Mini-Review

Assessing variations in manual pipetting: An under-investigated requirement of good laboratory practice

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A B S T R A C T

Pipettes are essential tools for biomedical and analytical laboratories, analogous to workstations for computer scientists. Variation in pipetting is a known unknown, as it is generally accepted that variations exist, but thus far, there have been limited studies on the extent of these variations in practice. In this mini-review, we highlight how manual pipetting is a key technique in the laboratory, and, although simple, inaccuracy and imprecision exist. If variations are not adequately addressed, errors can be compounded and consequently compromise data quality. Determination of the accuracy and precision of manual pipetting is straightforward, and here we review two common approaches that use gravimetry and spectrophotometry as readouts. We also provide detailed protocols for determination of accuracy and precision using manual single and multi-channel pipettes. These simple-to-use methods can be used by any laboratory for competency training and regular checks. Having a common protocol for evaluation of variation will also enable cross-laboratory comparison and potentially facilitate establishment of a reference value of acceptable ranges for operator error. Such a value could be of relevance to the scientific community for benchmarking and assuring good laboratory practice.

Introduction

Manual pipetting, a ubiquitous process in biomedical and analytical laboratories

Liquid handling is one of the most common processes in various academic, clinical, industrial and regulatory laboratory settings. Micropipettes are an indispensable tool for most laboratories that require handling of micro- to milli-liters of liquids. Generally, it is common knowledge that variations in pipetting exist [1]. Manufacturers of pipettes have assembled guides demonstrating good pipetting techniques and recommendations for regular checks and calibration. Guidelines are in place to ensure that the laboratory equipment is maintained and meets regulatory specifications for accuracy and precision, which are pre-requisites for laboratory certification in clinical [2], industrial, and regulatory environments. While these regulations cover the hardware, other potential sources of variation include the environment [3-5], operators [6-9], consumables [10], and technique [9,11] (Fig. 1A), all of which need to be monitored to ensure good manufacturing practice (GMP) and good laboratory practice (GLP). Indeed, pipetting precision assessments are commonly conducted in industry settings to ensure operator competency. On the other hand, the requirements for academic

laboratories are less stringent, and laboratory practices vary. Nonetheless, research in academia has contributed significantly to diagnostics and drug discovery, as well as non-healthcare sectors, such as food, fuel and other consumer products. Acquisition of research skills, including pipetting, starts in the education setting and understanding the technique, as well as precision and accuracy requirements, should also begin at this early stage.

Variations in manual pipetting, an under-investigated known unknown

The effects of inaccuracy and imprecision from manual pipetting should not be overlooked as errors are generally propagated in multi-step procedures [12] (Fig. 1D). Hence, factors which can contribute to variation (Fig. 1A) must be well-controlled. Furthermore, the analysis procedure will involve measurement methods that have an inherent range of variation and will contribute to the quality of an assay. Accuracy and precision are both critical measures in the validity of an assay or test. Accuracy is defined as how close a set of measurements are to their true value, while precision is defined as how close the various measurements are to each other. Variations for either or both measures can lead to artifacts, which may have detrimental impacts, such as skewed research findings, and, in the clinical setting, misdiagnoses.
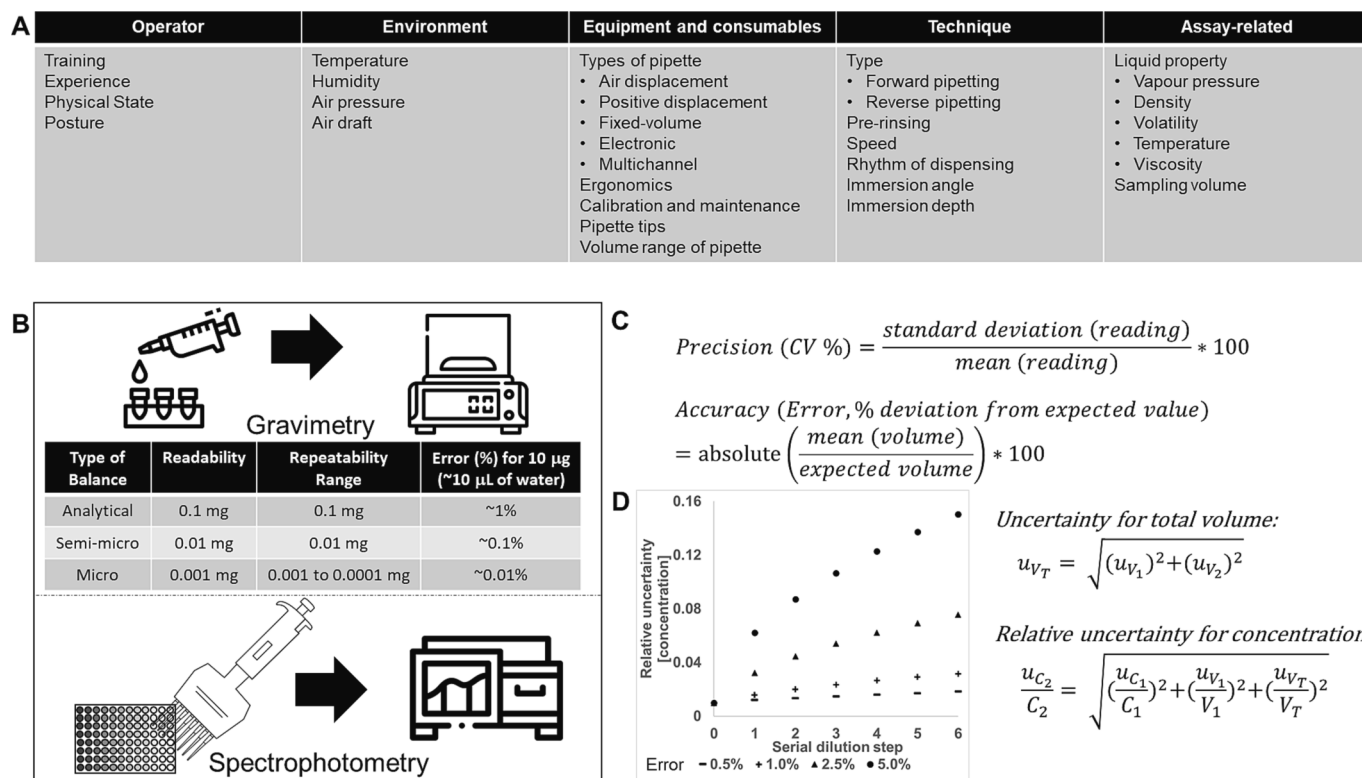

