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Optimization of Process Variables to Minimize Uncertainty in Micro-Volume Measurements

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Abstract—Calibration of micropipettes plays a significant role in ensuring the reliability and accuracy of volumetric results while conducting testing and research in the areas of biology, analytical chemistry and, pharmaceutical sciences, etc. Understanding the uncertainty elements and their contribution to measurements is the preliminary step in any calibration activity. In the study entitled 'Optimization of process variable to minimize uncertainty in micro-volume measurements', an attempt is made to analyze the role of uncertainty contributing process variables in volume calibrations based on ISO 8655. The objectives were to identify process variables contributing to the expanded uncertainty and optimization of the variables to minimize the uncertainty. The principles of Design of Experiment (DoE) was the tool applied for the study. The results of the study conclude the role of process variables in the controlled environment for micro-volume calibrations

Keywords—V**olume calibration, micro pipette, ISO 8655, optimisation of process variables, uncertainty estimation.**

INTRODUCTION

Ensuring minimum variability in results has an important role in assuring the reliability and accuracy of any experiment or test. Calibration is one of the metrological tools used in research and testing for quality control. Recently, the practice of implementing quality system platforms like Good laboratory practice (GLP), ISO 17025, etc; also insists the practice of traceable equipment calibrations. Calibration is defined in VIM-International Vocabulary of Metrology as 'operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication'[1].

Different micro volume liquid dispensing techniques are regularly used in routine testing and research areas of biology, analytical chemistry and pharmaceutical sciences. Its application ranges from simple pipetting and drug delivery using pipettes or infusion pumps to advanced techniques like flow cytometry and liquid chromatography [2]. Quality assurance requirements of such systems demands precise results with minimum errors / uncertainty.

Dispensing liquids in micro volumes may get affected by factors like changes in environmental conditions, competency level of operator, accuracy of measurement system etc. Drift in the performance of pipettes over years of use also can happen. In order to identify and reduce possible errors in intensive liquid handling process, it is necessary to assess the performance of the system in controlled and precise operating conditions. Mechanical calibration laboratories accredited for volume parameters are equipped for the purpose. [3].

Internationally accepted reference literature for the calibration of micro pipettes are ASTM (American Society for Testing and Materials) 1154: Standard Specification for Piston or Plunger

Operated Volumetric Apparatus and ISO (The International Organization for Standardization) 8655: Piston-operated volumetric apparatus -- Part 6: Gravimetric methods for the determination of measurement error[4,5,6]. Both describe gravimetric method of calibration in which, measurement of the weight of water and its conversion to volume using an accepted formula has to be performed. The theme for the study, ‘Optimization of process variable to minimize uncertainty in micro-volume measurements,’ is selected based on the ISO method. Identification of process variables contributing to the expanded uncertainty and optimization of the variables to minimize the uncertainty are the objectives of the study.Principles of Design of Experiment(DoE) is applied for the study.

OVERVIEW OF THE STUDY

The design of the experiment involves the following steps

- Selection/Identification of process variables i.e; elements contributing to uncertainty from the mathematical model.
- Defining the range of variables
- Conducting the DoE
- Arriving at transfer function and combination of process variables for volume estimations
- Estimating the microvolume using transfer function and actual mathematical model for V20.
- Analysing the data for error and it normality in distribution
- Conducting the DoE for the selected practical range of process variables
- Conducting the actual experiments at the combination of process variable suggested by DoE.
- Estimation of results using transfer function and actual experiment.
- Analysing the results

The details of the equipment used for the study are given in the table below.

TABLE 2
LIST OF EQUIPMENT

EQUIPMENT	SPECIFICATION
Electronic balance	Resolution : 0.01mg
Thermometer	Accuracy: ±0.2 °C
Thermohygrometer	Accuracy: ±0.5 °C; ±5%RH
Barometer	Accuracy: ± 0.5 °C
Portable Dehumidifier	12 liters /day
Micro pipette-100µl	Accuracy 0.8 µl

A calibrated electronic balance (Sartorius, ME 215 s) with a linearity of 0.1 mg was used for the measurement of the mass of water delivered by the micropipette. The temperature of water used in the volume calibration is recorded using the thermometer (Digisense thermometer). Temperature, relative humidity, and barometer of the calibration area are recorded using a pressure-temperature & RH monitoring device (Control company). Humidity levels of the air-conditioned environment were controlled by a portable dehumidifier (Novita / ND290) between RH levels of 50% and 90%. Temperature control is achieved using the temperature settings of the air conditioning unit. The test water used for the experiments conforms to grade 3 as specified in ISO 3696. For the purpose of carrying out experiments for volume calibrations, a Micropipette of 100µl nominal capacity (Finn pipette, Thermo Scientific) was used.

MODEL OF THE VOLUME MEASUREMENT

Volume measurement- V20, using piston pipettes based on gravimetric method can mathematically expressed as

V20= m x Z x Y

Where

m is the balance reading of the delivered water

Z is the combined factor for buoyancy correction and conversion from mass to volume

Y is the thermal expansion correction factor of the delivering device.

