	**998.308**	**3.20**	0	**99.717**	**-0.28**	**0.46**	0
	4.9680	**0.07**	0	**498.342**	**1.60**	0	**49.857**	**-0.29**	**0.25**	0
	0.9961	**0.03**	0	**201.193**	**0.71**	0	**19.882**	**-0.59**	**0.14**	0
2a (T=+4 deg)							99.491	-0.51	0.46	-0.35
							49.790	-0.42	0.25	-0.19
							19.858	-0.71	0.14	-0.12
2b (T=+37deg)							101.511	1.51	0.46	2.76
							50.943	1.89	0.25	3.07
							19.979	-0.10	0.14	0.49
3 (Solution B)	10.11	0.09	1.32	992.334	3.20	-1.32	99.918	-0.08	0.46	0.31
	5.08	0.07	1.19	494.938	1.60	-1.50	49.995	-0.01	0.25	0.39
	1.09	0.03	2.13	198.866	0.71	-2.32	20.209	1.05	0.14	1.65
4				996.071	3.20	-0.49	99.293	-0.71	0.46	-0.65
				497.006	1.60	-0.59	49.563	-0.87	0.25	-0.83
				200.072	0.71	-1.12	19.915	-0.42	0.14	0.17
5a (T=+4 deg)							99.386	-0.61	0.46	-0.51
							49.714	-0.57	0.25	-0.40
							20.407	2.04	0.14	2.65
5b (T=+37deg)							101.429	1.43	0.46	2.63
							50.931	1.86	0.25	3.04
							20.276	1.38	0.14	1.99
6							99.536	-0.46	0.46	-0.28
							49.755	-0.49	0.25	-0.29
							19.961	-0.20	0.14	0.40
7a (T=+4 deg)							97.906	-2.09	0.46	-2.78
							49.369	-1.26	0.25	-1.38
							20.149	0.75	0.14	1.35
7b (T=+37deg)							101.541	1.54	0.46	2.80
							50.481	0.96	0.25	1.77
							20.285	1.42	0.14	2.03
8a (T=+4 deg)							98.497	-1.50	0.46	-1.87
							49.526	-0.95	0.25	-0.94
							19.816	-0.92	0.14	-0.34
8b (T=+37deg)							101.248	1.25	0.46	2.35
							50.131	0.26	0.25	0.77
							19.812	-0.94	0.14	-0.36

The qualitative evaluation of the results for experiments #3 and #4 (influence of solution type and tip) for *small (1-10µL), medium (20-100 µL) and large* (200-1000 µL) volume pipettes has already been graphically depicted in the corresponding diagrams in Figures 5 and 7. As already explained in the previous paragraph, a quantitative evaluation of the effect of these parameters is not feasible, unless the complete nature and properties of the influencing factors are well described. This is not the case in the present investigation, therefore only a qualitative approach is adopted for these two experimental parameters. However, the effect of all other experimental factors is evaluated both qualitatively and quantitatively. The result of the evaluation for each experiment is presented in plots where the left y axis represents the qualitative outcome and the right y-axis the quantitative outcome.

4.3.1.1 Influence of one parameter: Temperature at two levels (+4 °C and +37 °C)

The influence of water temperature at two extreme levels (+4 °C and +37 °C) away from reference temperature (20 °C) was examined in experiments 2 a & 2 b (Table 4) and the result is depicted in Figure 12. The calibrations were performed with pre-rinsed tips according to the calibration protocol. This procedure is expected to establish the following condition for the case of warm and cold liquids, respectively. When pipetting a warm liquid, the captive air inside the tip is warmed up due to radial heat exchange with the tip walls which are almost adapted to the temperature of the warm liquid during pre-rinsing. As the piston travels further upwards outside the tip area, due to radial heat exchange again the captive air cools down since the pipette is colder and contracts. At the same time, heat exchange is also taking place across the interface between captive air and liquid (axial heat transfer). This axial heat exchange is counter acting the radial effect since the captive air in contact with the liquid surface becomes warmer and expands. Overall the radial effect is dominant, the captive air volume decreases and the resulting delivered volume increases. This is particularly true for the 100 μL and 50 μL points, while the effect is much less intense at the minimum volume. The situation is reversed for the case of a cold liquid. The results obtained are in line with the aforementioned behavior and also in agreement with previous findings [11,19]. An average temperature coefficient of the order of 0.1% / °K characterizes the shift in accuracy for the warm liquid data, but with the cold liquid data, although the absolute temperature difference is approximately the same (ΔT ≈ 15 °C), the effect is not symmetric in magnitude. Again the accuracy shift is slightly greater for the 100 μL and 50 μL points.

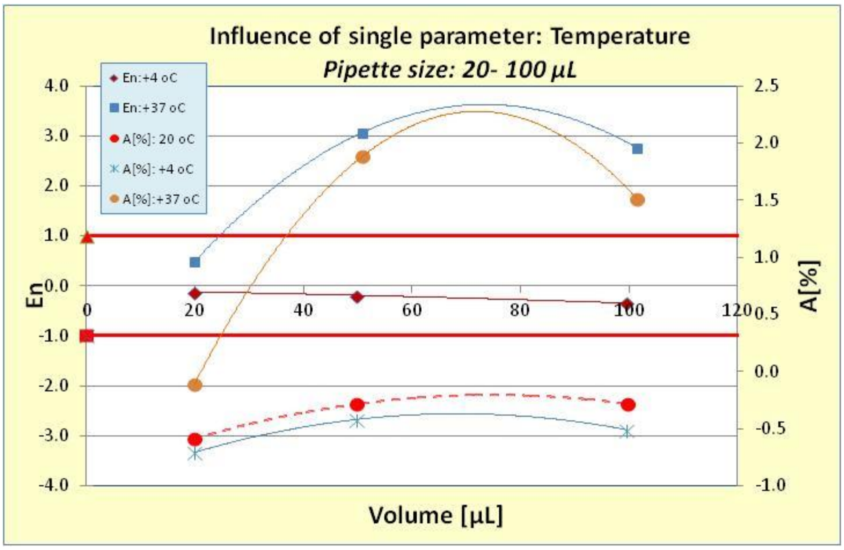


Figure 12. Influence of H_2O temperature in medium size pipettes

4.3.1.2 Influence of two parameters: Solution type & Temperature at two levels (+4 °C and +37 °C)

The combined influence of a liquid other than water at two different temperature extremes with respect to reference conditions (H_2O, 20 °C) was examined in experiments 5a and 5b (Table 4) and it is depicted in Figure 13. The liquid density at 20 °C was approximately equal to water's at the same temperature, while it is lower and higher at +4 °C and +37 °C, respectively. Provided that all other liquid properties are similar to the ones of water, the density alone is expected to enhance the effect of captive volume contraction in the warm case (lower hydrostatic pressure of the liquid column) and the effect of captive volume expansion in the cold liquid case (higher hydrostatic pressure of the liquid column). Here, the effect obtained in experiments 2a and 2b is repeated but only for 100 µL and 50 µL, while the result for 20 µL for cold solution is unexpected.

