



NATIONAL QUALITY INFRASTRUCTURE SYSTEM

HELLENIC INSTITUTE OF METROLOGY



# EURAMET Project 1484

Calibration of a variable volume 1000  $\mu\text{L}$  micropipette  
Calibration of a 100 ml flask

Final Report

Pilot laboratory

Hellenic Institute of Metrology  
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# Abstract

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*In the framework of the project "Regional Consultancy Fund for Quality Infrastructure South East Europe", PTB VH-No.:95330-02, the Hellenic Institute of Metrology (EIM) was commissioned with the implementation of an inter laboratory comparison (ILC) in small volume for up to 6 NMIs in South East Europe. Within this ILC EIM provides resource person, artifacts, organization and technical guidance for the project implementation acting as pilot laboratory. In particular EIM has been undertaken the tasks of preparing the protocol of the ILC, registering the ILC via EURAMET / TC Volume & Flow, issuing the A.T.A. Carnet for the circulation of the artifacts, providing the calibration of the artifacts and finally evaluating the results and preparing the DRAFT A, DRAFT B and final reports.*

*The ILC consists of two main tasks, the calibration of a variable volume 1000  $\mu\text{L}$  micropipette and the calibration of a 100 ml flask both using the gravimetric method. The participating NMIs in this ILC were IMBIH (Bosnia & Herzegovina), DPM (Albania) and CMI (Czech Republic).*

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## 1. Introduction

In the framework of the project "Regional Consultancy Fund for Quality Infrastructure South East Europe", PTB VH-No.:95330-02, the Hellenic Institute of Metrology (EIM) was commissioned with the implementation of an inter laboratory comparison (ILC) in small volume for up to 6 NMIs in South East Europe. The basic aim of this inter comparison was to provide support to developing NMIs in the major area of Balkans in their efforts to acquire international recognition through the submission of their CMCs in volume for publication in BIPM data base.

To this end, evidence regarding the technical competence of the corresponding NMIs in small volume calibration was provided by the outcome of an ILC organized by EIM in the gravimetric calibration of two artifacts typical for the activities of a volume laboratory, namely a 100 ml flask and a variable volume 1000 µL micropipette. The transfer standards were provided by EIM who performed the initial and final calibrations. The ILC was registered as EURAMET comparison 1484.

In this inter comparison participated four national laboratories (Greece/pilot, Czech Republic\*, Bosnia & Herzegovina and Albania). The inter comparison was launched in April 2019 and the measurements were completed in August 2019. A detailed technical protocol, specific instructions and an experimental protocol was provided by the pilot laboratory (EIM). Since two of the participating laboratories represent non EU countries the artifacts were accompanied by an ATA Carnet issued by the pilot.

\* CMI (Czech Republic) joined the project later with participation only in the part of the calibration of the 100 ml flask.

## 2. Participants, Transportation and Time Schedule

Each participant had two weeks to perform the measurements and two weeks to send the results and uncertainties accompanied by a brief report. The contact details of the participants and the time schedule are given in Table 1.

The measurements started in the middle of March 2019 and were completed by the end of September 2019.

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Table 1. List of participants and time schedule of inter-comparison

Laboratory	Responsible person	Contact details	Measurement period
EIM (start)	Dr Zoe Metaxiotou Head of Flow & Volume Laboratory of EIM	HELLENIC INSTITUTE OF METROLOGY Industrial Area of Thessaloniki Block 45, 57022 SINDOS GREECE e-mail: zoe @eim.gr	Week 11
IMBIH	Ernad Borovac Head of Department Laboratory for volume and flow	Institute of metrology of Bosnia and Herzegovina  Augusta Brauna 2 71 000 Sarajevo ernad.borovac@met.gov.ba	Week 14
DPM	Erinda Piluri	DPM erinda.piluri@dpm.gov.al	Week 20
CMI	Martina Vicarova Head of Department of primary metrology of physical chemistry	Czech Metrology Institute, Okruzni 31, Brno, CZ-638 00, Czech Republic, <a href="http://www.cmi.cz">www.cmi.cz</a>	Week 34
EIM (close)	Dr Zoe Metaxiotou Head of Flow & Volume Laboratory of EIM	HELLENIC INSTITUTE OF METROLOGY Industrial Area of Thessaloniki Block 45, 57022 SINDOS GREECE	Week 38