Z is given by 1/ρb x (ρb-ρa)/(ρw-ρa)

Where

ρw is the density of water;

ρa is the density of air;

ρb is the density of standard mass used to calibrate the balance. According to OIML (Organization Internationale de Metrologie Legale), ρb = 8000Kg/m3

Density of water is given by equation ρw =

$$= \sum_{i=0}^{4} a_i t_w^i$$

Where

tw is the water temperature in degree Celsius

with the constants in ITS-90 temperature scale

tw is the water temperature in degree Celsius

with the constants in ITS-90 temperature scale

- a0= 999.853 08 Kg/m3
- a1= 6.326 93 x 10-2 ^{o}C-1Kg/m3
- a2= 8.523 829 x 10-3 ^{o}C-2Kg/m3
- a3= 6.943 248 x 10-5 ^{o}C-3Kg/m3
- a4= 3.821 216 x 10-7 ^{o}C-4Kg/m3

The air density ρa =

$$\frac{k_1 \; pa + \phi(k_2 t_a + k_3)}{t_a + t_a 0} \; Kg/m^3$$

Where

- t a0 =273.15^{o}C
- p a is the pressure expressed in hPa
- ϕ is the relative humidity(RH) in%
- ta is the air temperature expressed in ^{o}C
- with the constants in ITS90 temperature scale
- k 1 = 0.34844 (Kg/m^3)^{o}C/hPa
- k 2 = 0.00252 Kg/m^3
- k 3 = 0.020582(Kg/m^3)^{o}C

The thermal expansion correction is given as

Y=1-ᶐc(td-t20)

Where

- ᶐc is the cubic expansion coefficient in ^{o}C-1
- td is the device temperature in ^{o}C
- t20=Reference temperature 20^{o}C at which the volume measurement is expected

Hence volume V20 is represented as a function of tw ta, pa, ϕ, ᶐc, td at a mass value ‘m’ corresponding to the selected volume .

V20=F(xi)=F(m,tw, ta, pa, ϕ , ᶐc, td, constants)

IDENTIFICATION OF UNCERTAINTY ELEMENTS AND SENSITIVITY COEFFICIENTS

The uncertainty associated with the V20 is identified based on GUM-Guide to the Expression of Uncertainty in Measurement

$$U^2\,(V_{20}) = \sum_i c_i^2 \;\text{ x } u^2(x_i) = \;\; \Sigma_i\left(\frac{\partial F}{\partial x_i} \right)^2 \text{x } u^2(x_i)$$

Where
u 2 (x i) are the standard uncertainty associated with each quantity m,t w , t a , ϕ , ᶐ c, and t d ;

The sensitivity coefficient is a multiplication factor to calculate the extent to which the estimated value of the measurement result is influenced by changes in the estimated value of the input variable. It can be determined from the model function F using equation or numerical methods. ci2 are the square of sensitivity coefficients corresponding to each uncertainty element. They are derived from the partial derivation of the function F, with respect to each quantity.

DERIVATION OF SENSITIVITY COEFFICIENTS FOR A PISTON-OPERATED PIPETTE/ MICROPIPETTE

Approximations taken for the derivation of sensitivity coefficients are;

- ρw − ρa≈ ρw, and ρb − ρa≈ ρb , (approximations in the order of 103)
- ρb − ρw≈ ρb (approximations in the order of 101)

A. *Sensitivity coefficient for a balance reading m and evaporation losses;*

To get the sensitivity coefficient with respect to ‘m’, partial differentiation of F is done.

$$\text{Cw} = \frac{\partial F}{\partial m} = \frac{1-\alpha c(t_{d} - 20)}{\rho W}$$

On applying approximations,

$$= \frac{1-\alpha c(t_{d-}20)}{\sum\limits_{i=0}^{4} a_i t_w^i}$$

B. *Sensitivity coefficient related to the water temperature;*

$$c_{t_\mathrm{w}} = \frac{\partial F}{\partial t_\mathrm{w}} = -\frac{m}{\rho_\mathrm{b}} \times \frac{1-\alpha_\mathrm{c}(t_\mathrm{d} - t_\mathrm{d20})}{(\rho_\mathrm{w} - \rho_\mathrm{a})^2} \times (\rho_\mathrm{b} - \rho_\mathrm{a}) \times \left(\sum_{i=1}^{4} i a_i t_\mathrm{w}^{i-1} \right)$$

$$c_{t_\mathrm{w}} = \frac{\partial F}{\partial t_\mathrm{w}} \approx -\frac{m}{\rho_\mathrm{w}^2} \times \frac{\partial \rho_\mathrm{w}}{\partial t_\mathrm{w}} = -\frac{m}{\rho_\mathrm{w}^2} \times \left(\sum_{i=1}^{4} i a_i t_\mathrm{w}^{i-1} \right)$$

C. *Sensitivity coefficient related to the air temperature*

$$c_{t_\mathrm{a}} = \frac{\partial F}{\partial t_\mathrm{a}} = \frac{m}{\rho_\mathrm{b}} \cdot \left[1-\alpha_\mathrm{c}(t_\mathrm{d} - t_\mathrm{d20})\right] \times \frac{\rho_\mathrm{b} - \rho_\mathrm{w}}{(\rho_\mathrm{w} - \rho_\mathrm{a})^2} \times \frac{\varphi k_2 t_\mathrm{a0} - k_1 p_\mathrm{a} - \varphi k_3}{(t_\mathrm{a} + t_\mathrm{a0})^2}$$

$$c_{t_\mathrm{a}} = \frac{\partial F}{\partial t_\mathrm{a}} \approx \frac{m}{\rho_\mathrm{w}^2} \times \frac{\varphi(k_2 t_\mathrm{a0} - k_3) - k_1 p_\mathrm{a}}{(t_\mathrm{a} + t_\mathrm{a0})^2}$$