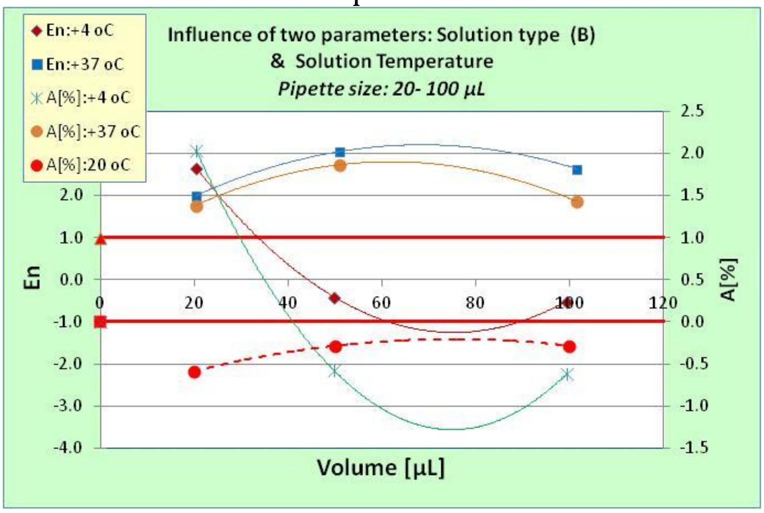


Figure 13. Combined influence of liquid type and temperature on medium size pipettes

4.3.1.3 Influence of two parameters: Liquid type & Tip

The combined influence of a liquid other than water (Solution B) and tip type was examined in experiment 6 (Table 4) and it is depicted in Figure 14.

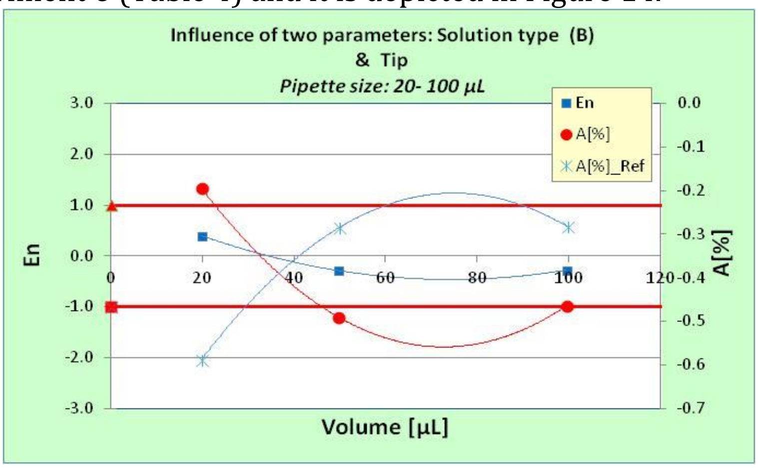


Figure 14. Combined influence of liquid type and tip on medium size pipettes

As can be seen in the figure above, although the combined effect of liquid type and tip has a moderate influence for 50 µL and 100 µL and a significant influence at 20 µL on the accuracy of the pipette compared to the reference conditions, overall the results are within the equivalence limits. This is rather expected, given that the liquid's density is similar to water's and the tips although not being the ones recommended by the manufacturer were of good quality. This is of course only true for this particular examination with this specific pipette size and no general statement can be formulated.

4.3.1.4 Influence of two parameters: Liquid (H_2O) temperature & Tip

The combined influence of water temperature and tip type was examined in experiments 8a and 8b (Table 4), while the parameter of temperature was set at two levels +4 °C and +37 °C, respectively. The results are depicted in Figure 15.

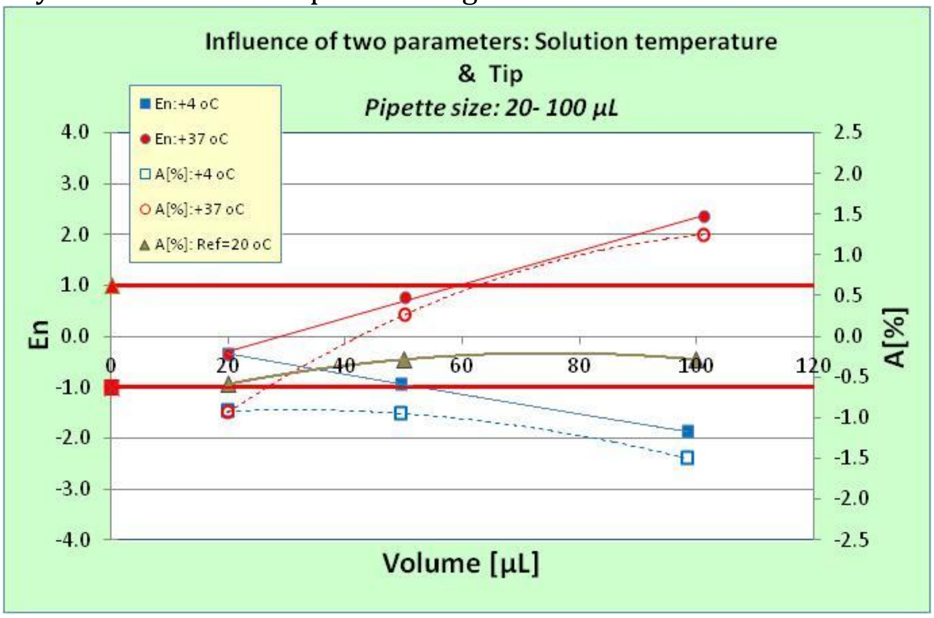


Figure 15. Combined influence of water temperature and tip on medium size pipettes

