Measurement Good Practice Guide No. 69

The Calibration and Use of Piston Pipettes

John Blues
National Weights and Measures Laboratory

David Bayliss
National Physical Laboratory

Mike Buckley
South Yorkshire Trading Standards Unit

Abstract: This publication is intended as an introduction and practical guide to the use and checking of piston pipettes by scientists, technicians and other laboratory workers. It covers single and multi-channel manual and automatic pipettes dispensing volumes ranging from 0.1 µl to 10 ml, but does not extend to medical syringes of the type used for giving injections.

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National Physical Laboratory
Teddington, Middlesex, United Kingdom, TW11 0LW

Website: www.npl.co.uk

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Contents

1 Introduction

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2 National and international standards

The current European Standard relating to pipettes is BS EN ISO 8655:2002 - *Piston-operated volumetric apparatus*. This also has the status of a British Standard. It is currently in six parts; a seventh is being prepared for publication. Most relevant to users of this guide are *Part 1: Terminology, general requirements and user recommendations* which includes a useful glossary of terms and definitions and *Part 6: Gravimetric methods for the determination of measurement error* which gives recommendations for testing and determining measurement errors of pipettes. It is recommended that all users of piston pipettes should obtain copies of these parts. Manufacturing requirements and tolerances for various types of pipettes and tips are given in *Part 2: Piston pipettes*. The previous British Standards BS 6018:1991, BS 7653-1:1993, BS 7532:1991 have been superseded by this standard, and German standard DIN 12650 has been withdrawn.

Two relevant standards published by the American National Standards Institute are: ASTM E542-01 - *Standard practice for the calibration of laboratory volumetric apparatus* and ASTM E1154-89(2003) - *Standard specification for piston or plunger operated volumetric apparatus*.

3 Classification and design

3.1 Pipettes

There are many types of piston pipette, but all work on essentially the same principle; hand or mechanical pressure on a piston or plunger working over a fixed length in a cylinder forces a pre-determined volume of liquid out of the orifice of the pipette.

They can be divided into two main groups:

3.1.1 Fixed volume

Those which are designed and supplied by the manufacturer to dispense a specific, fixed volume of liquid (defined as the nominal volume). This volume cannot normally be altered, although some types are designed so that they can be adjusted within small limits by the user to compensate for errors found during calibration or for use with liquids having physical properties differing from water.

3.1.2 Variable volume

Those in which the user can adjust the volume of liquid to be dispensed over a range specified by the manufacturer. In this case the nominal volume is defined as the upper limit of the manufacturer's designated volume range.

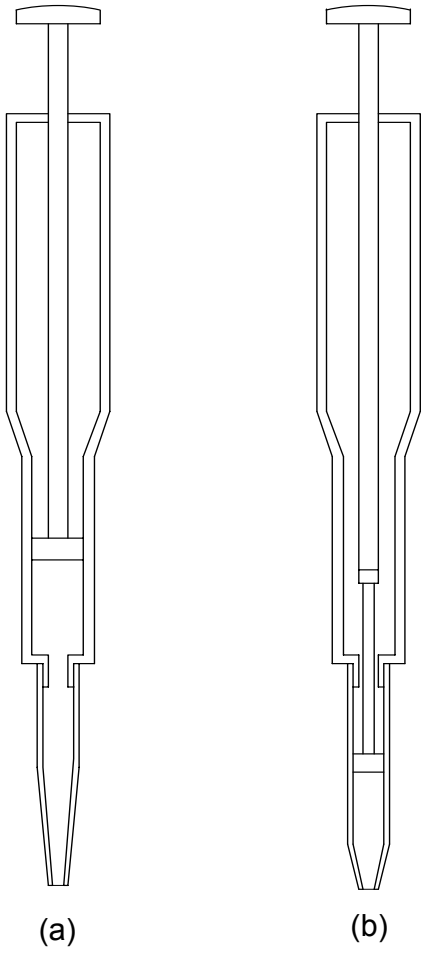

Figure 1. Schematic cross sections of (a) air displacement pipette and (b) positive displacement pipette

Both groups can be further sub-divided into 'type A' - air displacement pipettes (Figure 1a) which when filled have a pocket of air (termed the dead air volume) between the head of the piston and the liquid in the cylinder, and 'type D' - positive displacement (or direct displacement) pipettes (Figure 1b) in which the head of the piston is in direct contact with the liquid. Air displacement pipettes have the advantage that there is less risk of contamination when used repeatedly, but they are generally not as accurate as positive displacement pipettes, especially when small volumes of liquid are being dispensed, because of the compressibility of the dead air volume in the pipette.

Many air displacement pipettes incorporate two stop positions for the piston travel. This allows the dead air volume to 'blow out' any liquid remaining within the tip after the main body of liquid has been dispensed.

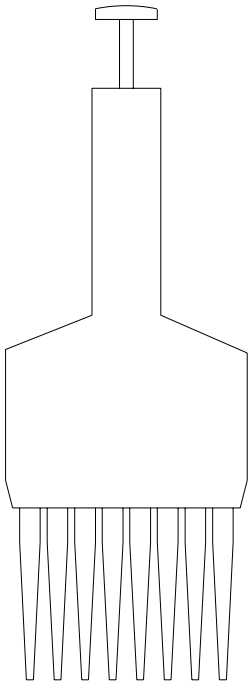

Figure 2. Multi piston pipette

Several pistons and cylinders can be combined into one unit to make a multi-channel pipette (Figure 2) capable of delivering equal volumes of liquid simultaneously through multiple tips. These are used extensively in biochemical and pathological laboratories to dispense accurate doses of a liquid into the wells of microtitre plates.