D. *Sensitivity coefficient related to the air pressure;*

$$c_{p_\mathrm{a}} = \frac{\partial F}{\partial p_\mathrm{a}} = \frac{m}{\rho_\mathrm{b}} \cdot \left[1-\alpha_\mathrm{c}(t_\mathrm{d} - t_\mathrm{d20})\right] \times \frac{\rho_\mathrm{b} - \rho_\mathrm{w}}{(\rho_\mathrm{w} - \rho_\mathrm{a})^2} \times \frac{k_1}{t_\mathrm{a} + t_\mathrm{a0}}$$

On applying approximations,

$$c_{p_\mathrm{a}} = \frac{\partial F}{\partial p_\mathrm{a}} \approx \frac{m}{\rho_\mathrm{w}^2} \cdot \frac{k_1}{t_\mathrm{a} + t_\mathrm{a0}}$$

If t_a = 20 °C is used:

$$c_{p_\mathrm{a}} = \frac{\partial F}{\partial p_\mathrm{a}} \approx 1{,}2 \times 10^{-9} \left(\frac{\mathrm{kg}}{\mathrm{m}^3} \mathrm{K} \right)^{-1} \times m \quad \mathrm{X} \ \frac{1}{\mathrm{hPa}}$$

E. *Sensitivity coefficient related to the relative air humidity ;*

$$c_\varphi = \frac{\partial F}{\partial \varphi} = \frac{m}{\rho_\mathrm{b}} \times \left[1-\alpha_\mathrm{c}(t_\mathrm{d} - t_\mathrm{d20})\right] \times \frac{\rho_\mathrm{b} - \rho_\mathrm{w}}{(\rho_\mathrm{w} - \rho_\mathrm{a})^2} \times \frac{k_2 t_\mathrm{a} + k_3}{t_\mathrm{a} + t_\mathrm{a0}}$$

$$c_\varphi = \frac{\partial F}{\partial \varphi} \approx \frac{m}{\rho_\mathrm{w}^2} \times \frac{k_2 t_\mathrm{a} + k_3}{t_\mathrm{a} + t_\mathrm{a0}}$$

If t_a = 20 °C is used:

$$c_\varphi = \frac{\partial F}{\partial \varphi} \approx -1 \times 10^{-10} \left(\frac{\mathrm{kg}}{\mathrm{m}^3} \% \right)^{-1} \times m$$

F. *Sensitivity coefficient for cubic expansion coefficient ạc of piston pipette*

$$c_{\alpha_\mathrm{c}} = \frac{\partial F}{\partial \alpha_\mathrm{c}} = -\frac{m}{\rho_\mathrm{b}} \times \frac{\rho_\mathrm{b} - \rho_\mathrm{a}}{\rho_\mathrm{w} - \rho_\mathrm{a}} \times (t_\mathrm{d} - t_\mathrm{d20})$$

$$c_{\alpha_\mathrm{c}} = \frac{\partial F}{\partial \alpha_\mathrm{c}} \approx -\frac{m}{\rho_\mathrm{w}} \times (t_\mathrm{d} - t_\mathrm{d20})$$

$$c_{\alpha_\mathrm{c}} = \frac{\partial F}{\partial \alpha_\mathrm{c}} \approx -10^{-3} \left(\frac{\mathrm{kg}}{\mathrm{m}^3} \mathrm{K} \right)^{-1} \times m \times (t_\mathrm{d} - 20 \ {}^\circ\mathrm{C})$$

G. *Sensitivity coefficient related to the temperature of piston pipette*

$$c_{t_d} = \frac{\partial F}{\partial t_d} = -\frac{m}{\rho_b} \times \frac{\rho_b - \rho_a}{\rho_w - \rho_a} \times \alpha_c$$

$$c_{t_d} = \frac{\partial F}{\partial t_d} \approx -\frac{m}{\rho_w} \times \alpha_c$$

If $\alpha_c = 10^{-5}\ K^{-1}$ is used:

$$c_{t_d} = \frac{\partial F}{\partial t_d} \approx 10^{-8} \left(\frac{kg}{m^3} K \right)^{-1} \times m$$

IDENTIFICATION OF POSSIBLE RANGES OF OPERATIONS OF PROCESS VARIABLES

Uncertainty contributions from balance reading- m, water temperature-tw, air temperature-ta, relative humidity of air- ϕ ,thermal expansion co efficient of pipettes- ᶐc, and device temperature-td; are identified as the process variables in the calibration of micro volume. Results of micro volume calibrations may get influenced by these variables. Each of these may range from a lower limit to upper limit depending on their environment specific to geographical positions. The probable range of each element in routine calibrations are listed in table 5.1 and their selection is discussed in section 5.1.1