According to Figure 15, the combined influence of water temperature and tip has an unexpected effect at the lower limit (20 µL) of the volume range of the pipette, while at all other volumes the effect is in accordance with the theoretical predictions described in the previous paragraphs. In particular, at 50 µL and 100 µL the pipette is delivering more and less volume with warm and cold water, respectively, compared to the reference conditions. Moreover, the temperature sensitivity is approximately the same for both warm and cold solution (≈0.08%/°K) and in rather good agreement with the case of temperature influence alone as described in §4.3.1.1. At 20 µL, on the contrary, the radial effect is very small rendering the axial effect dominant and this situation leads to the reversal of the phenomena.

4.3.1.5 Influence of three parameters: Liquid temperature, liquid type & tip

The simultaneous influence of three parameters (liquid temperature, liquid type and tip type) was investigated in experiment 7 (Table 4) and the results are shown in Figure 16.

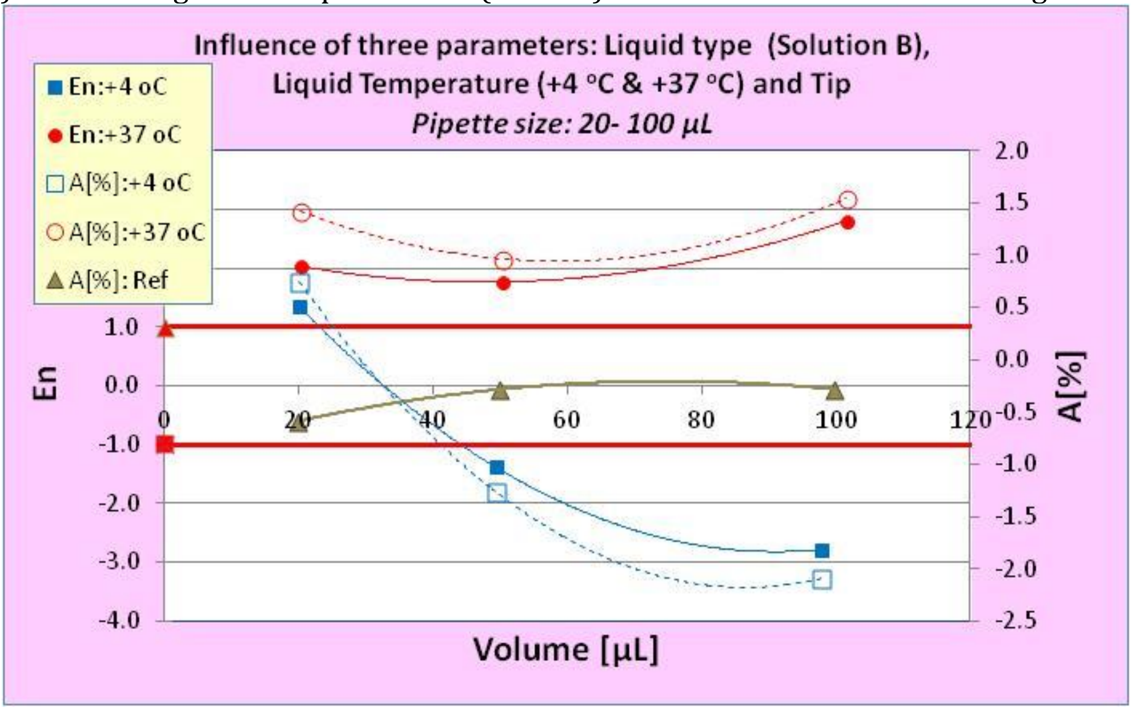


Figure 16. Simultaneous influence of liquid type, liquid temperature and tip type on medium size pipettes

It can be observed that the combined influence of the three parameters has an effect similar to the one obtained with the two parameters in the previous paragraph for the 50 µL and 100 µL volumes. This effect is in accordance to the theoretical predictions (increased delivered volume for warm solution and decreased for cold solution). Moreover, the effect seems also to be symmetric with approximately the same temperature coefficient slightly increased (≈0.1%/K) compared with the two parameter case. At 20 µL, on the contrary, more volume is delivered compared to reference conditions for cold liquid which again can be explained by the domination of the axial effect over the radial as obtained in Fig. 15.
Overall, the effect of the three parameters combined is significant along the complete measurement range of the pipette.

4.3.2 Group 2 investigation

One parameter only (temperature of water) was investigated at several different levels (5, 20, 25 and 36 °C) using two micropipettes of size 1 – 10 µL from two different manufacturers.

The two participants (BRAND & ZMK) duplicated their measurements to some degree. The summarized results of ZMK and BRAND are given in Tables 5 and 6, respectively.

Table 5. Type II experimental scheme results – Group 2-ZMK

EXPERIMENTAL DESIGN SCHEME II (a) - ZMK								
Pipette	**BRAND Transferpette 0.5-10 µL**			**Pipette**	**Eppendorf Reference 0.5-10 µL**			
EXPERIMENT #	**V**	**U**	**En**	**EXPERIMENT #**	**V**	**A[%]**	**U**	**En**
A Reference Conditions	9.998	0.030	0.00	B Reference Conditions	9.974		0.030	0.00
	5.041	0.023	**0.00**		4.947		0.023	**0.00**
	1.063	0.015	**0.00**		0.963		0.015	**0.00**
1 (Tw=+5.0 deg)	9.927	0.39	-0.18	(Tw=+5.0 deg)(Op	9.840		0.39	-0.34
	5.04	0.30	0.00		4.970		0.30	0.08
	1.105	0.20	0.21		0.977		0.20	0.07
2 (Tw=+20.0 deg)	9.984	0.070	-0.18	2 (Tw=+20.0 deg)	9.946		0.070	-0.37
	5.008	0.053	-0.57		4.989		0.053	0.73
	1.062	0.035	-0.03		0.996		0.035	0.87
5 (Tw=+22.5 deg)	9.996	0.030	-0.05	5 (Tw=+22.5 deg)	9.972		0.030	-0.05
	5.041	0.023	0.00		4.948		0.023	0.03
	1.065	0.015	0.09		0.964		0.015	0.05
9 (Tw=+25.0 deg)	9.975	0.070	-0.30	9 (Tw=+25.0 deg)	9.979		0.070	0.07
	5.017	0.053	-0.42		4.997		0.053	0.87
	1.039	0.035	-0.63		1.024		0.035	1.60
12 (Tw=+36 deg)	10.127	0.30	0.43	12 (Tw=+36,0 deg)	10.267		0.30	0.97
	5.029	0.23	-0.05		5.104		0.23	0.68
	1.019	0.15	-0.29		1.037		0.15	0.49