### 3. Transfer standards

**The transfer standard No 1** in this comparison is a 100 ml (class A) volumetric one mark borosilicate flask (Borosilicate Volumetric Flask, VOLAC Class A, 100ml, Serial Number: 84055) *and is provided by EIM.*

- Cubical thermal expansion coefficient of the material of the flask:  $9,6E-06 \text{ } ^\circ\text{C}^{-1}$

**The transfer standard No 2** in this comparison is a piston operated variable volume 1000  $\mu\text{L}$  micropipette (Eppendorf 1000  $\mu\text{L}$ , Serial Number: 370565Z) *and is provided by EIM.*

- Cubical thermal expansion coefficient of the material of the pipette:  $2,4 \times 10^{-4} \text{ } ^\circ\text{C}^{-1}$

## 4. The Measurement Procedure

Whenever possible, in order to establish a common basis for the comparison of the results, common practices were suggested to be used as following.

### 4.1. Experimental Method

All participating NMIs used the gravimetric method to determine the amount of water that the micropipette delivers at reference temperature of 20 °C, based on ISO 8655 [1], ISO 4787 [2] and EURAMET Guide 19 [3], according to equation (1):

$$V_{20} = (I_L - I_E) \times \frac{1}{\rho_w - \rho_A} \times \left(1 - \frac{\rho_A}{\rho_B}\right) \times [1 - \gamma(t - 20)] \quad (1).$$

where:

- $V_{20}$  is the delivered volume of the micropipette at 20 °C
- $I_L$  is the balance indication for the filled recipient, [g]
- $I_E$  is the balance indication for the empty recipient, [g]
- $\rho_A$  is the air density, [g/cm<sup>3</sup>]
- $\rho_B = 8.0$  g/cm<sup>3</sup> is the density of the reference weights used
- $\rho_w$  is the density of water at the temperature of measurement  $t_w$ , [g/cm<sup>3</sup>]
- $\gamma$  is the cubical thermal expansion coefficient of the material of the TS ( $2,4 \times 10^{-4}$  °C<sup>-1</sup>)
- $t_w$  is the water temperature during each measurement [°C]

The same formula as above was also used for the determination of the “dry contained” volume of the one-mark 100 ml flask,

where:

- $V_{20}$  is the “dry contained” volume of the flask at 20 °C
- $I_L$  is the balance indication for the filled flask, [g]
- $I_E$  is the balance indication for the empty and dry flask, [g]
- $\rho_A$  is the air density, [g/cm<sup>3</sup>]
- $\rho_B = 8.0$  g/cm<sup>3</sup> is the density of the reference weights used
- $\rho_w$  is the density of water at the temperature of measurement  $t_w$ , [g/cm<sup>3</sup>]
- $\gamma$  is the cubical thermal expansion coefficient of the material of the TS ( $9,6 \times 10^{-6}$  °C<sup>-1</sup>)
- $t_w$  is the water temperature during each measurement [°C]

For the calibration of the micropipette, in particular, the following unified approach in the calibration procedure was recommended to all participants:

- The receiving vial where the volume measured by the pipette is transferred should have a water film of at least 3 mm thickness.
- This vial should preferably be covered in order to minimize possible losses due to evaporation. Alternatively a moisture trap should be used.
- The water transfer from the pipette to the vial should be done under a slope of 30° – 60° while touching the vial wall with the tip. Any remaining drops in the tip wall should also be transferred in the vial.
- Before making any measurement a new tip should be attached to the pipette and be pre-rinsed five (5) consecutive times. After this preconditioning the tip should be replaced and the first run is performed with no further pre-rinsing of the new tip. The same tip is used for the next two runs as well and then is changed. The next four (4) runs are performed without pre-rinsing of the tip and then the tip is changed for the final three runs performed as previously. This sequence is followed for all set points and the tip is changed in between only in the case of grossly erroneous results.
- Ten consecutive runs should be performed at each calibration point according to the instructions above preferably starting the calibration from the highest volume.

#### 4.2. Water characteristics

The characteristics of the water used by the participants in the calibration of the artifacts are given in Table 2 and are complying with the requirements of ISO 3696 [4].