TABLE 5.1

IDENTIFIED PROCESS VARIABLE AND THEIR RANGE

Uncertainty element/ Process Variable	**Symbol**	**Range of operation/ calibration**
1.Balance reading	m	Measurements with no. of observations
2.water temperature	tw	n=5, n=10, n=15, n=20, n=25
3.air temperature	ta	15^{o}C, 20^{o}C, 23^{o}C, 27^{o}C and 30^{o}C
4.air pressure	pa	15^{o}C, 20^{o}C, 23^{o}C, 27^{o}C and 30^{o}C
5.relative humidity of air	ϕ	960 hPa, 980 hPa, 1000 hPa, 1013 hPa and 1030 hPa
6.thermal expansion coefficient of pipette tip	ᶐc	50% , 60%, 70%, 80% ,and 90%
7.device temperature	td	For Polyethylene, polypropylene, polytetrafluoroethylene, poly carbonate and polystyrene

Range of experimental levels and uncertainty contributions of process variables

A. *Balance reading-m*

For a nominal volume selected for calibration of a pipette, the results of measurements will be reported as average of 'n' trials with a spread indicated by standard deviation. Where n is the number of observations and it can be n=5, or n=10, or n=15, or n=20, or n=25.

B. *Water, air and device temperature (t w, t a and t d)*

The specifications provided by ISO 86556 for environment range from 15°C to 30°C in their temperature conditions. For the experimental purpose the lower limit is set as 18°C which may be practical in air conditioned controlled environment and 30°C as upper limit in a non air conditioned laboratory environment. Environment of Indian laboratories may range from 15°C to 30°C in their temperature conditions. Hence 5 level identified for the variable temperature are 15°C, 20°C, 23°C, 27°C and 30°C.

C. *Air pressure pa*

The air pressure level at a laboratory environment can range between 960hPa and 1030 hPa according to their geographical position. Hence 5 levels to cover this selected range are 960 hPa, 980 hPa, 1000 hPa, 1013 hPa and 1030 hPa.

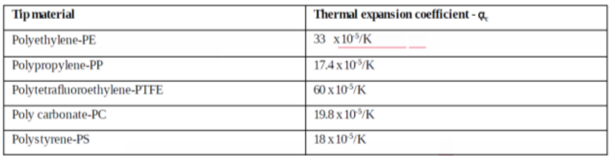
D. *Relative humidity of air ϕ*

Relative humidity > 50% is an essential requirement of piston pipette calibrations for minimizing the evaporation losses of microvolume calibrations. In India, humidity conditions reach more than 90% in humid localities like Kerala. Five RH levels, 50%, 60%, 70%, 80% ,and 90%, were selected for the experimental studies, though they are highly dependent on environmental temperature.

E. *Thermal expansion coefficient α_c*

Polyethylene, polypropylene, polytetrafluoroethylene, poly carbonate and polystyrene are the identified pipette tip materials in common use. Their thermal expansion coefficients are tabulated in table 5.1.E below [7].

TABLE 5.1.E:
THERMAL EXPANSION COEFFICIENT OF TIP MATERIAL



Tip material	**Thermal expansion coefficient - α_c**
Polyethylene-PE	33 x 10^{-5}/K
Polypropylene-PP	17.4 x 10^{-5}/K
Polytetrafluoroethylene-PTFE	60 x 10^{-5}/K
Poly carbonate-PC	19.8 x 10^{-5}/K
Polystyrene-PS	18 x 10^{-5}/K

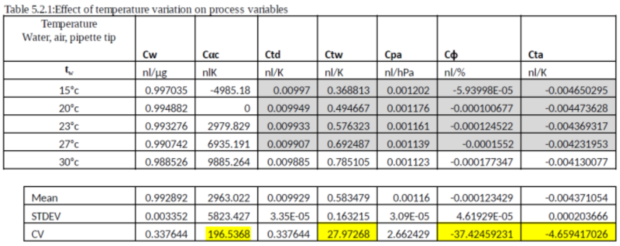
Estimation of Sensitivity coefficient values at identified levels of process variables and Coefficient of variation for each variable.

To study the influence of process variables in the final uncertainty measurement; steps given below are followed

- Sensitivity coefficient values are estimated for all process variables at the 5 different selected levels
- The coefficient of variation(CV) is estimated for the range of values corresponding to sensitivity coefficient for each variable

CV =standard deviation/average x 100%

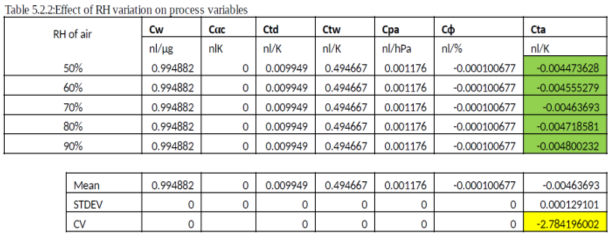
Table 5.2.1:Effect of temperature variation on process variables



Temperature Water, air, pipette tip	Cw	Cαc	Ctd	Ctw	Cpa	Cϕ	Cta
t_w	nl/µg	nlK	nl/K	nl/K	nl/hPa	nl/%	nl/K
15°c	0.997035	-4985.18	0.00997	0.368813	0.001202	-5.93998E-05	-0.004650295
20°c	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004473628
23°c	0.993276	2979.829	0.009933	0.576323	0.001161	-0.000124522	-0.004369317
27°c	0.990742	6935.191	0.009907	0.692487	0.001139	-0.0001552	-0.004231953
30°c	0.988526	9885.264	0.009885	0.785105	0.001123	-0.000177347	-0.004130077