Table 6. Type II experimental scheme results – Group 2-BRAND

EXPERIMENTAL DESIGN SCHEME II (a) - BRAND								
Pipette	Transferpette 0.5-10 µL			Pipette	Refrerence 0.5-10 µL			
EXPERIMENT #	V	U	En	EXPERIMENT #	V	A[%]	U	En
A Reference Conditions (Tw=+22.1 deg ΔT=0	9.969	0.04	0.00	B Reference Conditions	9.857	-1.430	0.04	0.00
	4.998	0.03	0.00		4.897	-2.060	0.03	0.00
	1.008	0.02	0.00		0.936	-6.400	0.02	0.00
1 (Tw=+5.1 deg)(Op 2)	9.850	0.12	-0.94	1 (Tw=+5.1 deg)(Op 2)	9.798	-2.020	0.12	-0.47
	4.99	0.09	-0.09		4.927	-1.460	0.09	0.32
	1.04	0.06	0.51		1.000	0.006	0.06	1.01
2 (Tw=+20.1 deg)	9.968	0.05	-0.02	2 (Tw=+20.1 deg)	9.903	-0.970	0.05	0.72
	5.015	0.04	0.34		4.953	-0.940	0.04	1.12
	1.009	0.03	0.03		0.958	-4.250	0.03	0.61
3 (Tw=+19.9 deg)	9.972	0.05	0.05	3 (Tw=+20.0 deg)	9.870	-1.300	0.05	0.20
	5.002	0.04	0.08		4.927	-1.460	0.04	0.60
	1.018	0.03	0.28		0.943	-5.700	0.03	0.19
4 (Tw=19.9 deg)	9.947	0.05	-0.34	4 (Tw=19.9 deg)	9.880	-1.200	0.05	0.36
	4.989	0.04	-0.18		4.921	-1.570	0.04	0.48
	1.016	0.03	0.22		0.944	-5.580	0.03	0.22
5 (Tw=+22.5 deg) (Op 2)	9.990	0.04	0.37	(Tw=+22.5 deg) (Op 2	9.907	-0.933	0.04	0.88
	5.022	0.03	0.57		4.950	-1.003	0.03	1.25
	1.034	0.02	0.92		0.939	-6.081	0.02	0.11
6 (Tw=+22.1 deg)	9.978	0.04	0.16	6 (Tw=+22.5 deg)	9.860	-1.400	0.04	0.05
	4.997	0.03	-0.02		4.908	-1.840	0.03	0.26
	1.009	0.02	0.04		0.938	-6.150	0.02	0.07
7 (Tw=+22.0 deg)	9.970	0.04	0.02	7 (Tw=+22.5 deg)	9.863	-1.370	0.04	0.11
	5.004	0.03	0.14		4.924	-1.510	0.03	0.64
	1.015	0.02	0.25		0.950	-5.020	0.02	0.49
9 (Tw=+25.3 deg)	10.006	0.05	0.58	9 (Tw=+25.1 deg)	9.890	-1.100	0.05	0.52
	5.009	0.04	0.22		4.910	-1.810	0.04	0.26
	1.018	0.03	0.28		0.944	-5.630	0.03	0.22
10 (Tw=+25.2 deg)	10.019	0.05	0.78	10 (Tw=+25.4 deg)	9.902	-0.980	0.05	0.70
	5.007	0.04	0.18		4.935	-1.300	0.04	0.76
	1.017	0.03	0.25		0.946	-5.370	0.03	0.28
11 (Tw=+25.3 deg)	9.990	0.05	0.33	11 (Tw=+25.4 deg)	9.906	-0.940	0.05	0.77
	4.999	0.04	0.02		4.933	-1.350	0.04	0.72
	0.995	0.03	-0.36		0.932	-6.820	0.03	-0.11
12 (Tw=+36 deg)	10.120	0.08	1.69	12 (Tw=+35.8 deg)	10.043	0.430	0.08	2.08
	5.055	0.06	0.85		5.002	0.040	0.06	1.57
	1.018	0.04	0.22		0.917	-8.260	0.04	-0.42
13 (Tw=+35.8 deg)	10.145	0.08	1.97	13 (Tw=+35.8 deg)	10.089	0.890	0.08	2.59
	5.083	0.06	1.27		5.007	0.150	0.06	1.64
	1.019	0.04	0.25		0.923	-7.670	0.04	-0.29
14 (Tw=+35.8 deg)	10.124	0.08	1.73	14 (Tw=+36.2 deg)	10.059	0.590	0.08	2.26
	5.083	0.06	1.27		5.005	0.090	0.06	1.61
	1.013	0.04	0.11		0.928	-7.170	0.04	-0.18

The results obtained from the above experiments are shown graphically in Figures 17, 18 and 19 for the three calibration points corresponding to 10 µL, 5 µL and 1 µL, respectively. The graphs show the influence of water temperature on the behavior of the micropipettes, simultaneously on qualitative (En/right y-axis) and quantitative (A[%]/left y-axis) level. The evaluation of the results is significantly facilitated by the information given in Table 7 which refers to the parameters that directly affect the relative magnitude of radial and axial heat exchange during aspiration.