**Table 2.** Water characteristics

NMI	Type	Density formula or table	Measured conductivity
EIM	Distilled	TANAKA	-
IMBIH	Distilled	TANAKA	-
DPM	Distilled	SPIEWECK	0.1 $\mu\text{S}/\text{cm}$
CMI	DEMI	TANAKA	1.2 $\mu\text{S}/\text{cm}$

### 4.3. Equipment

The primary equipment used by the participants for the gravimetric calibration of the artefacts is given in Table 3.

**Table 3.** Balance characteristics

<b>NMI</b>	<b>Type</b>	<b>Range</b>	<b>Resolution</b>
EIM	METTLER TOLEDO XS 205	220 g	0.00001 g
IMBIH	METTLER TOLEDO XS 205	81 g	0.00001 g
DPM	SARTORIUS GPC26-CW	20 g	0.001 mg
CMI	SARTORIUS CP 225D-OCE	80 g/ 220 g	0,00001 g/0,0001 g

### 4.4. Ambient conditions

The environmental conditions prevailing during calibration were recorded by the participants using appropriate traceable instruments for temperature, pressure and relative humidity measurement. The environmental conditions for each laboratory are given in Table 4 for the flask calibration and in Table 5 for the micro pipette calibration, respectively.

**Table 4.** Environmental conditions during calibration - Flask

<b>NMI</b>	<b>Air Temperature [°C]</b>	<b>Barometric Pressure [mbar]</b>	<b>Relative humidity [%]</b>
EIM	22 ± 0.2	1012 ± 1	35 ± 1
IMBIH	21.7 ± 0.2	945 ± 3	60 ± 2
DPM	21 ± 0.3	1005 ± 1	66 ± 1
CMI	19.8 ± 0.3	985 ± 1	60 ± 3



**Table 5.** Environmental conditions during calibration – Micro pipette

<b>NMI</b>	<b>Air Temperature [°C]</b>	<b>Barometric Pressure [mbar]</b>	<b>Relative humidity [%]</b>
EIM	23.3 ± 0.1	1013 ± 1	54 ± 1
IMBIH	21.1 ± 0.2	952 ± 2	45 ± 2
DPM	21 ± 0.5	1007 ± 1	60 ± 1
CMI	-	-	-

## 5. Results: Micropipette calibration

### *The stability of the TS*

EIM acting as the pilot laboratory made a calibration of the TS in the beginning and at the end of the comparison. The results are presented in table 6.

**Table 6.** Stability of 100 – 1000 µL micropipette – EIM results

<b>Calibration point</b>	<b>1000 µL</b>		<b>500 µL</b>		<b>100 µL</b>	
	V	U	V	U	V	U
<b>Start</b>	998.95	0.5	498.94	0.33	100.89	0.22
<b>Close</b>	1000.05	0.5	499.57	0.33	101.17	0.22

The values obtained by the pilot laboratory were tested for their consistency within the stated uncertainty limits by a combined F-test and T-test. The hypothesis to be tested was that the set of values obtained during the START and CLOSE series have the same mean. The outcome of these tests is shown in Table 7.

According to the outcomes of the above checks on a hypothesis level of  $\alpha=0.05$ , the two values obtained for each one of the calibration points belong to populations with the same mean (t-Test:  $p \geq 0.05$ ) but have different variances (F-test:  $p < 0.05$ ). This can be attributed to the environmental conditions prevailing during the two occasions and/or to handling effects. In particular the environmental conditions in the closing run were much better with respect to the prevailing relative humidity which definitely affects calibration. Based on the above analysis the two sets of values of the pilot lab are considered to be consistent. However, after the first consistency check the START value was excluded from the RV calculation and only CLOSE value was taken into account.

**Table 7.** Check of the consistency of the two series of results of the pilot laboratory at 1000 µL, 500 µL and 100 µL

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	Variable 2		Variable 1	Variable 2
Mean	1000.048543	998.8492833	Mean	1000.048543	998.8492833
Variance	0.036139132	0.276979774	Variance	0.036139132	0.276979774
Observations	10	10	Observations	10	10
df	9	9	Hypothesized Mean	0.9	
F	0.130475706		df	11	
P(F<=f) one-tail	<b>0.002825953</b>		t Stat	1.691194354	
F Critical one-tail	0.314574906		P(T<=t) one-tail	<b>0.059450848</b>	
			t Critical one-tail	1.795884814	
			P(T<=t) two-tail	<b>0.118901696</b>	
			t Critical two-tail	2.200985159	