Mean	0.992892	2963.022	0.009929	0.583479	0.00116	-0.000123429	-0.004371054
STDEV	0.003352	5823.427	3.35E-05	0.163215	3.09E-05	4.61929E-05	0.000203666
CV	0.337644	196.5368	0.337644	27.97268	2.662429	-37.42459231	-4.659417026

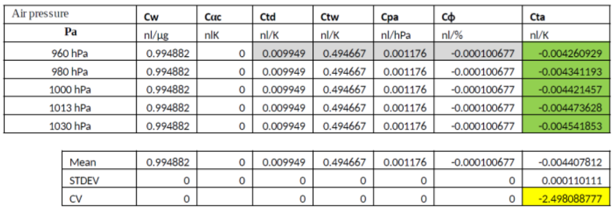
Table 5.2.2:Effect of RH variation on process variables



RH of air	Cw	Cαc	Ctd	Ctw	Cpa	Cϕ	Cta
	nl/µg	nlK	nl/K	nl/K	nl/hPa	nl/%	nl/K
50%	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004473628
60%	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004555279
70%	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.00463693
80%	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004718581
90%	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004800232

Mean	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.00463693
STDEV	0	0	0	0	0	0	0.000129101
CV	0		0	0	0	0	-2.784196002

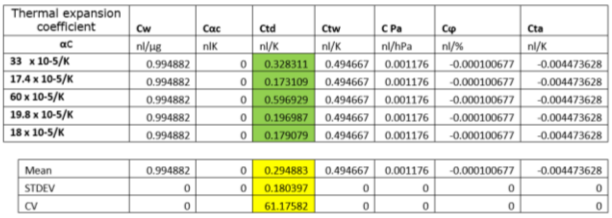
Table 5.2.3:Effect of air pressure variation on process variables



Air pressure	Cw	Cαc	Ctd	Ctw	Cpa	Cϕ	Cta
Pa	nl/µg	nlK	nl/K	nl/K	nl/hPa	nl/%	nl/K
960 hPa	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004260929
980 hPa	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004341193
1000 hPa	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004421457
1013 hPa	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004473628
1030 hPa	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004541853

Mean	0.994882	0	0.009949	0.494667	0.001176	-0.000100677	-0.004407812
STDEV	0	0	0	0	0	0	0.000110111
CV	0		0	0	0	0	-2.498088777

Table 5.2.4: Effect of variation in thermal expansion coefficient on process variables



Thermal expansion coefficient	Cw	Cαc	Ctd	Ctw	C Pa	Cφ	Cta
αC	nl/µg	nlK	nl/K	nl/K	nl/hPa	nl/%	nl/K
33 x 10-5/K	0.994882	0	0.328311	0.494667	0.001176	-0.000100677	-0.004473628
17.4 x 10-5/K	0.994882	0	0.173109	0.494667	0.001176	-0.000100677	-0.004473628
60 x 10-5/K	0.994882	0	0.596929	0.494667	0.001176	-0.000100677	-0.004473628
19.8 x 10-5/K	0.994882	0	0.196987	0.494667	0.001176	-0.000100677	-0.004473628
18 x 10-5/K	0.994882	0	0.179079	0.494667	0.001176	-0.000100677	-0.004473628

Mean	0.994882	0	0.294883	0.494667	0.001176	-0.000100677	-0.004473628
STDEV	0	0	0.180397	0	0	0	0
CV	0		61.17582	0	0	0	0

Based on the CV values; a correlation matrix is prepared for the process variables and sensitivity coefficients and is tabulated (Table 5.2.5).

TABLE 5.2.5
CORRELATION MATRIX OF PROCESS PARAMETERS

Correlation Matrix- Process Variables Vs. Sensitivity Coefficients					
Sensitivity Coefficients	**tw**	**td**	**ta**	**αc**	**pa**
	CV values (%)				
Ctw	27.97	27.97	27.97	X	X
Ctd	0.337	0.337	0.337	61.18	
Cta	-4.66	-4.66	-4.66	X	-2.5
Cαc	196.54	196.54	196.54	X	X
Cpa	2.66	2.66	2.66	X	X
Cϕ	-37.43	-37.43	-37.43	X	X
Note: X indicates negligible CV values					

From the above table; it is evident that the entire five variables are having influence in the volume calibrations. Temperature and thermal expansion co-efficients were the most significant contributors and air pressure and humidity are the least significant contributors in the volume estimations and calibrations.

A priority order is given to different variables based on their CV contributions as

a) Temperature (water, air and device)
b) Thermal expansion co-efficient
c) RH
d) Air pressure

EXPERIMENTS FOR VOLUME CALIBRATION

The experiment was planned for the 4 selected process variables namely temperature, relative humidity, thermal expansion co-efficient of the material used for pipette tip (t, pa, ϕ, α̣c,) at five selected levels. Assumed that at stabilized environmental conditions; water temperature, air temperature and device temperature reaches equilibrium temperature and took as equal for theoretical estimation purposes (tw= ta= td).

Using the equation of Volume function V20; volume capacity for a micropipette at 100µl nominal setting is calculated theoretically. Total 625 (for 4 variable at 5 levels - 54) combinations were prepared in Microsoft Excel spread sheets and calculated their corresponding volume values. The combination of process variables was selected based on the priority assignment done in section 5.2 (t, α̣c, ϕ, and pa).The spread of estimated volume results are represented in figure 6.