Table 7. Pipette parameters that define the magnitude of radial and axial heat exchange

Volume vs. tip dimensions for a 10 µL pipette			
Volume setting [µL]	10	5	1
Surface area of the liquid in a 10 µL tip [mm^2]	1.3	0.9	0.3

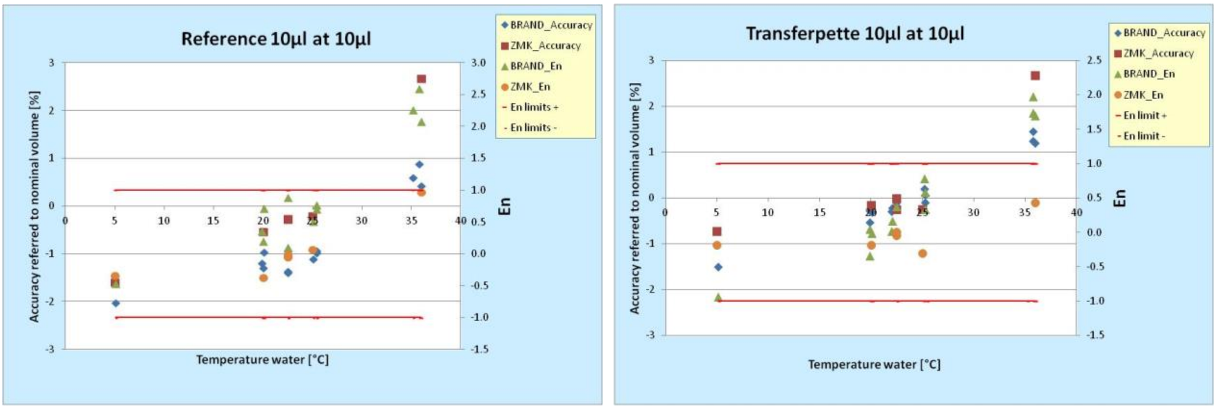


Figure 17. Influence of H_2O temperature at 10 µL

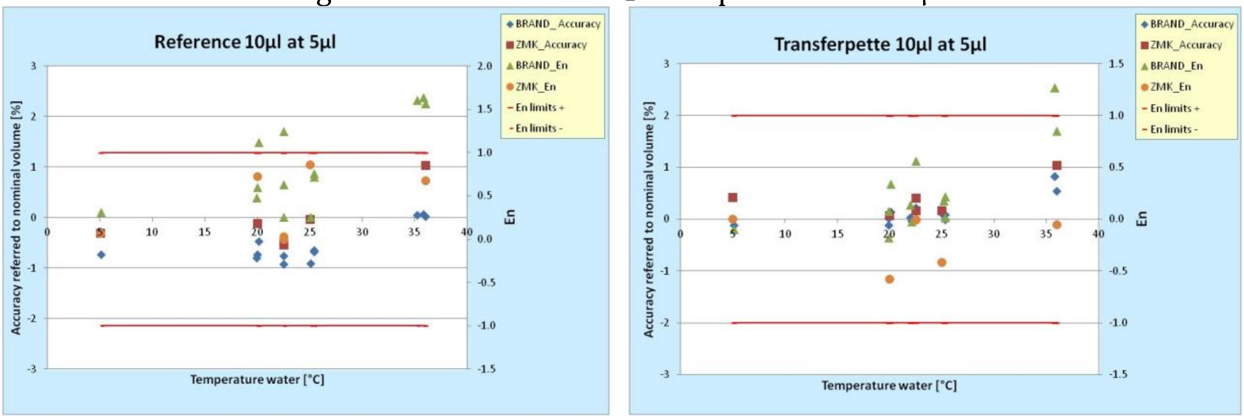


Figure 18. Influence of H_2O temperature at 5 µL

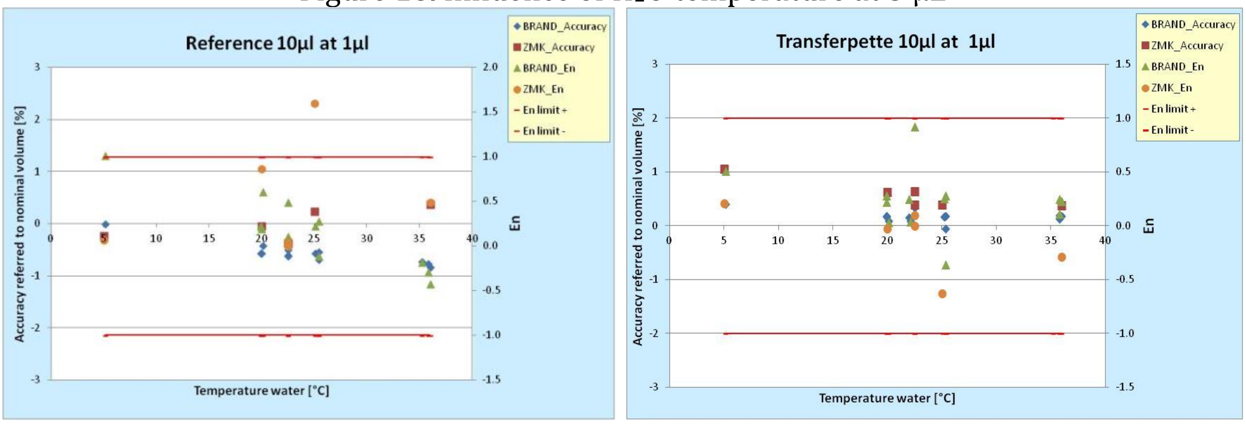


Figure 19. Influence of H_2O temperature at 1 µL

The observations that can be made on the results depicted in the above plots should be interpreted under the light of the following facts:

- The accuracy of the pipette to deliver the set volume is clearly dependent on the temperature of the liquid (the pipette and ambient air being at the same temperature, ≈20 °C), as theoretically predicted and experimentally established in previous investigations [11].
- The temperature dependence is a demonstration of a heat exchange process that takes place in air cushion pipettes between tip wall, captive air and pipette in both radial and axial direction.
- These two heat exchange processes are counter acting, therefore the overall temperature effect depends on their relative sizes.
- The radial heat exchange is proportional to the aspirated volume, while the axial heat exchange is proportional to the surface area of the liquid.
- In the case of a 1-10 µL pipette, at 1 µL the aspirated volume is 10 times smaller than the nominal volume, while the liquid surface area is approximately only 3 times less than the corresponding value at 10 µL. This means that while the radial heat exchange becomes 10 times less at 1 µL, the axial heat exchange becomes only 3 times less leading eventually to an inversion of the balance between these two counter acting effects.
- This relation between pipette accuracy referred to nominal volume (or simply delivered volume) and water temperature can be described by a linear model where the slope is decreasing as the aspirated volume is decreasing