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	Variable 2		Variable 1	Variable 2
Mean	499.5695319	498.9418894	Mean	499.5695319	498.9418894
Variance	0.033695226	0.208172622	Variance	0.033695226	0.208172622
Observations	10	10	Observations	10	10
df	9	9	Hypothesized Mean Difference	0.35	
F	0.161861946		df	12	
P(F<=f) one-tail	<b>0.006110981</b>		t Stat	1.785240917	
F Critical one-tail	0.314574906		P(T<=t) one-tail	<b>0.050</b>	
			t Critical one-tail	1.782287548	
			P(T<=t) two-tail	<b>0.10</b>	
			t Critical two-tail	2.178812827	

F-Test Two-Sample for Variances			t-Test: Two-Sample Assuming Unequal Variances		
	Variable 1	Variable 2		Variable 1	Variable 2
Mean	101.1688301	100.8907763	Mean	101.1688301	100.8907763
Variance	0.017099494	0.069551055	Variance	0.017099494	0.069551055
Observations	10	10	Observations	10	10
df	9	9	Hypothesized Mean	0.2	
F	0.245855275		df	13	
P(F<=f) one-tail	<b>0.024224723</b>		t Stat	0.838510244	
F Critical one-tail	0.314574906		P(T<=t) one-tail	<b>0.208447999</b>	
			t Critical one-tail	1.770933383	
			P(T<=t) two-tail	<b>0.416895998</b>	
			t Critical two-tail	2.160368652	

## The Pressure Correction

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Piston operated pipettes (air displacement) have an air-cushion which moves between the piston and the sample liquid and aspirates and dispenses the sample. With the decreasing atmospheric pressure the density of the air cushion decreases leading to a reduction in the dispensed volume of the micropipette.

If the dead volume and the capillary rise of the liquid column in the micropipette are known, the change in volume that results from calibration at a X2 location (with  $p_{L,X2}$  atmospheric pressure at location X2) compared to an X1 location (with  $p_{L,X1}$  atmospheric pressure at location X1) can be calculated using the following formula [5]:

$$\Delta V = -V_t \times \rho_w \times g \times h_w \times \left( \frac{1}{p_{L,X2} - \rho_w \times g \times h_w} - \frac{1}{p_{L,X1} - \rho_w \times g \times h_w} \right) \quad (2)$$

Where,

$\Delta V/\mu\text{L}$ : Volume change that results in the calibration at location X1 over a location X2

$V_t/\mu\text{L}$ : Volume of the air cushion

$g/(\text{m/s}^2)$ : Acceleration of gravity

$h_w/\text{m}$ : Rising height of the liquid column in the pipette tip

$p_{L,X1}/\text{Pa}$ : Atmospheric pressure at location X1

$p_{L,X2}/\text{Pa}$ : Atmospheric pressure at location X2

$\rho_w/(\text{kg/m}^3)$ : Water density at X2

For the case of the variable volume 100 – 1000  $\mu\text{L}$  micropipette the values for the rising height of the liquid column in the pipette tip used were 0.019 m, 0.034 m and 0.050 m for the 100  $\mu\text{L}$ , 500  $\mu\text{L}$  and 1000  $\mu\text{L}$  calibration points, respectively, while the volume of the air cushion was 2700  $\mu\text{L}$  [5].

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## The estimation of the ILC RV and the consistency check

To determine the reference value (RV) of this comparison the weighted mean (3) was selected, using the inverses of the squares of the associated standard uncertainties as the weights [6], according to the instructions given by the BIPM:

$$y = \frac{x_1/u^2(x_1) + \dots + x_n/u^2(x_n)}{1/u^2(x_1) + \dots + 1/u^2(x_n)} \quad (3).$$

To calculate the standard uncertainty  $u(y)$  associated with the volume  $y$  [6] equation (4) was used:

$$u(y) = \sqrt{\frac{1}{1/u^2(x_1) + \dots + 1/u^2(x_n)}} \quad (4).$$

The expanded uncertainty of the reference value is  $U(y) = 2 \times u(y)$ .