Figure 3. Examples of modern pipettes

An extensive range of commercially made pipettes is currently available (Figure 3). The design trend is to produce ergonomically designed pipettes which are easy to use and provide less strain on the operator with repetitive use. In addition to hand operated pipettes, many manufacturers produce semi-automatic electronically controlled pipettes which can reduce errors resulting from uneven aspiration and delivery. All are constructed so that, with normal usage, the warmth of the operator's hand is not transmitted sufficiently to cause a significant change to the temperature of the aspirated liquid.

3.2 Tips

All piston pipettes are fitted with some form of replaceable tip to minimise the risk of contamination. Although generic, independently made tips are available, it is good practice to use only tips recommended by the manufacturer of the pipette for the instrument in use, as these can be guaranteed to fit the pipette properly and have the correct internal volume. Tips fitted with filters are available which are valuable when radioactive or infectious materials are being used or where cross contamination from the air space cannot be tolerated.

Tips for air-displacement pipettes are designed to be a push fit onto the tip holder of the pipette. They are normally made of plastic and are intended for single use only. On no

account should any attempt be made to clean and re-use them. It is preferable for ease of use if they are obtained or placed in a tip rack box which will facilitate single handed manipulation of the pipette. This is especially important for multi-channel pipettes where all the tips should be the same height in order to draw up and dispense identical amounts of liquid.

Positive-displacement pipettes use a more complex tip consisting of an integral plunger and capillary. These are made from a variety of materials including plastic, metal and glass. They are frequently designed to be reusable after thorough cleaning and are often Teflon[1] coated to make the cleaning process easier by, for instance, using ultrasonic methods.

4 Good working practice

4.1 Laboratory

The laboratory should be designed and laid out to provide a comfortable working environment for the operator. If much repetitive work is to be undertaken, care should be taken by means of ergonomic planning and careful choice of suitable, easily manipulated pipettes to minimise the risk of Repetitive Strain Injury occurring.

To achieve optimum results, the laboratory should be draught free with the temperature maintained between 15 °C and 30 °C and stable to ± 0.5 °C. Relative humidity should be above 50%. The apparatus and samples should be allowed to stabilise in the laboratory before any work is carried out. Two hours is normally adequate for this.

4.2 Operation

Pipetting is a skilled operation which requires proper training and practice to achieve consistent and accurate results. Particular care should be taken to operate the piston of a non-automatic pipette in a smooth and regular manner. Drawing up the piston too quickly

[1] Teflon is a registered trademark of DuPont.

can result in the introduction of air bubbles resulting in an error in the amount of liquid dispensed.

A pipette is calibrated, and should be held in the vertical position when aspirating the liquid. Errors can occur if used at an angle, due to the different head of liquid. The depth to which the tip should be immersed within the liquid varies with the size, type and make of pipette. Any recommendations in the manufacturer's literature should be observed, but Table 1 is a general guide.

Pipette volume (µl)	**Immersion depth (mm)**
1 – 100	2 – 3
100 – 1000	2 – 4
1000 – 5000	2 – 5

Table 1. Guide to depth of tip immersion

To improve accuracy, air displacement pipettes are usually pre-wetted by filling them several times with the liquid being dispensed and expelling it to waste. This reduces the chance of air bubbles being aspirated, especially with viscous or hydrophobic liquids, and also allows the humidity of the dead air volume between the piston and liquid to stabilise – particularly significant when using liquids with a high vapour pressure. However, low volume air displacement pipettes of less than about 10 µl are usually designed to be used without pre-wetting. It is not normally necessary or desirable to pre-wet positive displacement pipettes.

After filling the pipette, any drops adhering to its tip should be carefully removed by touching it against the side of the vessel containing the liquid. If necessary, any surplus liquid still adhering to the outside of the tip can be carefully wiped off with a suitable material taking care to avoid contamination. Check that no further drops are forming at the orifice. This could be an indication of a poorly fitting tip or instability in the dead air volume, particularly in the case of a liquid with a high vapour pressure.

The procedure for dispensing a liquid is to touch the tip of the pipette against the wall of the receiving vessel just above the liquid surface at an approximate angle of 30° to 45° and draw it up for 8 mm – 10 mm after dispensing is complete. In all the procedures described above, it is important to consult the manufacturer's literature to check for any recommended deviation from the suggested practice.

5 Operational procedures

5.1 Conventional method

The usual method of use for a typical hand operated air displacement single channel pipette with two stop positions is illustrated below. The manufacturer's instructions should always be examined to see if any departure from the method shown is shown. Electronically controlled pipettes must always be operated in accordance with the manufacturer's recommendations.

a) Attach tip to tip holder taking care to avoid contamination. Hold pipette comfortably in one hand with thumb resting on plunger.

b) Press the plunger down smoothly to the first stop position. At this stage the pipette tip must not be immersed in the liquid.

c) Immerse the tip of the pipette in liquid to the correct depth (see 4.2 and Table 1). Keep the pipette in a vertical position and release the plunger in a smooth and uniform manner to aspirate the liquid.

d) Ensure that the plunger extends fully to its upper stop position and wait one or two seconds before withdrawing the tip of the pipette from the liquid.

e) Touch the pipette against the wall of receiving vessel at an angle of about 30° to 45°. Be careful to not immerse the tip in any liquid already in the receiving vessel.

f) Expel the contents of the pipette by pressing the plunger down steadily and evenly to first stop position while keeping the tip in contact with the wall of the vessel. Wipe the tip for 8 mm to 10 mm to remove any remaining liquid.

g) Push plunger down to second stop. This will force air through the tip to expel any remaining liquid.

h) Remove tip from the solution and release the plunger.