Indeed this is the situation depicted in the plots in Figures 17-19. At 10 µL the radial heat exchange is dominating, leading to higher volumes at high temperatures and lower volumes at low temperatures compared to the reference conditions. The over delivery at 36 °C is such that the delivered volume is outside the set equivalence limits (Figure 17).
At 5 µL the radial heat exchange is still dominant over the axial heat exchange but to a less extent. Again at 36 °C the temperature has a significant effect in the delivered volume being outside the equivalence limits as shown in Figure 18.
At 1 µL the axial heat exchange becomes dominant and this leads to a reverse situation as shown in Figure 19. In this case all results are equivalent and the effect of temperature in the range between +5 to +36 °C becomes insignificant according to the criteria set.

4.4 Influence of tip change and pre-rinsing

The aim of this investigation was to examine what happens if the tip is mounted on the micropipette and not pre-rinsed at all, then used once and discarded, <u>as the customer usually does</u>. The experiments were performed only with the small micropipette Transferpette S (1-10 µL) at the nominal volume at the two extreme temperatures +5 °C and +36 °C. The results of this investigation both on qualitative (En/right y-axis) and quantitative (A [%]/left y-axis) level are shown in Figure 20.

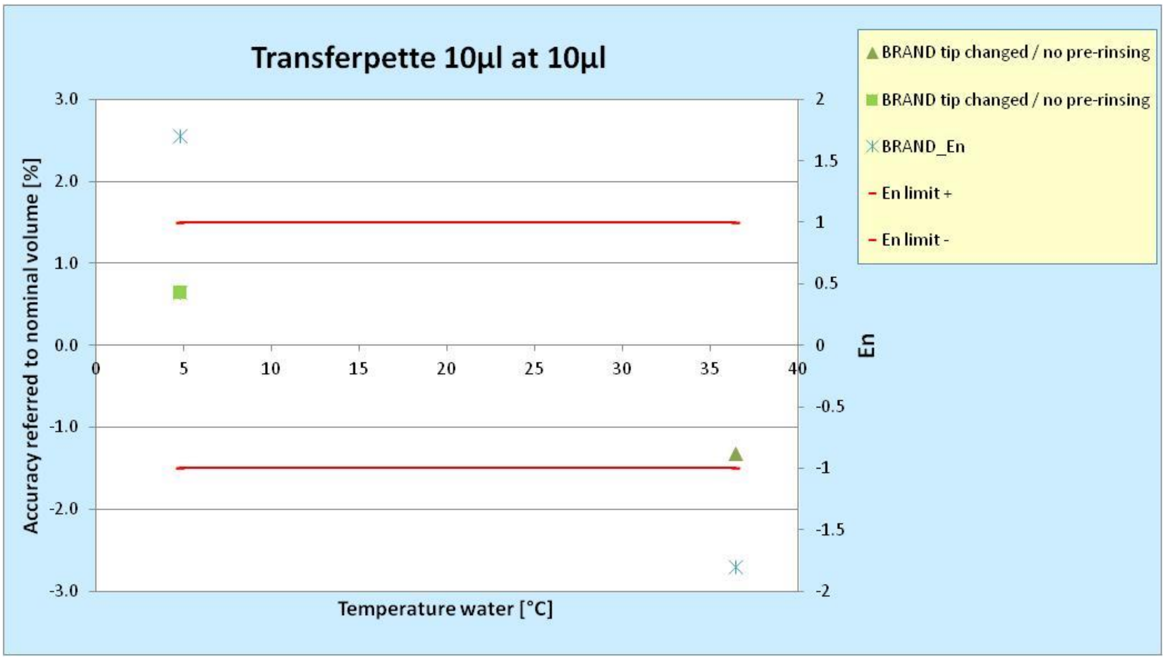


Figure 20. Influence of tip change and pre-rinsing

The situation depicted in the above diagram is completely in line with the theoretical predictions. In particular we see that absence of pre-rinsing leads to higher delivered volumes with cold liquid and lower delivered volumes with warm liquid. This is because in the absence of pre-rinsing all parts of captive air are at the same temperature (ambient ≈ 20 ^{o}C), while the air in contact with the liquid in the lower part of the tip contracts or expands when the liquid is cold or warm, respectively. This, in turn, causes an increase and a decrease in the delivered volume, respectively. In addition, in both cases above, the temperature has a pronounced effect on the results, which lay well outside the set significance limits.

4.5 Evaluation of influences with respect to EN ISO 8655-2 specifications

As stated in §4.1, the criteria adopted for the evaluation of the results were chosen not to be the specifications of the relevant EN ISO standard as being too high and modest for this investigation. However, it is definitely of great value, especially for the end user of air displacement pipettes, to know if the accuracy of these instruments is affected to such a degree by a certain parameter or combination of parameters that it does not comply anymore with the specifications set by ISO 8655-2. To this end the summarized effect of the parameters investigated which seriously affect the performance of the pipettes is presented in the following Table 8. The content of this table refers to medium size air displacement pipettes (20-100 µL).

Table 8. Parameter influence with respect to EN ISO 8655-2 specifications for 20 – 100 μL pipettes

Influence parameter of combination of parameters	**Compliance (YES) / Non Compliance (NO)**	
	High Temperature (+37 °C)	**Low Temperature (+4 °C)**
Liquid Temperature	**NO** (except at 20 μL)	Marginally YES
Liquid Temperature + Solution Type	**NO**	YES (**except at 20 μL**)
Solution Type + Tip	YES	YES
Solution Temperature + Tip	YES (**except at 100 μL**)	YES (**except at 100 μL**)
Solution Temperature + Solution Type + Tip	**NO**	**NO**

As shown in Table 8 all combinations involving temperatures of the working liquid at extreme values (either warm or cold) lead not only to significant influences but to overriding the MPE posed by the relevant standard.