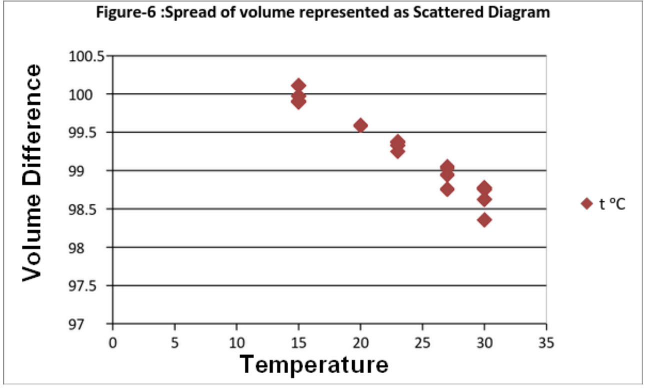


Figure-6 :Spread of volume represented as Scattered Diagram

Information on influence of the process variable temperature could be observed from figure 6. But analyzing the variations in volume at different combinations of process variables and establishing their correlation was very difficult in the total data set . Hence a practical range of process variable was selected for doing experiments to analyze the problem. Details are provided in following sections.

Selection of range for process variables

Range of Process variable – m

Number of observation, n>15 can assure a practically reliable results and n<5 may yield poor confidence level in reporting the measurement results. [8]. For the purpose of uncertainty estimation due to repeatability in measurements, n=10 is considered to provide sufficient information [9,10].Hence n is kept as constant (n=10) for the experiment.

Range of temperature- (tw/ ta/ td)

Out of the five temperatures (ranges from 15^{o}C to 30^{o}C) 20^{o}C, 23^{o}C and 27^{o}C are selected for the current study. The selection is based on international standards for volume calibration methods and volumetric apparatus, most of them specify volume capacity of volumetric wares at 20^{o}C [9]. But for tropical countries like India, 27°C may the standard temperature for volumetric operations [11]. In ideal conditions; water, air and device temperatures are assumed to get stabilized and equilibrated at environment temperature.

Range of air pressure- (pa)

The air pressure level at a permanent laboratory environment will not be a variable. In Trivandrum which is located at sea level; air pressure is 1013 hPa and kept constant for experimental purposes.

Range of relative humidity of air- (ϕ)

Different apex bodies of metrology ; specifies RH conditions from 35% to 85% for mechanical calibrations (NABL , India recommends 35% to 85% RH conditions and National Voluntary Laboratory Accreditation Program, NIST specified 40% to 60%)[12,13].RH greater than 50% is suggested by ISO 8655. Hence 50%, 60%, 70% and 80% RH levels are selected for the study to cover the possible uncertainty contributions of RH variable.

Range of thermal expansion co-efficient of material for pipette tip- (ᾳc)

The ᾳc for the commonly used pipette tip material is 17.4 x 10-5/K (poly propylene). Hence it was kept as constant for the purpose of experiments.

Combination of process variables for conducting experimental trials

Based on section 6.1, the control of only two process variables, temperature and RH, was possible. Hence, all possible combinations of the two variables were experimented with. Actual experiments were conducted in the following combination indicated in table 6.2.

TABLE 6.2
COMBINATION OF EXPERIMENTAL TRIALS

Sl.No	**Temperature °C**	**RH %**
1	20	50
2	20	60
3	20	70
4	20	80
5	23	50
6	23	60
7	23	70
8	23	80
9	27	50
10	27	60
11	27	70
12	27	80

Experimental trials - Estimation of Z-factor and nominal volume

In the experiment of volume calibration, gravimetric method of measurement was followed. The volume of water contained in the micropipette was delivered to a container placed on an electronic balance with 0.01mg resolution. To minimize the vibration effects in measurements' the balance was placed in an ant vibration table. The calibration environment was kept controlled within ± 1 °C temperature and ±2% RH conditions.

The mass value of the delivered volume of water by the micropipette was indicated in the balance and it was recorded. The measurement steps were repeated 10 times. All the related measurement conditions were recorded. It included the temperature of the air, the temperature of the water, the relative humidity of the air, and the mass value indicated by the balance. Based on the recorded temperature, RH, air pressure, thermal expansion coefficients, and mass indications, the Z x Y value and volume capacity (m x Z x Y) of the micropipettes were calculated.

Results from experimental trials

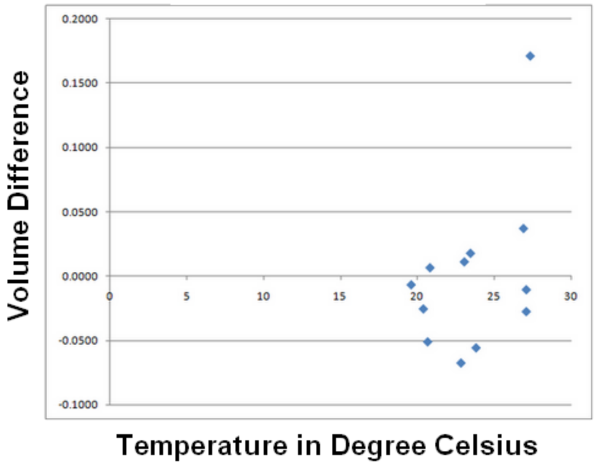
Based on section 6.3, volume estimations were completed and tabulated in Table 6.4 below. The results with theoretical estimations were compared with these results and plotted in Figure 6.4.