To identify an overall consistency of the results a chi-square test can be applied to all  $n$  calibration results [6].

$$\chi_{obs}^2 = \frac{(x_1 - y)^2}{u^2(x_1)} + \dots + \frac{(x_n - y)^2}{u^2(x_n)} \quad (5),$$

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

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

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

If the consistency check failed then the NMI 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 NMI. When the consistency check passes, for each NMI results,  $x_i$  the degree of equivalence  $d_i$  between each NMI and the KCRV ( $x_{ref}$ ) is calculated using the following formulas [6]:

$$d_i = x_i - x_{ref} \quad (6),$$

$$U(d_i) = 2 \times u(d_i) \quad (7),$$

where

$u(d_i)$  is calculated from

$$u^2(d_i) = u^2(x_i) - u^2(x_{ref}) \quad (8).$$

Discrepancy values can be identified if  $|d_i| > 2u(d_i)$ .

To calculate the degrees of equivalence  $d_{ij}$  between the NMIs the following formulas are used [6]:

$$d_{i,j} = x_i - x_j \quad (9),$$

$$U(d_{i,j}) = 2 \times u(d_{i,j}) \quad (10),$$

where

$u(d_{i,j})$  is calculated from

$$u^2(d_{i,j}) = u^2(x_i) + u^2(x_j) \quad (11).$$

The factor 2 in equations 7 and 10 corresponds to 95 % coverage under the assumption of normality.

The normalized error,  $E_{n,i}$ , describes the degree of equivalence of a laboratory related to the RV.

$E_{n,i}$  was calculated for each reported value of the participant as follows,

$$E_{n,i} = d_i / U(d_i) \quad (12).$$

If  $|E_{n,i}| \leq 1$ , the measurement is generally considered to be acceptable and the measured values are consistent.

In Table 8 are presented the corrected values of the participants for a standard atmospheric pressure of 1013.25 hPa using equation 2. These values were used for the determination of the reference value and consistency of the results.

The uncertainty values for the pilot laboratory are equal to the published CMC values of EIM in the BIPM KCBD. DPM (Albania) participated previously in a EURAMET intercomparison in the same field of measurement (100  $\mu$ L fixed pipette) with results marginally equivalent to the calculated RV and uncertainty claims much higher than the ones claimed in this ILC. For this reason the uncertainties of DPM used for the calculation of the current RV are modified accordingly in order to be in agreement with the previous comparison claims. In Table 8 both current and old claims for DPM are presented but for the calculations only the old uncertainty claims are considered.

**Table 8.** Final micropipette calibration results (Pressure corrected)

Volume Lab	1000 $\mu\text{L}$		500 $\mu\text{L}$		100 $\mu\text{L}$	
	V [ $\mu\text{L}$ ]	U [ $\mu\text{L}$ ]	V [ $\mu\text{L}$ ]	U [ $\mu\text{L}$ ]	V [ $\mu\text{L}$ ]	U [ $\mu\text{L}$ ]
<b>EIM - 1</b>	1000.05	0.5	499.57	0.33	101.17	0.22
<b>DPM - 2 (current)</b>	1000.40	0.34	500.05	0.24	101.70	0.25
<b>DPM (old)</b>	1000.40	1.0	500.05	0.75	101.70	0.50
<b>IMBIH -3</b>	999.17	2.02	498.98	1.12	101.38	0.27
<b>RV - 4</b>	1000.07	0.22	499.60	0.15	101.30	0.08