5 Discussion & Conclusions

A high degree of expertise from national metrology laboratories and manufacturers was combined to deliver a large set of data which comprise a wide experimental basis of evidence regarding the influence of various parameters involved in the measurement of small liquid quantities using air displacement pipettes in the range of 1 μL up to 1000 μL.
This experimental evidence was combined with existing theoretical approaches and previous research in this field in order to provide a solid background with the widest possible scientific consensus which will assist the scientific and metrological community to deliver reliable and accurate results through vital improvements in the calibration, operation and handling of micropipettes.
Moreover, the present work provides updated scientific knowledge in this field to be disseminated to relevant working committees and international organizations assisting them in a future reformulation of relevant ISO standards and guides.
Last but not least, the results of this work are expected to provide the basis for the formulation of a comprehensive guide aiming at assisting the final end users of micropipettes in the metrological, medical and analytical sector worldwide in their effort for more reliable results especially in critical micro volumes to the benefit of society.
The novelties of this investigation were the following:

1. The expertise and knowledge of both national metrology institutes and manufacturers were combined in this research work.
2. The investigation was designed in such a way that it resembles more to the laboratory use of micropipettes (e.g. in clinical labs) rather than to the conditions of a calibration in a national metrology institute laboratory according to specifications which are usually far from the daily routine in the actual working environment.
3. The aim of this approach was manifold:

- To examine how much a calibration result obtained under controlled standard laboratory conditions describes the performance of a pipette in real working conditions.
- To investigate the influence of basic experimental parameters usually present under typical working conditions and examine the single as well as the combined effect of those parameters in the delivered volume.
- To obtain both qualitative and quantitative measures of the impact of the parameters based on which the significance of these factors can be evaluated.
- Based on the accumulated knowledge obtained to reach a wide consensus regarding a re-evaluation of the measurement uncertainty budget among all interested parties in order for it to reflect in a more realistic way the volume measurement process with air cushion pipettes.

To this end several experimental schemes were applied with basic parameters being the type of liquid, the type of tip, the temperature of liquid, the experience of the operator and finally the presence or absence of pre-rinsing (in combination with different water temperatures) of the tip during calibration.
The main points and conclusions of this investigation are the following:

- Air cushion pipettes are measuring systems, not single instruments, comprised by the pipette body and the attached disposable tip. A critical component of this system is the captive air entrapped in the space between the tip's lower edge and the pipette piston.
- The physical phenomena taking place within this space involve complex interactions between air cushion, tip (shape, size and material), aspirated liquid characterized by its physical and thermal properties and ambient environment, while the complete system is also prone to additional effects originating from the operator's handling, skills and experience.
- Four parameters within this complex interaction system have been identified as most critical for the measurement result, namely operator's skills, tip type, liquid type and temperature difference between liquid and (pipette/air) system. Their influence, however, is not easily isolated from other influences, while at the same time most of these parameters are neither uniquely nor exhaustively characterized by a single property (e.g. a liquid is characterized by several properties like density, viscosity, surface tension).
- In this context, the influence of tip type was investigated only with respect to the origin of the tip being recommended or not by the manufacturer. Similarly, the influence of the operator was examined as being experienced or less experienced, while the type of liquid was defined only with respect to its density. In all the above cases the results were only qualitatively evaluated because any other attempt was lacking reasonable criteria.
- Good quality tips of origin other than the pipette manufacturer have shown to have an influence on the delivered volume. However, since no information was available about geometrical, physicochemical and constructional parameters of the alternative tips, it was not possible to attribute the observed behavior to a certain feature of the tips.
- Similarly, only qualitative indications about the influence of the operator were obtained, but no specific conclusions can be drawn since there was no strict and unified evaluation protocol of operator's skills that could provide quantitative evaluation criteria.

- Pipettes from different manufacturers demonstrate similar behavior on a qualitative basis, but have shown differences in absolute values of accuracy.
- A meaningful evaluation of the influence of liquid temperature can only be made under the light of the measurement routine used during calibration regarding the rinsing of the tip prior to aspiration. This preconditioning practice is entirely decisive for the outcome of this kind of investigation.
- The influence of temperature alone becomes significant mainly at nominal and intermediate volumes, while at the minimum value the effect is usually insignificant. The effect of temperature has higher impact in the warm side of the temperature range tested.
- The temperature sensitivity coefficients obtained through the course of this investigation were generally in agreement with previous investigations but somewhat lower.
- Any combination of temperature with solution type or tip type leads to volume deliveries at nominal volume outside the set equivalence limits.
- All three parameters combined, on the contrary, lead to volume deliveries which are not equivalent along the complete working range of the pipette and not just the nominal volume.
- Pre-rinsing of tips leads to higher delivered volumes with warm liquids and lower delivered volumes with cold liquids, the results obtained being in agreement with previous investigations. The situation is reversed in the absence of pre-rinsing. Absence of pre-rinsing means that the tip is filled only once and this aspired volume is delivered. Absence of pre-rinsing leads additionally to non-equivalent results at the nominal volume for 1 – 10μL pipettes.
- Last but not least the influences of the parameters or their combinations are not just significant according to the criteria adopted in this investigation, but in several cases are so severe that lead to performances outside the specifications set by the EN ISO 8655-2 standard.

6 Future recommendations

The findings in this investigation, as discussed in the previous paragraphs, definitely point out the need for the metrological and manufacturer community to further invest in joint similar research efforts in volume determination areas of even lower volume amounts (micro and nano liters) where the influences are expected to be even more pronounced. In addition, this type of investigation should be extended to different type of volume delivering instruments. Moreover, based on the present findings the uncertainty evaluation budgets should be revised accordingly in order to take into account the above mentioned effects.

7 Acknowledgments

The NMIs members of EURAMET TC-F who participated in this research project and in particular the pilot NMI (EIM) and Dr. Zoe Metaxiotou wish to thank the manufacturers and companies that jointed this project for their great support and share of their experience, their constructive criticism and contribution in this work. Special thanks to Dr. Barbara Werner for her hospitality and Herr Rainer Feldmann for his great contribution in the interpretation of the results and support along the course of this project.