Fig. 1. Assessing variations in manual pipetting. (A) Factors affecting pipetting accuracy and precision. (B) Two common methods for determination of accuracy and/or precision, gravimetry and spectrophotometry. For gravimetry, the choice of the balance needs to be considered, as measuring the mass of low volumes will be inaccurate, especially if reaching the readability and repeatability limits of the instrument. (C) Formulae for calculation of accuracy and precision. Low coefficient of variance (CV) and error mean high precision and accuracy respectively. (D) Representation of a simplified model of error propagation. In this case, a serial dilution experiment was considered, where the uncertainty of the diluted concentration (u_{C_2}) of the reagent is influenced by the uncertainty of the total volume (u_{V_T}), the previous concentration (u_{C_1}) and volume of the previous concentration of solution added (u_{V_1})[12]. If the error increases (0.5% to 5%), it is clearly propagated at every step of the procedure. More specific details and formulae for determination of uncertainty in gravimetric volume is described in the EURAMET guide[5].

With a strong need for quality assurance, reference values and materials, as well as reporting guides, are made available by various international bodies or networks, including National Institute of Standards and Technology (NIST), International Organization for Standardization (ISO) [13-16], Equator Network, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), Clinical and Laboratory Standards Institute (CLSI), and Food and Drug Administration (FDA). For instance, NIST offers NIST standard reference materials (SRM) for verification of important measurement results, development of new measurement methods and provision of a means to establish the traceability of results by benchmarking to a stated reference [17,18]. Increasingly, standardization and harmonization efforts are also taking place in academic settings, including international ring trials to address inter-laboratory variation of assays that can potentially be used for diagnostic purpose, and to establish reference values of biomolecules of relevance to human health [19-22]. Hence, it is of importance to ensure that during the analysis processes that involve liquid handling, accuracy and precision of pipetting are properly accounted for [23]. While there are a number of reports addressing the extent of variation, including the effects of operators and training [6,7,9,24-26], there are no reference values for permissible pipetting errors, except for manufacturer specifications of the equipment and the ISO 8655 guidelines for a piston-operated volumetric apparatus [13,14]. Moreover, the existing reports employed a range of different assays and/ or readouts and are not well-suited for cross-referencing. Having a reference value, based on standardized assays, for permissible operator variations would allow benchmarking of performance for GLP, and serve as a form of quality assurance.

Common methods of assessing pipetting accuracy and precision

The accuracy and precision of micropipettes can be evaluated using simple bioanalytical approaches, with the most common methods involving gravimetry and spectrophotometry [27] (Fig. 1B). The ISO 8655 also provides a set of guidelines for gravimetry-based [15] (ISO 8655-6) and photometry-based [16] (ISO 8655-7) assays which are used for piston-type pipette calibration.