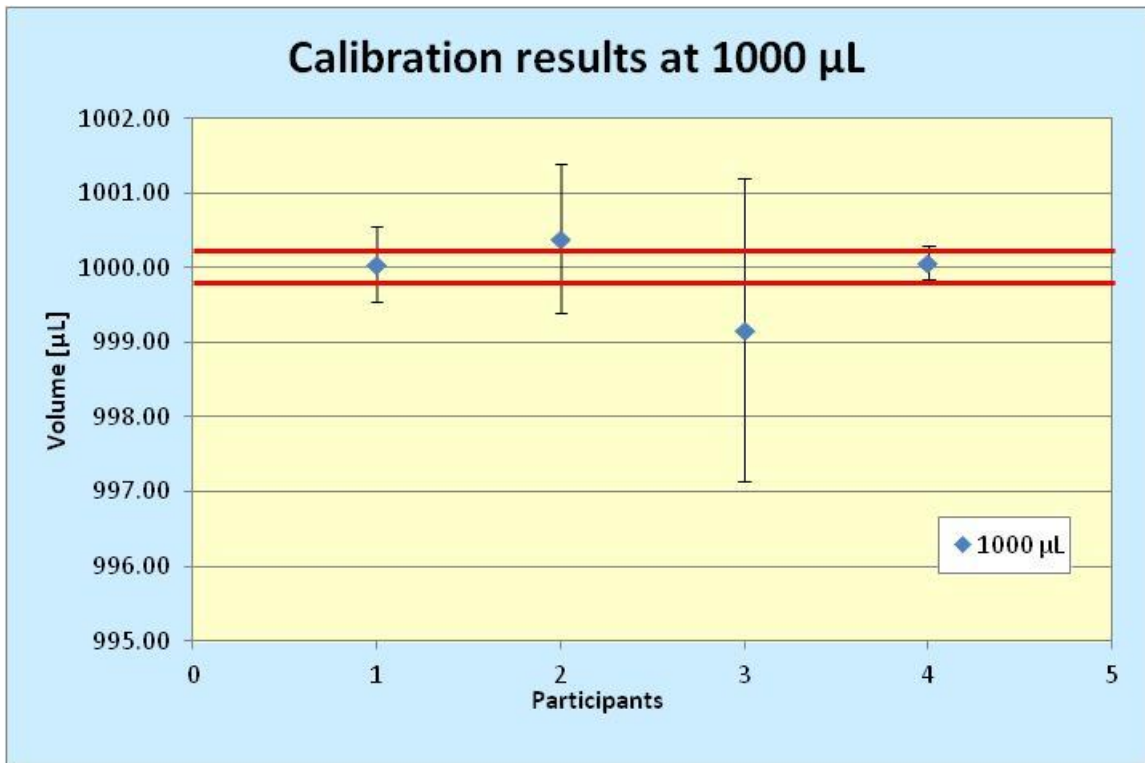
In Table 9 the degree of equivalence,  $d_i$ , and the normalized error,  $E_{n,i}$  for each laboratory are given as obtained by the application of the consistency tests. For the pilot laboratory CLOSE value is used for the calculation of the RVs.

**Table 9.** Degree of equivalence and normalized error

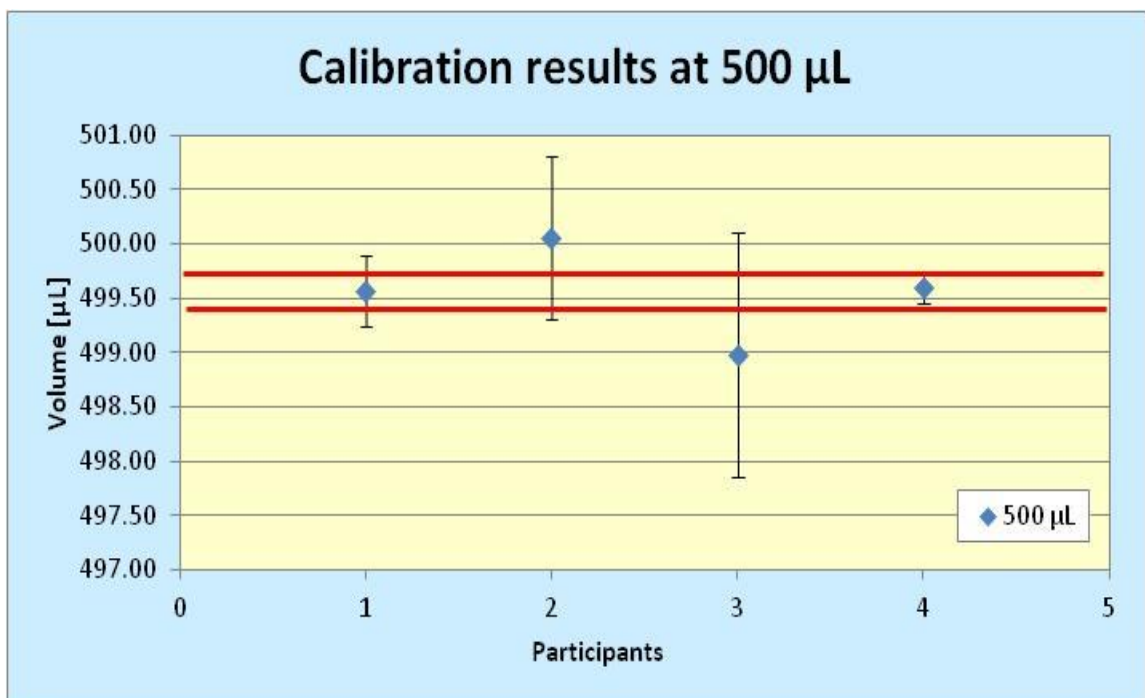
Volume Lab	1000 $\mu\text{L}$			500 $\mu\text{L}$			100 $\mu\text{L}$		
	$d_i$ [ $\mu\text{L}$ ]	U( $d_i$ ) [ $\mu\text{L}$ ]	$E_{n,i}$	$d_i$ [ $\mu\text{L}$ ]	U( $d_i$ ) [ $\mu\text{L}$ ]	$E_{n,i}$	$d_i$ [ $\mu\text{L}$ ]	U( $d_i$ ) [ $\mu\text{L}$ ]	$E_{n,i}$
EIM -1	0.03	0.45	0.06	0.03	0.30	0.11	0.13	0.20	0.63
DPM - 2	0.33	0.98	0.33	0.44	0.74	0.60	0.40	0.49	0.81
IMBIH -3	0.91	2.01	0.5	0.63	1.11	0.56	0.08	0.26	0.30

