Final Report

Calibration of micropipettes using the photometric method

EURAMET Project no. 1425

IPQ – Coordinator of the comparison

Elsa Batista

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Contents

1. Introduction

During the EURAMET TC F 2017 meeting the possibility of having a comparison on calibration of micropipettes using the photometric method was discussed in the volume subgroup [1]. Artel, one of the manufactures of a photometer volunteered to cooperate with EURAMET in this comparison. Since the majority of the laboratories do not have this method implemented in their laboratories it was decided to register this project as cooperation in research project. Artel supplied the photometer, the micropipettes and the tips. Training was given to the participants prior to the measurements.

The Volume and flow Laboratory of Portuguese Institute for Quality (IPQ) - National Laboratory of Metrology (NMI), acting as the pilot laboratory performed the initial and final measurements of the micropipettes.

Four micropipettes (transfer package) were tested at different volume capacities. The comparison schedule and participants are described in table 1.

Table 1 – Time table and participants list

NMI	Country	Responsible	Date for measurements
Artel	USA	George Rodrigues	August 2017
IPQ	Portugal	Elsa Batista	1 September - 17 September
FORCE	Denmark	Lise-Lotte Grue	18 September – 8 October
RISE	Sweden	Oliver Buker / Per Wennergren	9 October – 29 October
CMI	Czech Republic	Alena Vospělová/ Miroslava Benkova	30 October – 19 November
MIRS	Slovenia	Urška Turnšek	20 November – 10 December
GUM	Poland	Adam Urbanowicz	11 December – 2 January
EIM	Greece	Zoe Metaxiotou	3 January - 14 January 2018
IPQ	Portugal	Elsa Batista	15 January – 30 January
Artel	USA	George Rodrigues	February 2018

2. The instrument

The chosen instruments are 3 fixed volume Eppendorf pipettes (see figure 1) and 1 variable volume Rainin pipette (see figure 2). The micropipettes operate with an attached removable plastic tip in order to aspirate the liquid. Artel supplied the appropriate tips. The Photometer (PCS – figure 3), computer and software was also supplied by Artel. The participant laboratories were trained by Artel prior to the measurements.

The micropipettes used for this comparison are essentially of plastic material with a coefficient of thermal expansion of $2{,}4 \times 10^{-4}$ / ºC [2].

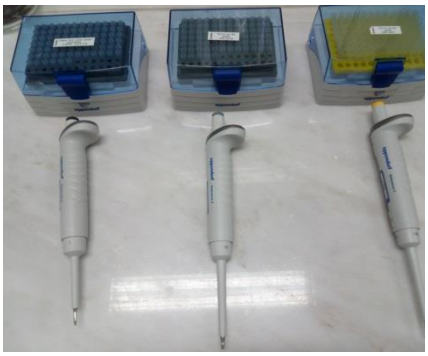

Figure 1- Fixed Micropipettes

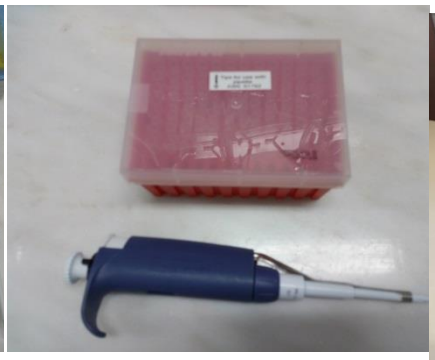

Figure 2 - Variable Micropipettes

Figure 3- PCS

3. Calibration method

3.1 Method description

The photometric method uses a high-resolution photometer and colorimetric solutions to determine the volume delivered by a micropipette [3]. It is a ratiometric method; the volume of liquid delivered is determined by comparison to a larger reference volume of diluent solution. The method is described in ISO 8655-7[1].
The basic principle behind photometric measurement is the conservation of mass. Two additional assumptions are also made to allow the photometric method to be used easily for volume measurements: conservation of volume and the Lambert-Beer Law [3].
In the dual-dye ratiometric photometry two colorimetric solutions are used. Each solution (one red, one blue) has an absorbance peak at a specific analytical wavelength. The basis of this technique is the following: an unknown volume of red dye is delivered into a vial containing a known volume and concentration of blue dye. The concentration of the red dye is also known, and the ratio between the two concentrations is a calibration factor for the method. After mixing, the change in absorbance of the resulting volume can be calculated as a ratio. The equation that describes this measurement principle is the following:

$$V_S = V_B \left(\frac{\frac{A_S}{A_B}}{K - \frac{A_S}{A_B}} \right) \quad (1)$$