5.2 Reverse pipetting

A variation of the conventional method, termed reverse pipetting, is often useful when dealing with liquids that have a high vapour pressure or those which are very viscous.

i) Press the plunger down fully, past the first stop position to the second stop position. At this stage the pipette tip must not be immersed in the liquid.

j) Immerse the tip of the pipette in the liquid to the correct depth. Keep the pipette in a vertical position and release the plunger in a smooth and uniform manner to draw up the liquid.

k) Ensure that the plunger extends fully to its upper stop position. Check the tip to make sure no drops are forming at the orifice.

l) Touch tip against wall of receiving vessel and expel the contents of the pipette by pressing the plunger down steadily and evenly, but only to the first stop position.

m) Touch pipette against wall of delivery vessel at correct angle (see 4.2). Be careful to not immerse tip in any liquid already in receiving vessel.

n) Note that some liquid will be left in the tip after dispensing. This should be returned to the original vessel.

6 Maintenance and care of pipettes

6.1 Cleaning and decontamination

Solvents used for cleaning should be appropriate for removing the liquid the pipette has been used with. The manufacturer's recommendations should be sought and followed carefully as some solvents might have a deleterious effect with the materials from which the pipette is made. Special care should be taken with automatic pipettes to ensure that any cleaning fluid used does not come into contact with the mechanism.

Many pipettes are designed to be autoclavable although they may require partial dismantling first. The manufacturer's instructions should be observed regarding the suitability of sterilisation media and the maximum temperatures and pressures which are permissible.

If a pipette has been in contact with any substances regarded as hazardous to health as defined in the current COSHH or Ionising Radiations regulations, it is the responsibility of the user to ensure that it is thoroughly decontaminated before it leaves the laboratory. A certificate of decontamination should accompany the pipette giving its make, model and serial number, and listing any hazardous materials that have been used with the device and any agents used to clean it.

6.2 Inspection

Pipettes should be periodically inspected to see that they are functioning correctly, and whether they have sustained any detrimental wear or damage. The mechanism should be tested to check that it is working correctly and that the action of the piston is smooth and positive. The tip holder should be carefully examined for marks or distortion, as it is essential for the measurement accuracy of the pipette that the tip is a good fit on the tip holder so that no leakage occurs.

Further checks should be made with a tip fitted and the pipette filled with liquid. Any sign of leakage could indicate leaking seals, O-rings, or an ill fitting or inappropriate tip. However, especially with a high vapour pressure liquid, it could be the result of a small

amount of the liquid changing to a gaseous state and causing an increase of pressure in the dead air volume.

6.3 Repairs

After any repair, a compliance statement should be obtained from the manufacturer or repairer indicating that the pipette still conforms to the requirements of BS EN ISO 8655-2:2002. A list of any parts replaced should also be obtained. The pipette should then be calibrated, preferably by a UKAS accredited laboratory, who will issue a Certificate of Calibration showing traceability to national standards which details the measurement results under standard conditions.

7 Testing and calibration

Although the methods used overlap to a considerable extent, a distinction should be made between testing and calibration. Testing is a routine operation to ensure that the performance of the pipette remains within pre-established acceptable limits. Calibration is an operation to determine the actual volume delivered by a pipette together with an uncertainty associated with that volume. Testing is normally performed by the user; calibration can be performed by the user or by a calibration laboratory specialising in such work.

Variable volume pipettes are designed to deliver accurate and repeatable volumes over their adjustable range, and should only require periodic calibration. However, some fixed volume pipettes can be adjusted over a small range to compensate for minor deviations from their nominal volume which become apparent during calibration. Similarly, some pipettes are designed to be adjustable so that they can dispense (after calibration) the correct nominal volume of a liquid other than water. In both cases details of the adjustment made (and the calibration liquid used, if appropriate) must be clearly affixed to the pipette itself, recorded on any certificate of conformity and incorporated in the user's quality manual.

7.1 User tests

Every pipette in use should be tested on a regular basis to determine the accuracy and repeatability of the volume of liquid it delivers. An assessment should be made to determine the frequency of testing appropriate to the use the pipette receives and the accuracy required from it. This should be at least once a year but wherever possible every three to four months. The factors to be considered include the amount of use, number of users, type of liquid dispensed and any manufacturer's recommendations. Details of the test procedure to be followed are described below and more fully in BS EN ISO 8655-6:2002.

7.2 Method of testing

Gravimetric methods are normally used to test the accuracy of a pipette. Other test techniques are available (e.g. titrimetric and photometric methods) but these are outside the scope of this publication.

The basic principal of gravimetric testing is to weigh the amount of pure water delivered in a single operation of the pipette, and divide the mass obtained by the density of the water, thus giving its volume. In practice, a number of repeat measurements are made to which corrections must be applied to compensate for any variation from standard temperature and atmospheric conditions, and any significant evaporation of the water during the test period.

Variable volume pipettes should be tested at three or more points over their designated range; usually at the maximum (nominal) volume, at 50% of the maximum volume and at the lower limit of their range.

7.3 Equipment

The laboratory or area where the testing is performed should be draught free and with a relative humidity above 50%. The temperature should be stable to within ± 0.5 °C and maintained between 15 °C and 30 °C - preferably as close to 20 °C as is reasonably practical.

The following measuring equipment is required:

a) An accurate top pan or analytical balance with a range and resolution appropriate to the volume of the pipette under test.

b) A thermometer. An electronic type with a resolution of 0.1 °C or better and a range appropriate to any likely variation in liquid temperature is adequate. It is essential that the measuring probe is submerged in the liquid to at least the minimum depth recommended by its manufacturer.

c) A hygrometer with a standard uncertainty 10% or better.

d) A barometer with a standard uncertainty of 0.5 kPa or better.

e) A timing device. A good quality stopwatch or stopclock is suitable.