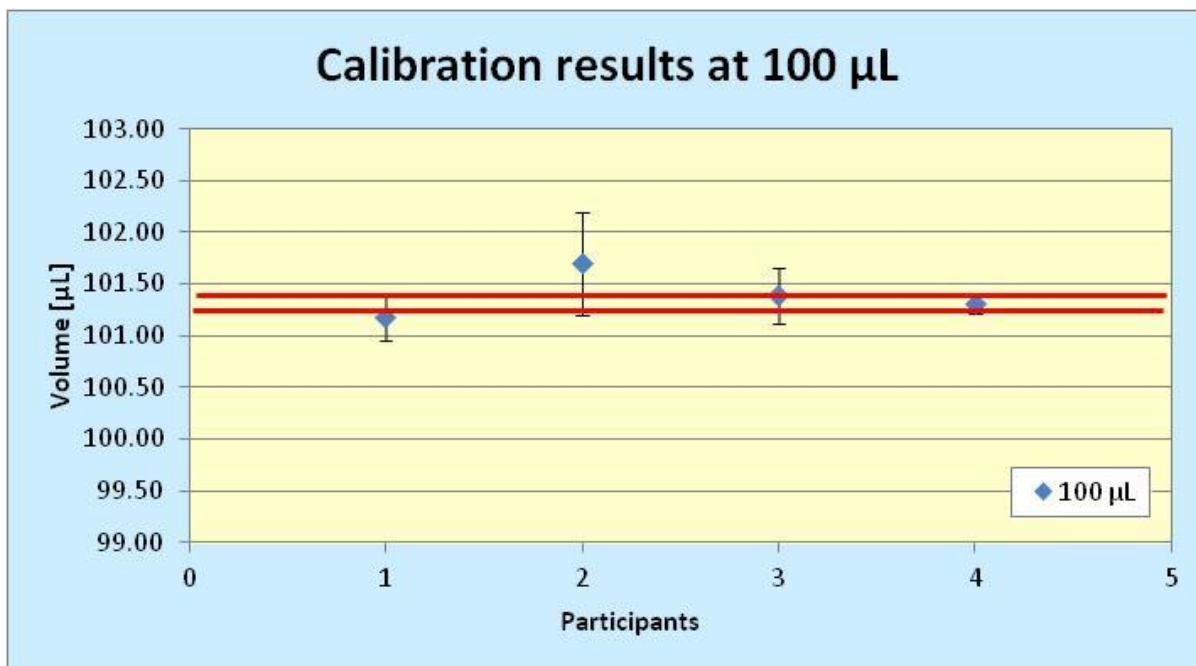
The overall results are presented graphically in figures 1, 2 and 3 for the three points of calibration of the micropipette, respectively.

