PIPETTING PERFORMANCES BY MEANS OF THE ANDREW ROBOT

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CONTENTS

1. ABSTRACT

Andrew, from Andrew Alliance, is a novel pipetting robot since it uses conventional single channel pipettes designed for manual operations. We describe an exhaustive study of pipetting performances in a conventional laboratory environment by relying on the unique reproducibility of the Andrew system. We perform photometric and gravimetric measurements in the same conditions of real experiments, and compare the performances according to the guidelines and concepts described in the ISO-8655 international standard. The sources of systematic and statistical errors are analyzed and measured, guidelines and suggestions are reported.

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2. INTRODUCTION

Liquid handling is a very recurring, basic laboratory procedure performed across many industries: biological research, diagnostics, drug manufacturing, food quality control, and environmental testing being the more important. Often, performances of accurate and reproducible liquid handling are mission critical, and imply tedious manipulations; recurrent operator training; cross-verification and quality assurance by data duplication, blind controls, operator supervision; monitoring and regulation of environmental parameters; last but not least, periodic verification of the tools involved. Liquid handling is performed both manually and by means of automated solutions called liquid handling workstations (1): when it is performed manually, it is achieved by means of tools called pipettes (or micropipettes) capable of liquid volume definition for quantitative aspiration and dispensing. Here, we focus on a unique solution, the Andrew robot, consisting of an automated liquid handler using manual pipettes (2). There are two principal ways to assess volumetric performances for liquid transfers: by means of gravimetric methods (e.g. scales weighting the liquid samples) and by means of photometry (e.g. exploiting suitable light detectors quantitatively measuring the amount of dyes or light emitters transported in a pipetting operation). In this document we exploit both techniques, with the purpose of showing that specific experimental configurations are sensitive to different aspects that could be relevant in assessing the performances of daily operations. Since Andrew is intrinsically reproducible, we have the unique opportunity to simulate typical errors and create "what-if" scenarios that highlight the risks of incorrect pipette manipulations occurring daily in all laboratories. This analysis is unprecedented, since Andrew is the first available solution that allows a laboratory to validate pipetting performances in a fully unattended and objective manner.

General methods and guidelines describing procedures for evaluation of pipetting performances are documented in the International Standard norm ISO-8655, covering multiple aspects in relation to different experimental configurations and procedures (3). However, these methods are not designed to evaluate the performances of pipettes in the actual operational environment, when used by actual operators with real samples: the specific methods are designed to assess the theoretical performances of the pipetting tools in ideal conditions (controlled temperature, humidity and pressure; specific operator knowledge; precise procedures and defined liquids). The ISO norms are ultimately designed to allow various independent accreditation services (UKAS in the UK, COFRAC in France or A2LA in the USA as examples) to certify the performance of a pipette according to an internationally recognized directive, in some cases to comply with a mandatory legal requirement. Additionally, pipettes needs to be verified regularly since their performances can change over time. The frequency depends on the "Mean Time Between Failures" (MTBF) induced by use or other procedures like sterilization or maintenance: as a reference, the American Standard Test Method norms ASTM E1154-89 recommends that pipettes receive a comprehensive evaluation at least on a quarterly basis (4). However, it is logical that even frequent verifications do not guarantee any user or laboratory that the process performed at a given day is going to be precise or reproducible: many more parameters dominate the pipette performances in real life, including environmental factors like temperature, human-related aspects the operator posture or fatigue, or simply the liquids involved. So, liquid handling performances ultimately remain responsibility of the laboratory, implying a continuous verification and monitoring of the tools and methods involved. We are convinced that it is desirable for those organization to access experimental methods that can validate pipette performances in the same conditions pipettes are used daily, with a limited investment of time and costs. These methods could allow verification the performances of pipettes with a short turn-around time and precisely when it is needed, allowing to decide if a pipette can be used or should be replaced by another one, or undergo maintenance by the manufacturer or a service laboratory.

The ISO-8655 norms clearly indicate reference performances for pipettes – independently from the manufacturer or field of application – across a large range of liquid volumes. The upper limits indicated by the ISO-8655 norms are reported below, separately for systematic and random errors, and in terms of absolute or relative errors:

Nominal volume	Maximum permissible systematic error		Maximum permissible random error	
[µl]	[%]	[µl]	[%]	[µl]
1	± 5	± 0.05	± 5	± 0.05
2	± 4	± 0.08	± 2	± 0.04
5	± 2,5	± 0.125	± 1.5	± 0.075
10	± 1.2	± 0.12	± 0.8	± 0.08
20	± 1	± 0.2	± 0.5	± 0.1
50	± 1	± 0.5	± 0.4	± 0.2
100	± 0.8	± 0.8	± 0.3	± 0.3
200	± 0.8	± 1.6	± 0.3	± 0.6
500	± 0.8	± 4.0	± 0.3	± 1.5
1000	± 0.8	± 8.0	± 0.3	± 3.0
2000	± 0.8	± 16.0	± 0.3	± 6.0
5000	± 0.8	± 40.0	± 0.3	± 15.0
10000	± 0.6	± 60.0	± 0.3	± 30.0

TABLE 1: MINIMAL PIPETTING PERFORMANCES AS INDICATED BY THE NORM ISO-8655-2 (2002)

The norms additionally allow unambiguous evaluation, for a given pipette type, of the target performances to be achieved as a function of the maximum dispensing volume. In the remaining part of the present document, we describe a general solution to exploit the exceptional accuracy and precision of pipettes by means of a robot called Andrew. Useless to say, Andrew allows validating the pipette performances by using procedures that are exactly the same procedures performed during the daily work of the robot, so providing an exact knowledge of the expected performances in the everyday operations. With Andrew, we compare specific experimental data obtained by gravimetric and photometric procedures, allowing to understand advantages and disadvantages for the two methods.