Best practice dictates that the equipment should be calibrated periodically by an accredited calibration laboratory[2]; this will also enable the best value and uncertainty to be assigned if the pipette is calibrated by the user.

The liquid used for testing is pure water conforming to grade 3 of ISO 3696, *Water for analytical laboratory use – Specification and test methods.* This can be bought in or prepared by the user using suitable deionising or distillation apparatus.

Other equipment required includes a reservoir vessel, typically a glass beaker, of adequate capacity to contain all the water likely to be needed for a complete test run, and a weighing vessel of a suitable size to sit comfortably on the balance pan. If a small volume pipette of 50 µl or below is being tested, a cover to the weighing vessel should be used to prevent excessive evaporation during a measurement run from affecting the result. Both vessels must be cleaned, rinsed thoroughly in deionised or distilled water and dried before

[2] The United Kingdom Accreditation Service (UKAS) is the only national accreditation body recognised by the UK government to assess, against internationally agreed standards, organisations that provide certification, testing, inspection and calibration services.

use. A proprietary cleaning agent such as Decon 90[3] used in accordance with the manufacturer's instructions is suitable for glassware.

Before testing, the pipette should be dismantled (as for cleaning) and reassembled in accordance with the manufacturer's or supplier's instructions.

7.4 Test procedure

Reference should be made to BS EN ISO 8655-6:2002, which describes in detail a gravimetric procedure for testing piston pipettes. An outline of the procedure is described below.

a) For most air displacement pipettes, a new tip should be fitted and water from the reservoir vessel aspirated and expelled to waste five times. This allows the humidity in the dead air volume to stabilise. For some low volume pipettes and direct displacement pipettes, this pre-wetting operation is omitted unless specified in the manufacturer's or supplier's instructions.

b) Add water to the weighing vessel to a depth of at least 3 mm. Immediately before starting a test run record the temperature of the water, and the ambient air pressure and relative humidity. Replace the lid (if used) on the weighing vessel. Record the balance reading or null the balance.

c) Fill the pipette with water from the reservoir vessel and dispense it into the weighing vessel. The water should be aspirated and dispensed in the same way as conventionally used for routine work. Use the 'blow out' feature (see 3.1) if fitted to expel any remaining liquid. Record the new balance reading. The time taken for a complete cycle should be kept to a minimum consistent with the smooth and careful transfer of the water – preferably less than 60 seconds.

d) Repeat the operations described in the last paragraph a further nine times. At the end of each cycle record the balance reading.

[3] Decon is the registered trade mark of Decon Laboratories Ltd.

e) At the conclusion of the tenth cycle, record the temperature of the liquid in the weighing vessel and the total elapsed time.

f) If evaporation is likely to be significant, e.g. when testing small volume pipettes, the balance reading should be recorded once again after leaving the weighing vessel on the balance pan for a further period equal to the elapsed time of the test run. If a cover has been used on the weighing vessel this should be left in position. Dividing the mass of water lost in this period by 10 will give a working approximation of the average mass loss per cycle.

7.5 Calculations

a) Calculate the mass of water delivered for each cycle by subtracting the balance reading recorded at the end of the previous cycle from the reading recorded at the end of the cycle. If appropriate, add the mass of water lost by evaporation as determined in the previous paragraph.

b) To convert each mass value to a volume at 20 °C (the standard reference temperature for the calibration of pipettes) it must be divided by the density of water corrected to 20 °C. A correction for air buoyancy, which varies with the air density, must also be applied.

   Both these factors are taken into account by multiplying the each mass value by a correction factor *Z*. A table specifying the value of *Z* for a temperatures range of 15 °C and 30 °C and air pressure range of 80 kPa to 105 kPa is given as Annex A in BS EN ISO 8655-6:2002. The correction factor is also given as an equation in ISO/TR 20461:2000.

c) Add together each of the 10 volumes calculated in the previous paragraph and divide by 10 to give the mean volume at 20 °C. This can be expressed in microlitres or millilitres.

8 Uncertainties

8.1 Introduction

The following is a guide to the main components which will require consideration when compiling an uncertainty budget for the test or calibration of a pipette. Some contributions will vary with each calibration and can only be derived from the user's or calibrator's own test data.

More detailed information on Internationally agreed methods for the evaluation of uncertainties can be found in the *Guide to the Expression of Uncertainty in Measurement* [1], while the procedures required for UKAS accreditation are described in *The Expression of Uncertainty and Confidence in Measurement for Calibrations* [2]. Both publications are consistent in their approach to the calculation of uncertainties.

Uncertainty evaluations are conventionally divided into two classes. *Type A* evaluations are of uncertainties calculated by statistical methods (typically based on the standard deviation of a set of measurements) and are usually, but not invariably, due to random variations in a measured quantity. *Type B* uncertainties are mainly systematic errors derived from all other sources, for example calibration certificates of thermometers, barometers etc., published information such as the uncertainty on the density of water at a specific temperature, and environmental instability.

8.2 Uncertainty contribution of the pipette

The micropipette can be considered as a delivery measure which dispenses a quantity of distilled water at each operation. It may be a fixed volume or a variable volume type. The range of volumes considered here is from 20 µl to 1 ml. The calibration process involves weighing the discharged water and computing its volume from a knowledge of its mass and density. The range of weight of each discharge corresponding to the volume range above is approximately 20 mg to 1 g.

The volume dispensed from the pipette each time it is operated will vary because the instrument will not be perfectly repeatable, i.e. there is a *random error* of the volume

discharged. A plot of a number of operations against the volume dispensed each time will show a spread of volumes forming a statistical *probability distribution*. In this case the probability distribution of the volumes discharged is a *normal distribution* (sometimes called a *Gaussian distribution*) forming a bell shaped curve (Figure 4), which can be characterised by a mean value ($\overline{V}$), and a parameter associated with the spread of the volumes - usually quantified by the *experimental standard deviation,* or *random error of measurement* (s_r).