**Figure 1.** Inter comparison results at 1000  $\mu\text{L}$  point



**Figure 2.** Inter comparison results at 500  $\mu\text{L}$  point



**Figure 3.** Inter comparison results at 100  $\mu\text{L}$  point



## 6. Results: 100 ml flask calibration

The summarized results of the calibration of the 100 ml flask are given in Table 10 along with the reference value as obtained after the consistency check. The same results are shown graphically in Figure 4.

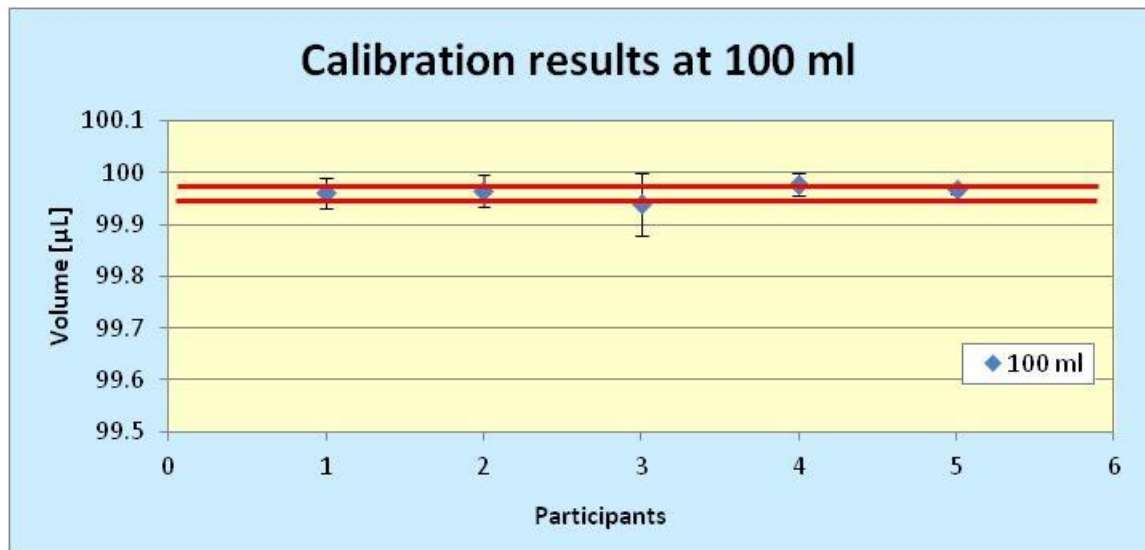
**Table 10.** Final 100 ml flask calibration results

<b>Volume</b>	<b>100 ml</b>	
<b>Lab</b>	V [ml]	U [ml]
<b>EIM - 1</b>	99.96	0.03
<b>IMBIH - 2</b>	99.97	0.03
<b>DPM - 3</b>	99.94	0.06
<b>CMI - 4</b>	99.98	0.02
<b>RV - 5</b>	<b>99.97</b>	<b>0.01</b>

In Table 11 the degree of equivalence,  $d_i$ , and the normalized error,  $E_{n,i}$  for each laboratory are given as obtained by the application of the consistency tests.

**Table 11.** Degree of equivalence and normalized error (flask)

<b>Volume</b>	<b>100 ml</b>		
<b>Lab</b>	$d_i$ [ml]	$U(d_i)$ [ml]	$E_{n,i}$
<b>EIM - 1</b>	0.01	0.03	0.26
<b>IMBIH - 2</b>	0.00	0.03	0.09
<b>DPM - 3</b>	0.03	0.06	0.47
<b>CMI - 4</b>	0.01	0.02	0.45

**Figure 4.** Inter comparison results for a 100 ml flask calibration

## 7. Conclusions

The results presented in this report refer to the application of the gravimetric method for the calibration of a variable volume 100 – 1000 µL micro pipette and the calibration of a one mark 100 ml borosilicate glass flask. All participants used traceable equipment and established methods for the calibration of the artifacts aiming to deliver results which are equivalent to the reference value of this Euramet ILC estimated after the application of the appropriate consistence checks.

All participants succeeded in both calibrations and obtained equivalent results with the RV as obtained in the present ILC for the micro-pipette at three delivered volumes (1000 µL, 500 µL and 100 µL) and the one mark 100 ml glass flask.

## 8. Literature

- [1] ISO 8655-1/2/6:2002, Piston-operated volumetric apparatus.
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