Where,
A_S/A_B is the absorbance ratio measured in the Photometer
K is the calibration factor for the dyes
V_B is the volume of the blank solution
Vs is the volume delivery to be determined

3.2 The measurement procedure

Materials/equipment:

1. PCS Instrument Serial number 20030 and Computer with PCS Software, or equivalent

2. Eppendorf Reference 2 100 µL pipette ASN 02517, SN: R20260F, Yellow tips
3. Eppendorf Reference 2 10 µL pipette ASN 02518, SN: R20748F, Light gray tips
4. Eppendorf Reference 2 1 µL pipette ASN 02519, SN: R19948F, Dark gray tips
5. Rainin 0.1-2.5 µL pipette ASN 01792, SN: R18496F, LTS tips
6. PCS Blank and Calibrator A (CAL A) vials
7. PCS Range 2,3,5 & 6 solutions
8. PCS Calibrator Kit
9. Kim Wipe or equivalent

Each laboratory was assigned its own solutions and blank vials in a separate box labeled with the correspondent institute name.

Procedure

1. Pipette calibration through periodic use of the PCS Instrument ensures traceability of dispensed volumes to the International System of Units (SI).
2. The appropriate frequency of calibration is dependent upon accuracy requirements, frequency of use, number of operators using the pipette, nature of the liquids dispensed (corrosiveness, solvent power, etc.), and recommendations made by the manufacturer.
3. For fixed-volume POVA, this calibration procedure is performed with the collection of 10 data points.
4. For variable-volume POVA, this calibration procedure is performed with the collection of 10 data points each at the high, middle, and low volumes. Typically the volumes are 100%, 50%, and 10% of nominal. For this study the 2,5 µL pipette will only be calibrated at 0,1 µL.
5. The accuracy and precision acceptance requirements are primarily dependent upon the requirements placed on the POVA by the applications for which it is used. See Artel Lab Report Issue 5, Setting Tolerances for pipettes in the laboratory (Doc # 19A3230) for guidelines on maximum permissible errors.

4. Evaluation of the measurement results

4.1 Reference value

To determine the reference value the formula of the weighted mean is used, by means of the inverses of the squares of the associated standard uncertainty are the weighting factors [4]:

$$y = \frac{x_1 / u^2(x_1) + \ldots + x_n / u^2(x_n)}{1/u^2(x_1) + \ldots + 1/u^2(x_n)} \tag{2}$$

To determine the standard uncertainty *u(y)* associated with *y* the following expression is used:

$$u(y) = \sqrt{\frac{1}{1/u^2(x_1) + \ldots + 1/u^2(x_n)}} \tag{3}$$

4.2 Consistency determination

To identify an overall consistency of the results a chi-square test can be applied to all *n* calibration results.

$$\chi^2_{obs} = \frac{(x_1 - y)^2}{u^2(x_1)} + \ldots + \frac{(x_n - y)^2}{u^2(x_n)} \quad (4)$$

where the degrees of freedom are: $\nu = n - 1$

The consistency check is regarded as failed if: $\Pr\{\chi^2(\nu) > \chi^2_{obs}\} < 0{,}05$. The function *CHIINV(0,05; n-1)* in MS Excel was used. The consistency check was failing if *CHIINV(0,05; n-1)* < $\chi^2_{obs.}$

If the consistency check did not fail then y was accepted as the KCRV x_{ref} and $U(x_{ref})$ was accepted as the expanded uncertainty of the KCRV.

If the consistency check failed then the laboratory with the highest value of $\frac{(x_i - y)^2}{u^2(x_i)}$ is excluded from the next round of evaluation and the new reference value, reference standard uncertainty and chi-squared value is calculated again without the excluded laboratory.

5. Measurement results

5.1 Micropipette stability

The volume measurements obtained by IPQ in the beginning of the comparison (IPQ-1) and in the end of the comparison (IPQ-2) are presented in the following table.

Table 2 – Volume measurement results

	Nominal volume	Volume (µl)	U_{exp} (µl)	ΔV (µl)
IPQ – 1	100	100,11	0,49	0,23
IPQ - 2	100	100,34	0,29	
IPQ – 1	10	9,945	0,028	0,008
IPQ - 2	10	9,953	0,026	
IPQ – 1	1	1,014	0,009	0,003
IPQ - 2	1	1,011	0,009	
IPQ – 1	0,1	0,098	0,013	0,006
IPQ - 2	0,1	1,004	0,007	

From the obtained results its can be verified that the micropipettes were stable. Only the first results from IPQ were used to determine the reference value.