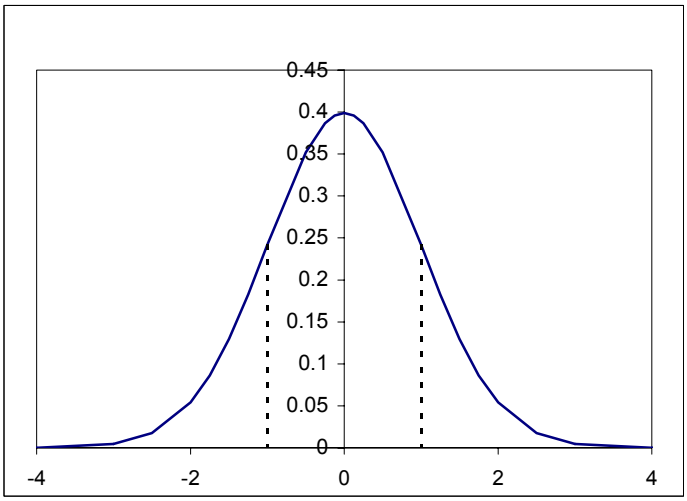

Figure 4. Normally distributed data with a mean value of zero and a standard deviation of ±1.

The standard deviation encompasses approximately 68% of the measurement values (the area under the curve between the dotted lines in Figure 4). It can be calculated using the formula below, although in practice a scientific calculator or spreadsheet is usually used:

$$s_r = \sqrt{\frac{\sum_{i=1}^{n}(V_i - \overline{V})^2}{(n-1)}}$$

This can also be expressed as a percentage termed the *coefficient of variation* (*CV*)

i.e. $$CV = 100\frac{S_r}{V}$$

The conventional term used in pipetting (BS EN ISO 8655-6:2002) for the difference between the experimentally determined mean volume of samples dispenses and the

nominal volume (V_s) is termed the *systematic error of measurement* and is assigned the symbol e_s.

i.e. $$e_s = \overline{V} - V_s$$

or expressed as a percentage of the nominal volume

$$e_s = \frac{100(\overline{V} - V_s)}{V_s}$$

Thus two fundamental quantities are established from the calibration of a pipette:

- a mean value for the volume of liquid it dispenses, exhibiting a systematic error or offset from the manufacturer's quoted nominal volume
- a random uncertainty associated with the spread of the volumes it dispenses, relative to the mean volume, when used repeatedly.

8.3 Other uncertainty contributions

A number of other components (Table 2) each having its associated uncertainty have to be taken into account when considering the various factors that make up the uncertainty budget for a pipette calibration. Other probability distributions will be encountered, the two most common being *rectangular distribution* (sometimes called *uniform distribution*) and *triangular distribution*.

A rectangular distribution (Figure 5) represents a group of measurements in which the values are assumed to lie randomly between two limits without ever falling outside them, as for example, the temperature of the water.

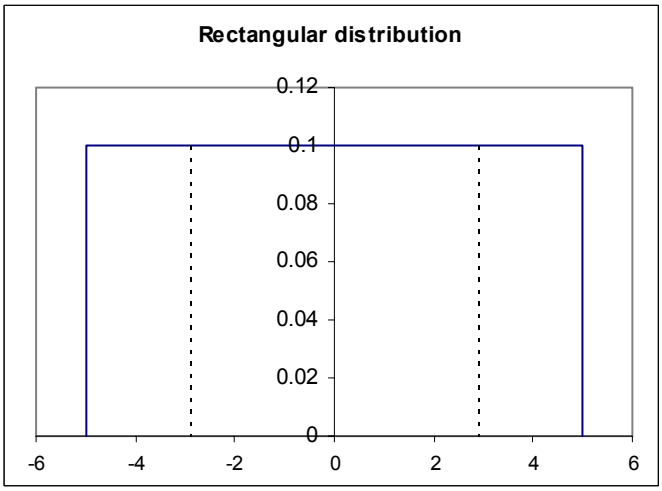

Figure 5. Rectangular distribution with a mean value of zero and limits of ±5. One standard deviation ±2.89.

A triangular distribution (Figure 6) is the result of adding two rectangular distributions. An example is the uncertainty due to rounding errors.

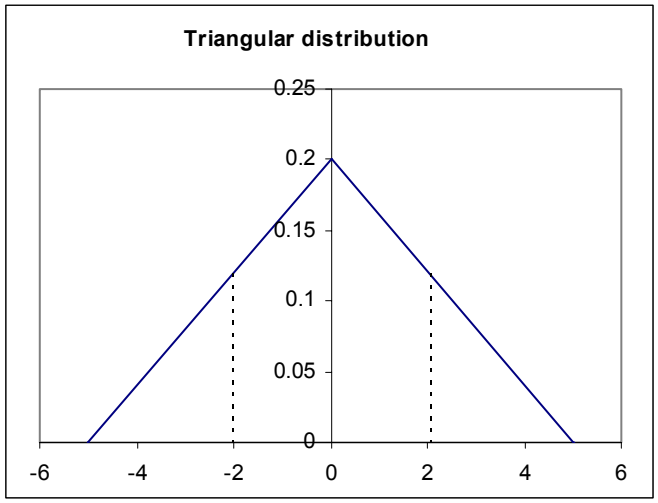

Figure 6. Triangular distribution, with a mean value of zero and limits ±5. One standard deviation is ±2.04.

8.4 Input quantities

8.4.1 Confidence level

All the *input quantities* (i.e. the values of the uncertainty components) must be reduced to the same *confidence level* in order to eventually sum all the individual *ouput quantities*.