The gravimetric approach is based on the principle of measuring the mass of a fixed volume of liquid, most commonly water, to assess pipette accuracy and precision. Accuracy is determined by taking the average of repeat measurements and comparing these to the expected value, while precision is derived by calculating the coefficient of variation of the readings (Fig. 1C). Considering the non-linear property of manual pipettes, the error when handling small volumes is not necessary linearly correlated with the error when handling large volumes. Hence assessing manual pipetting variations should involve multiple volume ranges, which, in fact, are used in the calibration process [25,28]. The gravimetry method is straightforward to implement and most laboratories will have at least an analytical balance to perform this test. Fig. 2 shows the variation in pipetting accuracy and precision of two types of liquids, water and chloroform, based on data collected from 10 junior academic researchers who had received supervision during their onboarding training. As expected, variations did exist, and the degree of error depended on the type of liquid and volumes pipetted, amongst other factors. When factoring in the permissible error from the manufacturer and ISO 8655, a significant number of measurements exceeded these limits (Fig. 2, orange and red dashed lines), which brings into question what should be the cutoff for evaluating operator performance.
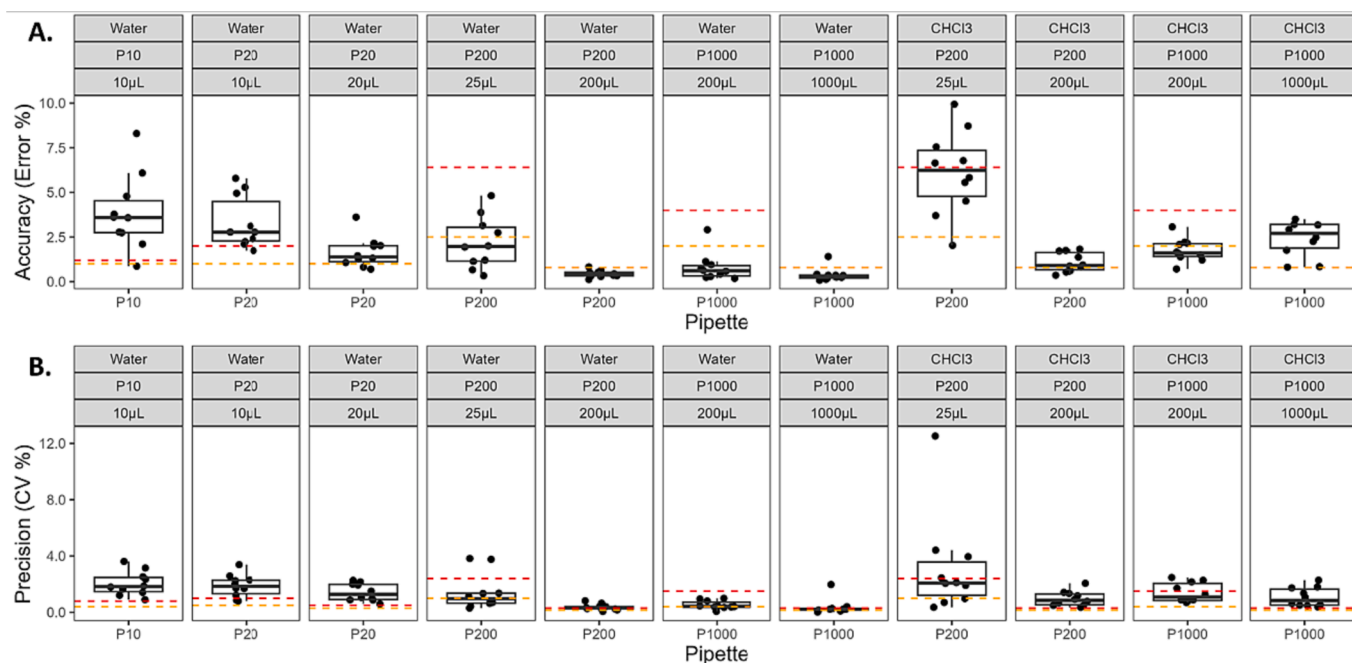
Fig. 2. Variations in manual pipetting of water and chloroform using air displacement pipettes. To determine the accuracy and precision, various volumes of two types of liquids, water and chloroform (CHCl3), were weighed with an analytical balance (readability: 0.1 mg, repeatability, 0.1 mg) by 10 operators (with supervision). The liquids were handled using a range of single channel pipettes, with six repeat measurements. Accuracy (defined by error %, which is calculated based on the deviation from expected mass) (A) and precision (defined by CV %) (B) were calculated and represented as boxplots, with the orange dashed lines representing the error cutoff from the manufacturer, and pink dashed lines representing the error cutoff from ISO8655. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Critically, the observation that the errors for low volumes (20 μL or less) of water mostly failed the ISO 8655 cutoff could be due to the limits of the analytical balance used (Fig. 1C), and not necessarily related to the operators or the pipettes. This warrants consideration for setting cutoffs based on the specification of the measurement tools, since not all laboratories own a microbalance, which offers greater sensitivity and precision for mass measurements.