TABLE 6.4
RESULTS FROM VOLUME CALIBRATION EXPERIMENTS FOR ALL COMBINATIONS OF RH & TEMPERATURE.

Temperature	**RH**	**Volume (from experiment)**	**volume (theoretical)**	**Difference (after bias correction)**
°C	%	µl	µl	µl
19.61	50.36	100.17	99.59	**-0.0066**
20.68	60.2	100.22	99.59	**-0.0509**
20.4	69.77	100.19	99.59	**-0.0253**
20.83	80.92	100.16	99.59	**0.0066**
23.46	50.53	99.94	99.38	**0.0178**
22.84	59.91	100.02	99.38	**-0.0673**
23.82	69.14	100.01	99.38	**-0.0556**
23.06	79.88	99.94	99.38	**0.0112**
26.9	50.16	99.59	99.05	**0.0371**
27.08	60.37	99.64	99.05	**-0.0104**
27.08	60.37	99.66	99.05	**-0.0274**
27.34	80.65	99.46	99.05	**0.1710**

Figure 6.4 indicates the spread of error in volume measurements by experimental method against volume at typical environment conditions calculated theoretically. The volume at temperature 27°c and RH 80% is having a notable deviation from other points. At all other conditions the volume measurement are within control. But the individual influence of each the process variable could not be derived here. Hence the tool DoE is applied for the study.

Figure 6.4:Comparison of volume from experiment and Theoretical estimations



DESIGN OF EXPERIMENTS (DOE)

Sir RA Fisher introduced the method of designing experiments by conduct of experimental trials with different combinations of multiple factors involved. [14,15]. This technique is known as the factorial design of experiments.

A full factorial design will use all possible combinations for an available set of factors. It may result in large number of experimental trials, on having many factors affecting the experimental results. To reduce the number of experiments to a practical level, only a small set from all the combinations is selected. The method of selecting a limited number of experiments which produces the optimum result is known as a partial fraction experiment.

DoE in Volume calibration:

Using DoE, full information of all influential factors can be derived with optimum number of experiments. Skilled selection of combinations of design variables for the conduct of experiments can result in efficient solutions. The minimum number of experiments that are required to conduct the design of optimization experiments can be calculated based on DoE approach. A statistical tool Minitab was used to conduct the DoE for the volume calibration experiments of micropipettes. Based on the results of DoE for a defined range of process variables a transfer function is derived for the volume calibrations. This simple transfer function was used to estimate the volume values for theoretically selected set values of process variables. This is then proved to be equivalent to the original mathematical model of the volume measurement function V20. For this purpose, the error between the volume estimations; resulted from both the derived transfer function from DoE and the original mathematical function were mathematically analyzed and their normality in distribution was studied.

DoE for the theoretical Range of Process Variables:

The design of the experiment for the range selected as per Table 7.2.1 is carried out using Minitab software. A transfer function is derived for the Z factor calculations for the combinations of process variables suggested by DoE analysis. The results of the analysis are tabulated in Table 7.2.2.

A histogram (figure 7.2.1) is plotted for the residual errors of transfer function values and theoretical values of the Z factor. This indicates a normal distribution of the transfer function. Figure 7.2.2- Pareto chart indicates the influence of process variables [16].

TABLE 7.2.1
SELECTION OF RANGE FOR DOE

Uncertainty element/ Process Variable	**Symbol**	**Range of operation/ calibration**

Temperature	t	15°C, 20°C, 23°C, 27°C and 30°C
Air pressure	pa	960 hPa, 980 hPa, 1000 hPa, 1013 hPa and 1030 hPa
Relative humidity of air	ϕ	40% , 60%, 70%, 80% ,and 90%
Thermal expansion coefficient of pipette tip	αc	For Polyethylene, polypropylene, polytetrafluoroethylene, poly carbonate and polystyrene

TABLE 7.2.2
RESULTS FOR DOE

t	**RH**	**αc**	**pa**	**V**	**z**
15	40	0.000174	960	99.889	0.99889
30	40	0.000174	960	98.778	0.98778
15	90	0.000174	960	99.889	0.99889
30	90	0.000174	960	98.777	0.98777
15	40	0.000330	960	99.969	0.99969
30	40	0.000330	960	98.620	0.98620
15	90	0.000330	960	99.969	0.99969
30	90	0.000330	960	98.619	0.98619
15	40	0.000174	1030	99.899	0.99899
30	40	0.000174	1030	98.781	0.98781
15	90	0.000174	1030	99.898	0.99898
30	90	0.000174	1030	98.780	0.98780
15	40	0.000330	1030	99.976	0.99976
30	40	0.000330	1030	98.627	0.98627
15	90	0.000330	1030	99.976	0.99976
30	90	0.000330	1030	98.626	0.98626
30	40	0.000174	960	98.778	0.98778
15	90	0.000174	960	99.889	0.99889