5.2. Volume results, 100 µl

The obtained results for the 100 microliter micropipette are the following:

Table 3 - Volume results 100 μl

Participant	Volume (μL)	*U* (μL)
ARTEL	99,93	0,49
IPQ 1	100,11	0,29
FORCE	100,24	0,35
RISE	99,38	0,29
CMI	100,36	0,27
MIRS	100,12	0,29
GUM	100,1	0,48
EIM	99,96	0,35
IPQ 2	100,34	0,29

5.3. Volume results, 10 μl

The obtained results for the 10 microliter micropipette are the following:

Table 4 - Volume results 10 μl

Participant	Volume (μL)	*U* (μL)
ARTEL	9,972	0,094
IPQ 1	9,945	0,028
FORCE	10,04	0,11
RISE	9,901	0,035
CMI	9,921	0,055
MIRS	9,948	0,026
GUM	10,009	0,096
EIM	9,923	0,035
IPQ 2	9,953	0,026

5.4. Volume results, 1 μl

The obtained results for the 1 microliter micropipette are the following:

Table 5 - Volume results 1 μl

Participant	Volume (μL)	*U* (μL)
ARTEL	1,018	0,031
IPQ 1	1,014	0,009
FORCE	1,025	0,023
RISE	1,008	0,013
CMI	1,003	0,027
MIRS	1,036	0,008
GUM	1,028	0,024
EIM	1,002	0,013
IPQ 2	1,011	0,009

5.5. Volume results, 0,1 μl

The obtained results for the 0,1 microliter micropipette are the following:

Table 6 - Volume results 0,1 μl

Participant	Volume (μL)	*U* (μL)
ARTEL	0,081	0,044
IPQ 1	0,098	0,013
FORCE	0,100	0,025
RISE	0,112	0,017
CMI	0,131	0,008
MIRS	0,107	0,005
GUM	0,117	0,016
EIM	0,103	0,020
IPQ 2	0,104	0,007

5.6. Determination of the reference value, 100 µl

The obtained reference value is 100,15 µl. The obtained expanded uncertainty $U = 2 \times u(y)$ of the reference value is 0,13 µl. In order to have consistent results the value from RISE had to be excluded from the calculations.
The calculated value $\chi^2(\nu) = 12{,}59$ is larger than $\chi^2_{obs} = 4{,}90$, the observed value, therefore the results are then consistent with each other and with the reference value from a statistical point of view.
All the measurement results, the reference value and its uncertainty are presented in the following figure 4:

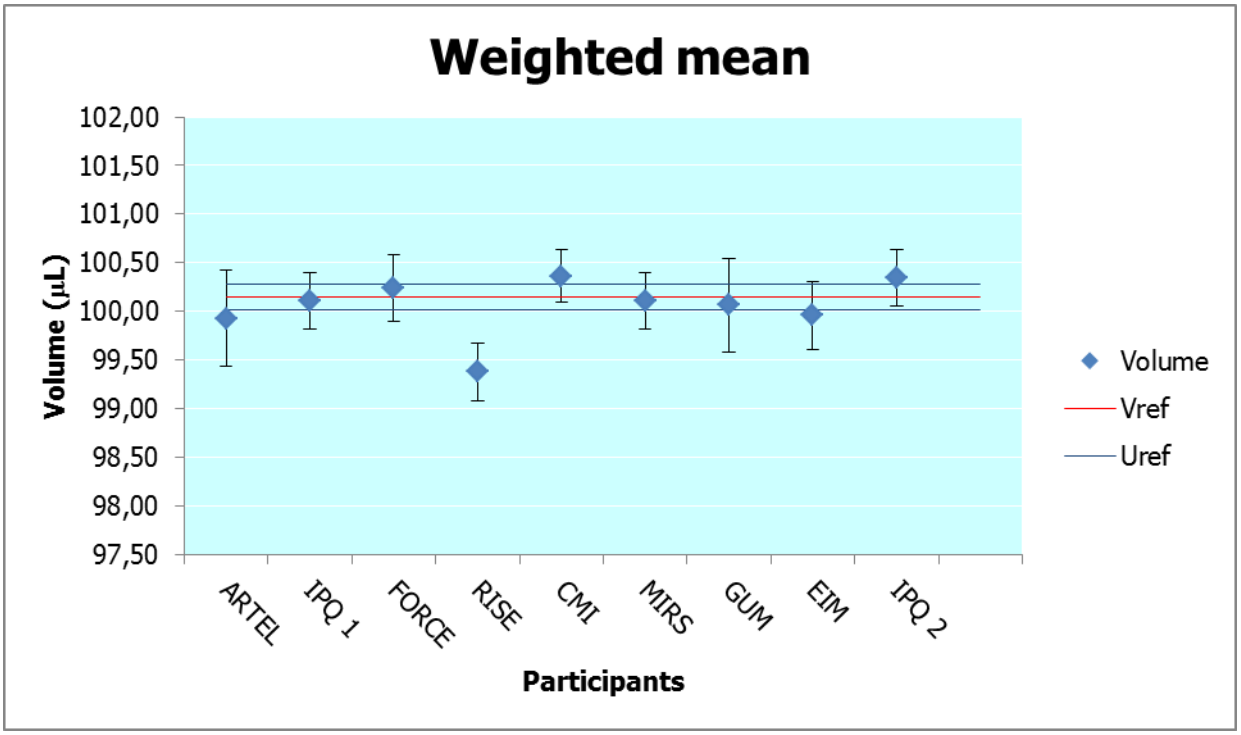

Figure 4 – Volume results with reference value – 100 µl

From this figure it can be observed that the volume result of RISE is the only inconsistent value.

5.7. Determination of the reference value, 10 µl

The obtained reference value is 9,937 µl. The obtained expanded uncertainty $U = 2 \times u(y)$ of the reference value is 0,014 µl.
The calculated value $\chi^2(\nu) = 14{,}067$ is larger than $\chi^2_{obs} = 12{,}49$, the observed value, therefore the results are then consistent with each other and with the reference value from a statistical point of view.
All the measurement results, the reference value and its uncertainty are presented in the following figure 5:

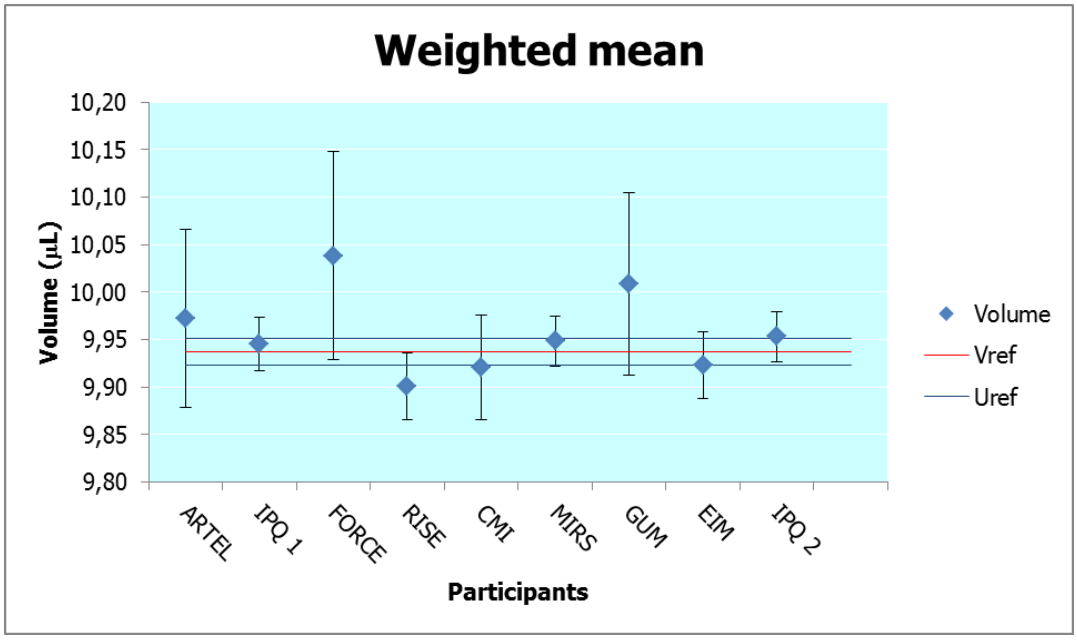

Figure 5 – Volume results with reference value – 10 µl

5.8. Determination of the reference value, 1 µl

The obtained reference value is 1,0116 µl. The obtained expanded uncertainty $U = 2 \times u(y)$ of the reference value is 0,0058 µl. In order to have consistent results the value from MIRS had to be excluded from the calculations.
The calculated value $\chi^2(\nu)$ = 12,59 is larger than χ^2_{obs} = 6,61, the observed value, therefore the results are then consistent with each other and with the reference value from a statistical point of view.
All the measurement results, the reference value and its uncertainty are presented in the following figure 6:

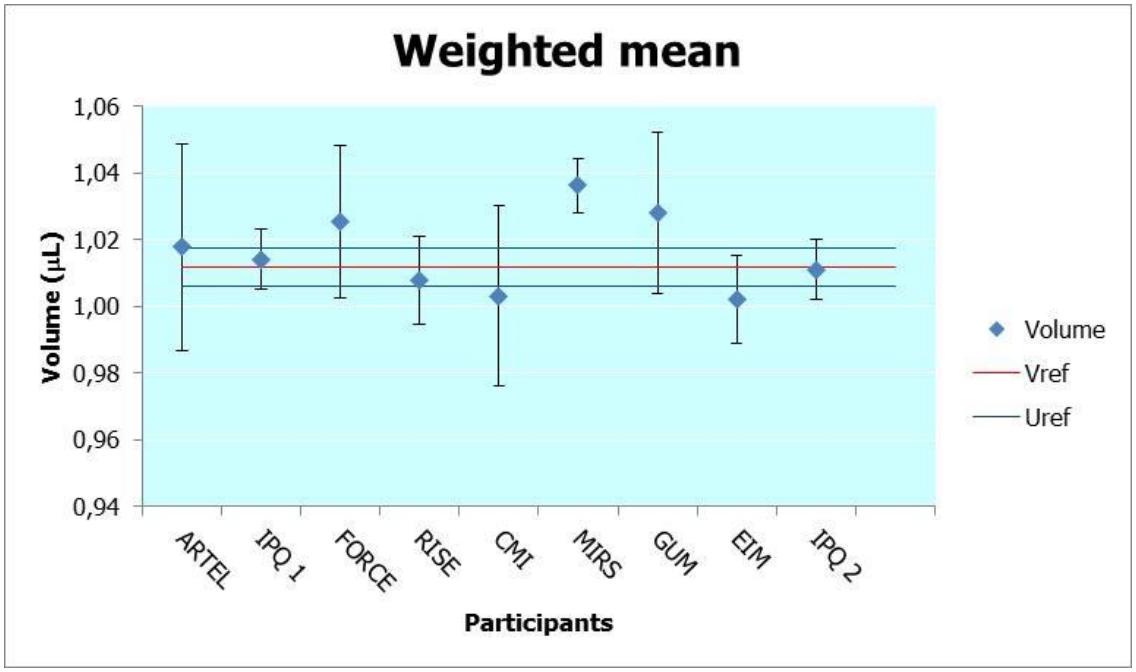

Figure 6 – Volume results with reference value – 1 µl

From this figure it can be observed that the volume result of MIRS is the only inconsistent value.

5.9. Determination of the reference value, 0,1 µl

The obtained reference value is 0,1067 µl. The obtained expanded uncertainty $U = 2 \times u(y)$ of the reference value is 0,0042 µl. In order to have consistent results the value from CMI had to be excluded from the calculations.
The calculated value $\chi^2(\nu)$ = 12,59 is larger than χ^2_{obs} = 5,64, the observed value, therefore the results are then consistent with each other and with the reference value from a statistical point of view.
All the measurement results, the reference value and its uncertainty are presented in the following figure 7:

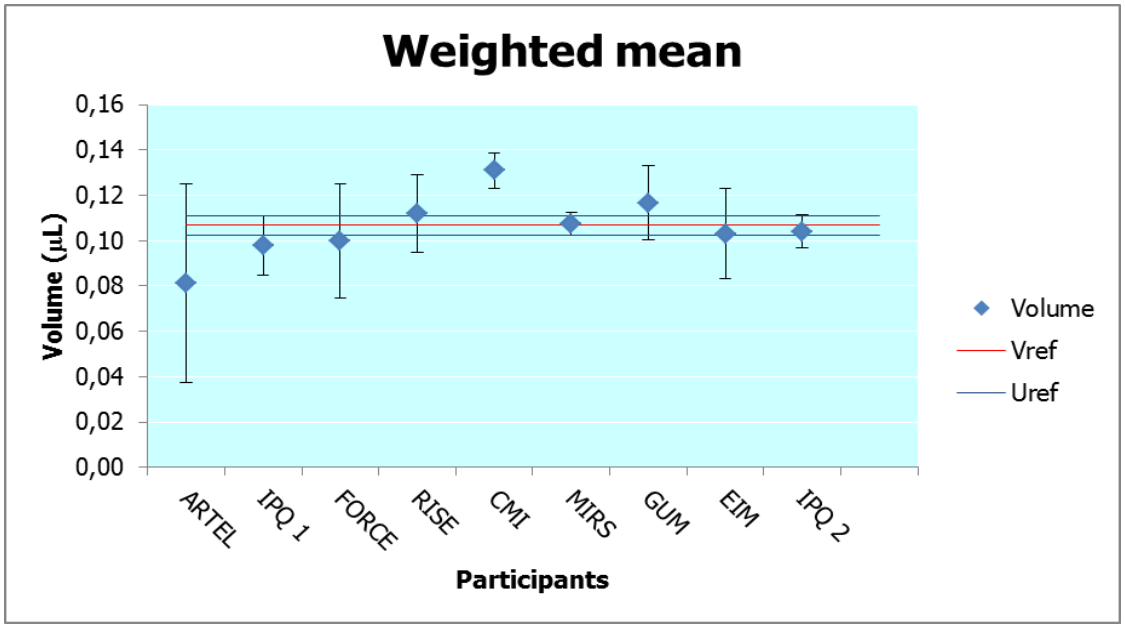

Figure 7 – Volume results with reference value – 0,1 µl

From this figure it can be observed that the volume result of CMI is the only inconsistent value.

6. Uncertainty calculation

The uncertainty of pipette calibration for all participants was estimated following the Guide to the Expression of Uncertainty in Measurement (GUM) [5].
The main contributions for the standard uncertainty of the photometric method are: the repeatability of the measurements, the photometer calibration, the photometer resolution, the solutions and the reproducibility.

Table 7 - Uncertainty components in the calibration of a micropipette using the photometric method

Source / Symbol	Standard uncertainty component	Evaluation process	Evaluation type	Distribution
PCS	u(PCS)cal	Calibration	A	Normal
	u(PCS)res	Resolution	B	Rectangular
Solutions	u(Sol)cal	Calibration	A	Normal
Repeatability	urep	Mean standard deviation	A	Normal
Reproducibility	urepr	Mean standard deviation	A	Normal

Some variation can be found in the declared expanded uncertainty by some participants and this is mainly due to the repeatability of the measurements.

7. Conclusions

IPQ has implemented the photometric method at its volume laboratory in 2016 [6], following a bilateral comparison in the frame of EURAMET project 1353 which supported the publication of CMCs at BIPM webpage. This method allowed IPQ to increase the range and to reduce uncertainty claims in the calibration of micropipettes with a volume lower than 100 μL. In order to verify that this can be accomplished by other laboratories, a pilot study was performed by seven Europeans NMI's. Four micropipettes were calibrated at different nominal volumes.
The obtained results were 88% consistent with the reference value for all micropipettes. There was some variation found in the expanded uncertainty declared by the participants and that was mainly due to the repeatability of the measurements. For the majority of the participants this was a first contact with a new method so it is expected that some variation in the results and the uncertainty would arise due to limited or non-existent experience in this method.

The photometric method has certain advantages compared to the gravimetric method which make it favourable especially at smaller volumes. In particular, due to its principle of operation the photometric method offers low uncertainties at the low volume range. On the contrary the gravimetric method's highest uncertainty component arises from the balance itself, has a fixed value and weighs significantly in the low volume range. Moreover, the photometric method is not affected by environmental conditions usually prevailing in the laboratory like temperature, humidity, static electricity, vibrations, etc. However, it has a higher cost of consumables therefore it becomes less favourable compared to the gravimetric method in the range over 100 microliters due to higher uncertainty and it cannot be applied for the determination of volumes higher than 5000 microliters.

8. References

[1] ISO 8655-7:2005 Piston Operated Volumetric Apparatus – Part 7: Non-gravimetric methods for the assessment of equipment performance

[2] ASTM E542-01(2012) - Standard practice for calibration of laboratory volumetric apparatus

[3] George Rodrigues, Bias and transferability in standards methods of pipette calibration, Artel, June 2003

[4] Cox M.G., The evaluation of key comparison data, *Metrologia*, 2002, **39**, 589-59

[5] BIPM et al, Guide to the Expression of Uncertainty in Measurement (GUM), 2nd ed., International Organization for Standardization, Genève, 1995

[6] EURAMET project 1353, Volume comparison on Calibration of micropipettes – Gravimetric and photometric methods, www.EURAMET.org