The gravimetry method is well-suited for training and determination of the error range for handling diverse liquids including those that are difficult to pipette due to their viscosity (e.g. glycerol) or volatility (e.g. isopropanol, ethanol). As can be observed, when operators were tasked with pipetting chloroform, a highly volatile liquid used in bioanalysis [8,29-31] and molecular biology [32], the errors were generally higher than for water (Fig. 2, *p*-value < 0.05 for inaccuracy and precision). Moreover, unlike water, which exhibited a non-linear inverse correlation between volume and error, the correlation between volume and error is less clear for chloroform. This was expected as chloroform has a high vapour pressure and, hence, exhibits a higher evaporation rate as well as a tendency to drip with forward pipetting using air-displacement pipettes. The latter can be overcome technically by pre-wetting the pipette tip by aspirating and dispensing the liquid a few times to saturate the air cushion. Alternatively, other liquid handlers, including glass syringes and positive displacement pipettes can be used for volatile organic solvents as well as viscous liquids. With both syringes and positive displacement pipettes, the liquid is in direct contact with the piston and there is no air cushion, thus pipetting performance will not be affected by the physical properties of the sample. Hence, these apparatuses are well-suited for handling volatile solutions as well as hot and cold liquids in contrast to air displacement pipettes. However, each laboratory will need to consider the available options based on their applications as a syringe is not suitable for reuse without washing, and the pipette tips for positive displacements pipettes are costly. For processes requiring high throughput, automated liquid handling systems, which implement measures such as active anti-droplet control that

compensate for pressure changes due to evaporation, have also enabled pipetting of highly volatile liquid with improvement of analytical reproducibility [8], and will require alternative measures for performance testing [33-35].

Clearly, the gravimetric method is useful for testing a range of manual pipettes, as well as liquids. The degree of pipetting variation cannot be based on assumption and must be systematically tested based on the laboratory's applications. Importantly, several considerations need to be in place when using this approach. First, multiple factors (Fig. 1A) can affect the results, including changes in the testing environment, such as temperature, humidity, and atmospheric pressure, which need to be controlled [5]. To address this, the measured mass can be converted with a Z-factor, which considers the effects of the environmental temperature and air pressure on the liquid density [3-5,36]. In addition, these environmental factors can affect evaporation rates, which will have significant impact, especially for small volumes [15]. The results can also be influenced by the sensitivity and precision of the weighing scale used, which have an inherent error, as summarized in Fig. 1C, that also needs to be factored in for result evaluation. To ensure the test is properly conducted to minimize error contribution from the evaluation methods, calibration and performance checks of the balance will need to be performed with calibration weights. A drawback of the gravimetic analysis method is that the throughput is low and evaluation is tedious for testing of multichannel pipettes.

Photometry-based assays can address the throughput issue, as tests for both single- and multi- channel pipettes can be implemented in a 96-well format and the readout can be carried out using a plate-reader (Fig. 1B). A wide range of reagents, such as Orange G [37], potassium dichromate, copper (II) sulfate, and methylene blue, which can absorb or transmit light over a specific wavelength, are readily available. The involvement of multiple steps of pipetting and serial dilution is common in practice, particularly for sample preparation and bioanalysis of sub-micro quantities of samples. While serial dilutions involve multiple steps, they allow use of the optimal pipetting volume range to improve

Table 1

Reproducibility of serial dilution using Cu(II)SO4 and methylene blue solutions (final volume: 150 µL). Both single- and multi-channel pipettes were used, and the inter-day and inter-operator variations were assessed. Precision (CV %), as well as linearity of the serial dilution (R^2 and slope) can be assessed with this approach.