The confidence level, as its name implies, is a statement of the statistical likelihood (in percentage terms) that a determined value lies within specified limits. For instance, a calibration certificate might certify that a pipette delivers 100.65 µl ± 0.05 µl with a confidence level of approximately 95%. This means the probability is that 19 times out of 20 it will deliver somewhere between 100.60 µl and 100.70 µl of liquid.

8.4.2 Divisor

A measurement uncertainty expressed in terms of a standard deviation (as shown in Figure 4) is termed the *standard uncertainty*. For a normal distribution, one standard deviation is equivalent to a confidence level of approximately 68 %. The standard uncertainty can be multiplied by a coverage factor (*k*) to give an *expanded uncertainty*. Twice the standard deviation (expressed as *k*=2) of a normal distribution gives a confidence level of approximately 95%.

To convert input uncertainty values derived from other probability distributions to a standard uncertainty, a *divisor* is applied as shown in Table X.

Distribution	Divisor
Normal	1 or 2
Rectangular	√3
Triangular	√6

Figure 7. Divisors for probability distributions

A normal distribution has a divisor of either 1 or 2 depending on the level of confidence of the value quoted. For example, a certificate of calibration might give the uncertainty as ‘k = 1’ (~68% confidence level) or ‘k = 2’ (~95% confidence level) in which case the divisor is the ‘k’ number. Other values are sometimes used, for example k = 3 (~99.7% confidence level).

8.5 Output quantities

8.5.1 Sensitivity coefficient

To be able to combine the all the uncertainty components together they must be in the same units – in the case of pipettes this is usually microlitres (µl). The sensitivity coefficient (c_i) is a multiplication factor which converts the uncertainty in the value of an input quantity (such as mass or temperature) to a corresponding uncertainty in the output quantity (volume), taking into account any change in the units of measurement (e.g. mg to µl).

i.e. u_i (source unit) x $c_i = u_i$ (µl)

8.5.2 Degrees of freedom

The number of values in the final calculation of a statistic that are free to vary are termed *degrees of freedom.*

For Type A components, if (v_i) are the degrees of freedom for individual uncertainty contributions, and (n) is the number of measurements used to evaluate the contribution, then

$$v_i = n-1$$

For Type B components, (v_i) is usually taken to be infinite.

8.5.3 Combined uncertainty

After the uncertainties for each individual contribution have been evaluated as standard uncertainties expressed in the same unit (e.g. µl), an overall or *combined standard uncertainty* for the mean volume of the pipette as determined by the calibration must be calculated. This is done by adding all the standard uncertainties in quadrature, i.e by squaring each one, adding them together and finding the square root of the sum.

8.6 Certification

Users should establish individual process specifications for the use of each pipette and define acceptance criteria.

A Certificate of Conformity should be issued for each pipette tested that conforms to the defined acceptance criteria.

A Calibration Certificate or Report should be issued for each pipette calibrated by the user that conforms to the defined acceptance criteria. This should report the values and associated uncertainties determined from the calibration. The coverage factor (k) and/or the confidence level of the reported uncertainty must be clearly indicated on the certificate.

It should also be stressed that in normal usage, additional uncertainties will be introduced, particularly if the liquid being pipetted is not the same as that used when the pipette was calibrated.

8.7 Detailed considerations

There is a complication in the assessment of the uncertainties associated with the use of pipettes in that there are two clear sources of type A component; those associated with the repeatability of the balance and with the repeatability of the pipette. Since the pipette output can only be seen experimentally by way of the balance readings both components contribute to the overall uncertainty. However, because a balance with a resolution appropriate to the volume of the pipette being calibrated should be used (see BS EN ISO 8655-6:2002), the uncertainty contribution due to the balance will be small relative to that of the pipette. Although it would be possible to separate the components by deconvolution to determine a figure for the pipette alone, this is unnecessary as s_r is defined in terms of the weighing results and is therefore a figure for the system rather than the pipette.

8.7.1 Inaccuracy

The broad contributors to the uncertainty of the calibration will be the device itself, the liquid used, the balance, the process or procedure and the environment. Each of these can be subdivided into the components listed in Table 2.

Broad contributory area	Detailed component
Pipette	Temperature of the device Thermal expansion coefficient of the device
Reference liquid (pure water)	Density – from published formulae or tables
Balance	Repeatability Resolution Linearity Calibration (or standard weight)
Process and procedure	Operator contributions
Environment	Evaporation Air buoyancy

Table 2. Uncertainty contributions

The only Type A components contributing to a pipette calibration are the uncertainty of the volumes delivered and the uncertainty of the weighings. Their combined uncertainty will be representative of the system rather than the pipette but will be taken as reflecting the pipette alone. This will yield a slightly worse uncertainty than a more rigorous analysis would produce. Thus the Type A contributions will be taken as the (n-1) weighted standard deviation of the calculated discharged volumes, divided by the square root of n and assigned (n-1) degrees of freedom.

8.7.2 Imprecision

The imprecision is defined in terms of an expression used to compute it from experimental results and the uncertainty may be made arbitrarily small by including sufficient decimal places in the calculation.

9 Sample uncertainty budget for a micropipette

Details to be considered when compiling of a typical uncertainty budget for a 50 µl pipette are described in the example below.

9.1 Units

The units used in the computation of the uncertainties for the micropipette will be nanolitres (nl). The sensitivity to the output to any mass related parameter will be taken as:

1 ml per gram	1µl per mg	1 nl per µg

Table 3

9.2 Contributions and sensitivity coefficients

9.2.1 Temperature of the device

The temperature of the pipette is assumed to be known to within ±1 °C and assigned a rectangular distribution

9.2.2 Expansion coefficient of device

The pipette is made of a plastic material with a nominal linear coefficient of thermal expansion of $\alpha = 10 \times 10^{-5}$ °C^{-1}. This equates to a cubic coefficient of thermal expansion[4] of:

$$\gamma = 3 \times 10^{-4} \text{ °C}^{-1}$$

The true volume is assumed to assumed to be known to ±10 % of the nominal volume. This component is assigned a rectangular distribution.