8 Literature

[1] Kornelia Ewald, "Liquid Handling Today, Fundamentals of Dispensing", G.I.T. Laboratory Journal 05-06/2008, pp 42–44, GIT VERLAG GmbH & Co. KG, Darmstadt.

[2] GILSON Guide to Pipetting, Second Edition.

[3] Rodrigues G., and D. Rumery, "Accuracy of Liquid Delivery Using Pipettes – Reproducibility of Calibration is the missing piece", NCSL International Workshop and Symposium, 2015.

[4] Thermo Scientific, Good Laboratory Pipetting Guide.

[5] Expert Report DKD-E 8-1: Experimental study on the calibration of piston-Operated pipettes with air cushion, Dr. Barbara Werner et al.,2013.

[6] G. Rodrigues, Bias, Uncertainty and Transferability in Standard Methods of Pipette Calibration, 2013.

[7] Verification Procedure for Accuracy and Precision, Procedure LT802292/F - © 2007 Gilson SAS, 2007.

[8] Defining accuracy & precision, ARTEL Lab report, Issue 4.

[9] Setting Tolerances, ARTEL Lab report, Issue 5

[10] Guideline DKD-R 8-1: Calibration of piston-operated pipettes with air cushion, 2011.

[11] R. Feldmann and Karl Heinz Lochner, Influences on volume in piston-operated air-displacement pipettes, Accred Qual Assur (2016), 21, 69-82.

[12] A. Björn Carle et al., Best Practices for the Use of Micropipettes", AMERICAN LABORATORY, 1, 2014.

[13] Measurement Good Practice Guide No. 69, "The Calibration and Use of Piston Pipettes", J. Blues et al.,NPL, 2004.

[14] A. Björn Carle et al., Errors in Liquid Delivery – The Importance of Laboratory Conditions on Pipetting, ARTEL

[15] A.B. Carle et al., "Liquid Delivery Errors – The Impact of Laboratory Conditions on Volumetric Measurements ", ARTEL.
[16] Christoph Spälti et al., "Influence of altitude on the dispensed volume of a piston pipette with air cushion" , 2011.
[17] Rodrigues, G., "Climb Every Mountain**:** Extreme pipetting: the effect of barometric pressure on data integrity"**,** *Pharmaceutical Formulation & Quality,* **2007**, 40-43.
[18] Muriel Art et al., "The Tip of the Iceberg: How Pipette Tips Influence Results", Eppendorf Application Note No 354, 2016.
[19] Expert Report DKD-E 8-2: Analysis of influencing parameters on calibration of piston-operated pipettes with air cushionsK. H. Lochner, R. Feldmann, J. Pfohl, (2013).
[20] E. Batista et al., "A Study of Factors that Influence Micropipette Calibrations", NCSLI Measure J. Meas. Sci., Vol. 10 No. 1, 2015.
[21] K. Shirono, M. Shiro, H. Tanaka, and K. Ehara, "Evaluation of "method uncertainty" in the calibration of piston pipettes (micropipettes) using the gravimetric method in accordance with the procedure of ISO 8655-6," Accred. Qual. Assur., vol. 19, no. 5, pp. 377-389, October 2014.

APPENDIX I

Characterization of solutions other than water used in the course of EURAMET Project 1295

Instituto Português da Qualidade

REPÚBLICA PORTUGUESA
ECONOMIA

PORTUGUESE INSTITUTE FOR QUALITY
Rua António Gião, 2
2829-513 CAPARICA Portugal
Tel. 212 948 186
Fax. 212 948 188
metrologia@ipq.pt
www.ipq.pt

Tabela de Resultados
Table of Results

Glicerol A	
Temperatura *Temperature* °C	Massa Volúmica *Sample Density* kg/m^3
17,001	1101,260
20,000	1099,932
22,999	1098,568

Tabela de Resultados
Table of Results

Glicerol B	
Temperatura *Temperature* °C	Massa Volúmica *Sample Density* kg/m^3
17,001	998,794
20,001	998,221
22,999	997,554

Tabela de Resultados
Table of Results

Glicerol C	
Temperatura *Temperature* °C	Massa Volúmica *Sample Density* kg/m^3
17,002	1089,085
20,001	1087,841
22,999	1086,553

Rudolph Research Analytical
55 Newburgh Road
Hackettstown
NJ 07840

RUDOLPH RESEARCH ANALYTICAL

Date & Time: Wednesday, Feb 10 2016, 10:34 AM

This sample was measured on 2911 Plus, Serial Number DDM3222, manufactured by Rudolph Research Analytical, Hackettstown, NJ, USA.

Method Name : Artel Density
Sample ID : Triton X/DI 1 liter
OPERATORS : T Schafer
Sample Temp. : 20.000 Deg C

B

Parameters

P1 - Density
P2 - Relative Density
P3 - Period of Oscillation

No.	P1 Density g/cm^3	P2 Relative D..	P3 Period of .. ms	Date Time
1	0.998213	1.000007	2.9271401	Feb 10 2016 10:34 AM
2	0.998211	1.000005	2.9271388	Feb 10 2016 10:34 AM
3	0.998211	1.000005	2.9271389	Feb 10 2016 10:34 AM
Mean:	0.998212	1.000006	2.9271393	
SD:	0.000001	0.000001	0.0000007	

CHEMICAL METROLOGY LABORATORY (EXHM/GCSL-EIM)
GENERAL CHEMICAL STATE LABORATORY-HELLENIC METROLOGY INSTITUTE
5TH ATHENS CHEMICAL SERVICE, 16 TSOCHA STREET, 115 21 ATHENS, GREECE
tel: +30 210 6479136-8 fax:+30 210 6479114 email: metrology@gcsl.gr

DMSO	water	solution	DMSO	density #1	density #2	density #3	density #4	average density	density uncertainty (u)
mass (g)	mass (g)	mass (g)	conc (% wt)	g/mL at 20 °C (measured in a Anton Paar DMA 5000 digital pycnometer)					
4.38548	39.46932	43.85480	10.00	1.011175	1.011177	1.011177	1.011173	1.011176	9.57E-07
14.69591	34.29046	48.98637	30.00	1.040789	1.040787	1.040783	1.040782	1.040785	1.65E-06