Reagent	Pipette Type	Inter-day (Single Operator) CV (%)	Inter-day (Single Operator) R^2	Inter-day (Single Operator) Slope	Inter-operator CV (%)	Inter-operator R^2	Inter-operator Slope
Cu(II)SO4 Inter-day (n = 3) Inter-operator (n = 5)	Multi-channel (12 channels)	0.816 ± 0.107	0.99964 ± 0.00003	1364.1 ± 11.2	1.622 ± 1.526	0.99982 ± 0.00033	1362.6 ± 26.0
	Single-channel (n = 6)	1.385 ± 0.629	0.99991 ± 0.00004	1381.3 ± 14.9	1.465 ± 0.190	0.99965 ± 0.00036	1375.0 ± 47.4
Methylene Blue Inter-day (n = 4) Inter-operator (n = 5)	Multi-channel (12 channels)	0.996 ± 0.126	0.99884 ± 0.00045	14.479 ± 0.123	2.836 ± 1.762	0.99761 ± 0.00180	14.466 ± 0.509
	Single-channel (n = 6)	1.554 ± 0.406	0.99894 ± 0.00066	14.564 ± 0.362	2.166 ± 0.818	0.99864 ± 0.00056	14.415 ± 0.346

accuracy and precision in contrast to a one step dilution involving a high dilution factor; this is important since the error range is generally lower when handling liquids at the maximum volume capacity of manual pipettes (Fig. 2).

Using a photometry-based approach, users can test their precision by serially diluting a reagent in a 96-well plate format. Coefficients of variation for the readings can be obtained to evaluate precision, while regression analysis can be performed to determine the goodness of fit. With a reference material where the concentration is known, the accuracy can be further determined. Table 1 shows the inter-day and inter-operator reproducibility of serial dilution of two relatively inexpensive reagents, copper (II) sulfate and methylene blue, with measurement carried out using a spectrophotometer. As observed with the low inter-day CV (mean <1 % for multichannel pipette, and mean <2 % for single channel pipette) for the assays, they can be amenable to routine training and performance checks.

To ensure the quality of photometric measurement, regular maintenance, performance checks, and calibration of the spectrophotometer is required [38,39]. Furthermore, when conducting the above assay(s), a simple way to assess the precision of the photometric measurement is to perform multiple readings of the same plate. It should also be noted that, to achieve the highest accuracy and precision, the absorbance value range of most spectrophotometers is between 0.1 and 1.0 OD. This is based on the principle that 90 % of light will be absorbed by the sample at 1.0 OD, and any further increase in sample concentration will only reflect minor changes in absorbance with the reduced percentage of transmitted light and, hence, readings will be less accurate above this range.

Detailed protocols of the two described assays and a template for basic data analysis are provided in Supplementary Material (S1 to S3), which individual laboratories can adapt for training and evaluation of variation in pipetting performance. Result interpretation must take into considerations the performance of the respective evaluation methods, as outlined above, and best practice in ensuring instrument maintenance and performance checks is crucial in fact for all experimental nature. These approaches are a starting point, and ultimately each laboratory will need to conduct performance tests on the assays and methods used in the laboratory, such as quantitative polymerase chain reaction (qPCR) [40] and mass spectrometry [8], which are more complex analyses involving more steps and reagents. For instance, in mass spectrometry-based analysis of peptides, small molecules and lipids, organic solvents are frequently used from sample preparation through bioanalysis [8,17,20,22]. Hence, careful consideration of the type of pipettes used, as well as performance testing using the relevant solvents should be in place to achieve maximal accuracy and precision. Moreover, sample matrix presents another source of challenge during liquid handling.

Biological fluids such as plasma, which are commonly handled in clinical testing laboratories, as well as human research, are compositionally more complex and sample viscosity depends on multiple components [41,42]. Ultimately, technical replication using the exact matrix will provide a better representation of the variations.

Conclusion

Generation of reliable data starts with good practice. Variations in pipetting exist, but the extent to which they introduce error into an assay can be limited via close control over the hardware, environment and operator(s). Possession of any skill, even a common one such as pipetting, and understanding of a technique should not be assumed, and training [6,43] and evaluation of performance need to be in place. One key outstanding question is the acceptable range of variation introduced by pipetting, which needs to be collectively addressed at the community-level.

We have provided a set of protocols as a starting point for laboratories to introduce training and assessment of variation. With further refinements and improved study design, it is foreseeable that such shared protocols could be used for inter-laboratory evaluation of pipetting variation. Precision and accuracy are critical in diverse arenas, and in the medical community the STAndards for the Reporting of Diagnostic accuracy studies (STARD) [44] aims to improve the accuracy and completeness of reporting of studies of diagnostic accuracy. Records of performance testing to establish a baseline for the equipment can be put in place to reduce the likelihood of error contribution from pipettes. Overall, greater awareness of variations introduced during pipetting, as well as training, along with regular testing and maintenance of these ubiquitous liquid handlers is important to ensure the validity of any study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2023.09.001.

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