FIGURE 7.2.1
HISTOGRAM OF RESIDUAL ERROR OF TRANSFER FUNCTION WITH THEORETICAL VALUE

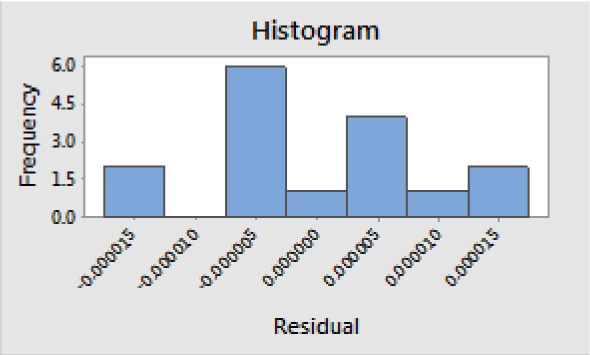
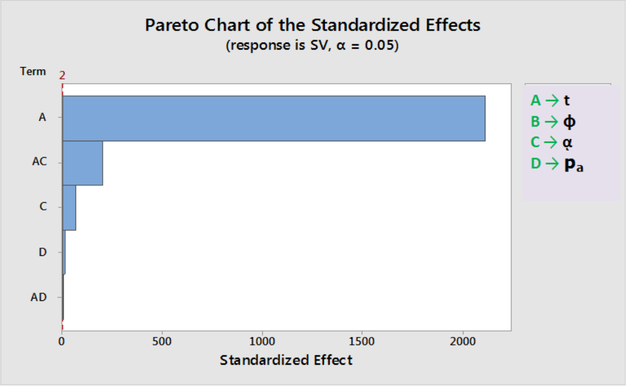


FIGURE 7.2.2
PARETO CHART INDICATING THE INFLUENCE OF PROCESS VARIABLES. A INDICATES TEMPERATURE, B INDICATES RH, C INDICATES EXPANSION COEFFICIENT AND D CORRESPONDS TO PRESSURE.



DoE for the Practical Range of Process Variables:

The design of the experiment for the range selected as per Table 7.3 is carried out using Minitab software. A transfer function is derived for the Z factor calculations for the combinations of process variables suggested by DoE analysis. The results of the analysis are tabulated in Table 7.3.

Derived transfer function is given as

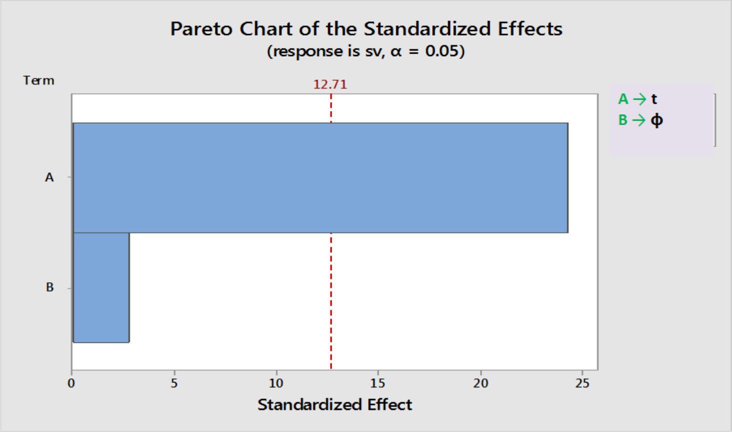
z = 1.01234 - 0.000763 Temp - 0.000020 RH

The transfer function indicates clearly that the effect of RH is not significant in the selected range of 50% to 80% of RH. A Pareto chart (figure 7.3) plotted indicates the influence of process variables in volume calibrations. This gives clear evidence of the effect of temperature and the insignificance of RH.

TABLE 7.3
PRACTICAL RANGE OF PROCESS VARIABLES FOR DOE

Uncertainty element / Process Variable	Symbol	Range of operation/ calibration
temperature	t	20^{o}C, 23^{o}C,and 27^{o}C
relative humidity of air	ϕ	50% , 60%, 70% and 80%
air pressure	pa	1013 hPa
thermal expansion coefficient of pipette tip	αc	polypropylene

Figure 7.3
Pareto chart indicating the influence of process variables. A indicates temperature-t and B indicates RH-□.



CONCLUSIONS

In the study 'Optimization of process variable to minimize uncertainty in micro-volume measurements, an attempt is made to analyze the role of uncertainty contributing process variables in volume calibrations based on ISO 8655. All the relevant process variables were identified by modeling the volume function and deriving their sensitivity coefficient by partial differentiation of the volume function with respect to each process variable. Their influence was studied based on actual experiments and the Design of Experiments tool. The study concluded that precise control of process variable - temperature with an RH above 50% could optimize the uncertainty in micro-volume measurements. A simple transfer function derived using the DoE can be used for volume estimations in an optimized environment conditions of temperature and RH.

The design of experiments indicates that all the process variables are not significantly contributing to the uncertainty of volume calibration results except the variable temperature. In micro-volume calibrations, the most significant process variables contributing to the uncertainty are variations in temperature and thermal expansion coefficient. But for a specific pipette, the thermal expansion coefficient can be kept constant. Precise control of temperature at the reference temperature (20^{o}C for V20) conditions is the optimized condition for micro-volume calibrations. Error and uncertainty in measurement may increase significantly as the environment temperature deviates largely from the reference temperature. The process variable RH does not have a significant role in contributing to uncertainty. In the study, a simple transfer function was derived based on DoE that can be used for volume estimations in the controlled and optimized environment of calibration laboratories.

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