[4] The coefficient of cubic expansion is calculated by multiplying the coefficient of linear expansion by x3.

9.2.3 Repeatability of the pipette

The repeatability of the best fixed volume pipette available to the user is used to determine the best measurement capability (BMC). For all practical work the repeatability determined on the day is used to establish the uncertainties. As described in (8.7), the effects of the repeatability of the balance are included within this figure.

9.2.4 Water

The water density is calculated from Kell's equation [3] truncated to include terms only up to the square of the temperature[5]. The equation used is that applicable to air-free distilled water with the temperature corrected in accordance with the ITS-90[6]. The critical parameter is the temperature of the water. Other minor contributions include the difference between the composition of the water on which Kell's work was based and that used for this calibration, and the effect of truncating the formula, but these are taken to be negligible.

Because the thermal expansion of water is non-linear, assigning a volumetric thermal expansion coefficient is not strictly appropriate. However, for uncertainty estimation an expansivity (calculated at 20 °C) of 210 ppm $°C^{-1}$ is used:

$$0.21 \text{ nl } \mu l^{-1} \text{ } °C^{-1}$$

The estimated uncertainty in the water temperature is taken as ± 0.3 °C.

9.2.5 Evaporation

The design of the weighing cell is such that the discharged water is under a near saturated atmosphere yielding negligible evaporation over the measurement period.

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[5] Several papers have been published on the absolute density of water since Kell's work in 1975, the most recent being by Tanaka *et al* in 2001 [4]. Although any differences from Kell's values are insignificant in the context of the method of pipette calibration described in this guide, it is good practice to work to the latest published information.

[6] International Temperature Scale of 1990

9.2.6 Repeatability of the balance

See 9.2.3 above.

9.2.7 Resolution of the balance

The balance resolution is 10μg. Since the weighing result is calculated from a difference of readings from a digital device, a triangular distribution has been assigned to this element.

9.2.8 Linearity of the balance

The effects of departures from linearity will increase the observed imprecision and produce systematic effects on the measured inaccuracy. The latter will be dependent upon the weighing range used for the measurements in question. However, since the mass increment for each operation of the pipette is small in relation to the capacity of the balance, any uncertainty contribution due to non-linearity is usually negligible.

9.2.9 Calibration of the balance

Taken to be the limit of error on the weight used to span the machine assigned pro rata for the range of weighing in question. For the same reasons described in the previous paragraph, this contribution is normally negligible.

9.2.10 Operator effects

For micropipetting, the technique and level of skill associated with the operator can form a significant component of the uncertainty budget. With a thorough understanding of the process, good training and practical experience these operator effects can be reduced to a consistent minimum. To establish a practical basis for the uncertainty component, extensive repeat testing was carried out by the operators involved. The results did not indicate any clear bias between operators, gave no clear indication of volume dependence or displayed any particular characteristic distribution over the range of pipettes used. The spread of inter-operator differences was in the range ±150 nl; therefore a rectangular distribution was used with a semi-range of 150 nl in all cases.

9.2.11 Atmospheric buoyancy

Because the water delivered to the receiving vessel is weighed in air, but the density of water is defined in terms of mass per unit volume, a correction for the effect of air

buoyancy is used. This correction is based on a calculated air density (using the measured ambient values of barometric pressure and air temperature) and an assumed density for the material being weighed of 8 000 kgm^{-3}. The magnitude of the correction amounts to about 0.1 %, thus for larger pipettes the correction is of the same order as the total uncertainty. The uncertainty on the correction is small enough to be considered as an insignificant contribution to the uncertainty budget.

Table 4. Example of an uncertainty budget

Contribution				Calculation				
Parameter		Probability distribution	Value	Divisor	uncertainty (source unit)	Sensitivity coefficient	Standard uncertainty (nl)	Degrees of freedom
Device	Temperature	Rectangular	1 °C	√3	0.5774 °C	300	173.22	∞
	Expansion coefficient	Rectangular	0.0003 $°C^{-1}$	√3	$1.732x10^{-4}$ $°C^{-1}$	2×10^{6}	346.40	∞
	Repeatability	Normal	0.12 g	1	0.12	316.228	37.95	9
Water	Temperature	Rectangular	0.3 °C	√3	0.1733 °C	210	36.37	∞
	Density calculation	Rectangular	Insignificant	√3	0	1	0.00	∞
Balance	Repeatability	Normal	0.030 mg	1	0.030 mg	1	0.03	∞
	Resolution	Triangular	0.010 mg	√6	0.0041 mg	1	0.00	∞
	Calibration	Rectangular	0.025 mg	√3	0.0145 mg	1	0.01	∞
	Linearity	Rectangular	0.030 mg	√3	0.0174 mg	1	0.02	∞
Operator	Repeatability	Rectangular	150 nl	√3	86.6026 nl	1	86.60	∞
Weighing vessel	Evaporation	Rectangular	Insignificant	√3	0	1	0.00	∞
Air	Density	Rectangular	Insignificant	√3	0	1	0.00	∞

10 Appendix A: Measurement tests

This Appendix summarises a series of tests undertaken using both hand operated and electronic piston type volume pipettes. The results of the measurements are all based upon a series of ten consecutive volumes of water dispensed in accordance with the general principles contained in British Standard Specification BS EN ISO 8655.

Measurements were made to ascertain:

- The repeatability and accuracy of the pipettes when used by:
  a) trained calibration personnel and
  b) personnel who were not specifically trained to use a pipette but who followed the manufacturer's instructions
- The effect of temperature
- The effects of using different liquids
- Variations in the measured dispensed volumes

It should be noted that the results summarised here are not based on a comprehensive study but reflect a limited number of tests carried out during a limited time.

10.1 Measurement data

10.1.1 Using different personnel

For these tests, measurements were made by a number of trained calibration personnel who followed the same documented calibration procedure, together with other staff who were not specifically trained to undertake the test but who followed the manufacturer's instructions supplied with the pipette (Table A1).

A controlled series of measurements were made using a 100 µl fixed volume pipette. The mean values and standard deviations obtained are summarised in Table A1.

	Mean Value (µl)	**Standard Deviation (µl)**
Calibration Operator A	99.74	0.03
Calibration Operator B	99.73	0.05
Calibration Operator C	99.74	0.04
Untrained Operator 1	99.81	0.75
Untrained Operator 2	99.52	1.39

Table A1.

Conclusion: The results from the untrained operators had far larger standard deviations. The results for Operator 2 were affected by poor control of the 'last drops'.

Measurements were made on a variable volume pipette in which a rotary indicator with a fiducial scale was used to set the volume dispensed. The scale divisions were at 0.2 µl intervals. The nominal volume could be set by approaching the required value from both above and below the scale mark.

The mean dispensed volume realised by the calibration operators was 100.26 µl with a standard deviation of 0.21 µl. For untrained operators the mean volume was 100.57 µl with a standard deviation of 1.91 µl. In all cases the nominal value was set from both above and below the scale mark. The test was repeated by the untrained operators, but with the scale mark set from the same direction each time. Although the mean volume was similar, the standard deviation was reduced to 1.31 µl.

Conclusion: A systematic approach to setting the nominal value improves repeatability.

Measurements were made using a variable volume pipette fitted with a fixed stop digital indicator. Results demonstrated similar mean volumes for both trained and untrained operators, with standard deviation for trained operators being 0.17 µl compared to 0.96 µl for untrained staff. Similar test were carried out on pipettes of different volumes and by alternative manufacturers.

Conclusion: No significant differences were found for pipettes of different designs, or produced by different manufacturers. However, standard deviation attained by trained operators was considerable better than that of untrained staff.

10.1.2 Effects of temperature

Measurements were made at different temperatures in the range 15 ºC to 27 ºC by trained staff. Within the calibration uncertainties, no significant temperature effects were observed.

The effect on the pipette of warmth from the operator's hand was measured. A series of measurements from immediate handling showed a change of up to 3 % in the measured volume during the first five to seven measurements (depending on model). After an initial period of five to seven measurements the effects of the local heating from an operator's hand was no longer noticeable.

Measurements were also made with an operator wearing a protective glove such as used in a testing laboratory. This reduced this instability to around three initial measurements.

Conclusion: Warmth from an operator's hand can have a significant effect when a pipette is initially operated[7]. This should be taken into account when planning a calibration procedure.

10.1.3 Effect of using different liquids

Measurements were made using the same pipette (rinsed between fluids in distilled water) for dispensing different liquids within a density range of approximately 700 kgm^{-3} to 1 700 kgm^{-3} (Table A2). The liquids chosen were non-aggressive and known to make accurate and reliable density standards.

[7] BS EN ISO 8655-2:2002 states that for pipettes complying with their specification, the effect of hand warmth may be ignored.

Measurement results for the same liquid varied with pipette used, but in general there was a correlation between the pipettes from the same manufacturer. For these tests, the density of distilled water was taken to be 1 000 km m^{-3}, and the error in the volume dispensed was assumed to be zero.

	Approximate density of liquid			
	700 kgm^{-3}	**1 000 kgm^{-3}**	**1 300 kgm^{-3}**	**1 700 kgm^{-3}**
	Value Error (%)			
Manufacturer A1	-0.9	0.0	+1.3	+1.6
Manufacturer A2	-1.0	0.0	+1.5	+1.8
Manufacturer A3	-0.9	0.0	+1.2	+1.7
Manufacturer B1	-1.3	0.0	+4.6	+9.7
Manufacturer B2	-1.5	0.0	+5.2	+9.1
Manufacturer B3	-1.4	0.0	+5.1	+11.3
Manufacturer C1	+2.4	0.0	+9.4	+23.1
Manufacturer C2	+2.7	0.0	+10.1	+24.7
Manufacturer C3	+2.1	0.0	+8.7	+25.1

Table A2

Conclusion: Significant measurement errors can occur when liquids other than water are measured. These errors are manufacturer dependent and not necessarily linear over the density range.

10.1.4 Summary

Fixed volume pipettes performed better than variable volume pipettes, whether used by trained or untrained operators. The use of an established method which provides advice about the ‘last drops’ gives improved repeatability.

Measurements using variable volume pipettes depend on the repeatability of setting the nominal volume to be dispensed. Fixed step digital indicators are better than variable scale pipettes, although the effects of setting hysteresis must be considered.

Some pipettes gave much larger standard deviations than others. In some cases this was due to poor ergonomic design as the pipette was found to be too uncomfortable to hold for long periods (i.e. 10 determinations).

Pipettes from all manufacturers were found to be generally reliable when new. After three months occasional use, however, pipettes from two manufacturers showed poor repeatability and a noticeable change in the mean value. In general, these pipettes tended to show a worse repeatability due to wear.

11 References

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[4] TANAKA, M., GIRARD, G., DAVIS, R., PEUTO, A. and BIGNELL, N. Recommended table for the density of water between 0 °C and 40 °C based on recent experimental reports. *Metrologia*, 2001, **38**, 301-309.