2.1. GRAVIMETRIC METHOD: A STANDARD FROM ANCIENT TIMES

Weighting method for validating quantities has a tradition without rivals: balances employing reference weights have been in use since at least 1878 B.C. (5).

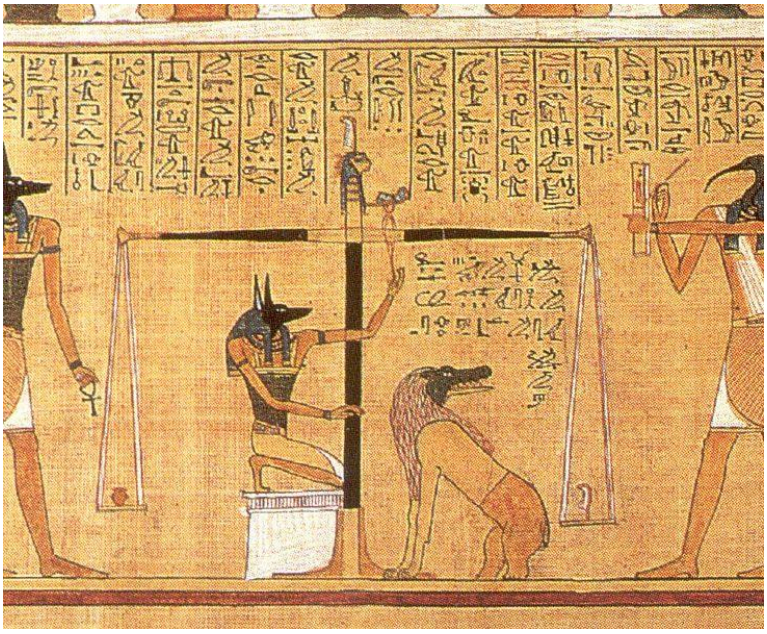

FIGURE 1: BALANCE SCALE FROM THE EGYPTIAN BOOK OF THE DEAD

Weighting a sample of liquid that has been aspirated and transferred from a source to a weighting destination is theoretically a very straightforward, direct way to assess the volume of the liquid, through the knowledge of its weight at a given temperature. A number of details and guidelines are described thoroughly, for example, by the "Measurement Good Practice Guide No. 69, July 2004" from the National Physics Laboratory (6).

In particular, the previously mentioned document reviews also in details the sources of uncertainties and the estimation of systematic errors. Especially at small weights, the experimental procedure can get very complex due to the continuous evaporation of the liquid and because of the special requirement of

high precision balances sensitive to micrograms (called microbalances). It is easy to highlight the principal value of the gravimetric method: laboratory scales are one of the few available instruments with an intrinsic and standardized absolute calibration, and therefore any indirect measurement based on weight is also a calibrated result. However, it is also easy to highlight the challenges: these measurements have to be performed by expensive balances in very precise environmental conditions (controlled temperature within 0.5°C, special heavy marble tables to be insensitive to vibrations, humidity above 50%, no air drafts) and with an accurate and reproducible procedure that's extremely time consuming and difficult to automate. For this reason, the ISO-8655-6 calibration is typically performed in dedicated external laboratories designed and organized for the purpose, implementing the complex procedure to the advantage of third party customers with a turnaround time as short as feasible by an external service.

2.2. PHOTOMETRIC METHOD: THE PRINCIPLE

The principle of a photometric evaluation of a volume relies mainly on the absorbance of a sample *with known absorbance* in a known optical configuration and at a known wavelength. The Lambert-Beer law determines the light Transmission *T* for a beam of light traversing a liquid, and it can be expressed as:

$$T = \frac{I}{I_0} = 10^{-\varepsilon l c}$$

Where ε is the extinction coefficient of the absorbing sample, l is the length of the light beam across the liquid (path length), and *c* is the concentration of the sample inside the liquid (typically, a dilution buffer). Absorbance is consequently defined as

$$A = \varepsilon l c$$

And A is typically the variable that's reported by conventional laboratory absorbance photometers.

Many different experimental conditions can be envisaged to exploit the Lambert-beer law principle and correlate an absorbance measurement with an unknown sample volume. The most obvious geometry consists of a cuvette where the light beam transverses a *constant* path length, independent from the volume of the liquid. In this configuration, mixing two liquid volumes with different absorption properties induces a change of concentration, detectable by the photometer through the measurement of the light transmission. For example, the norm ISO-8655-7 suggests different experimental conditions to achieve a volume measurement, by diluting an existing dye with a transparent buffer or, vice-versa, by adding a dye to a transparent liquid (7).

In this context, we would like to highlight the form of the lambert beer law in relation with the transmission of a light-absorbing sample of volume V and with an initial concentration c when introduced into a container of **constant cross-section S**, initially containing a Volume V_{Buf} of non-absorbing buffer. If the light beam travels in the direction orthogonal to the container cross-section, the addition of sample modifies simultaneously the concentration and the path length of the light beam. So the absorption becomes

$$A = \varepsilon \frac{V+V_{Buf}}{S} \times \frac{c \times V}{V+V_{Buf}} = \frac{\varepsilon c}{S} \times V$$

It can be noted that the final absorbance is proportional to the sample Volume V, being ε, S and the initial concentration c constant parameters. We can implement this configuration into a clear-bottom microplate well that's measured by means of a vertical light beam, provided that the well has a constant cross-section (typically, with a circular or rectangular shape). In this geometry, the light beam absorbance measurement is directly proportional to the volume of the dye loaded with a pipette onto the same well. Also, it should be note that the factor $c \times V = Q$ corresponds to the amount of dye molecules that have been loaded into the well: in other words, if we assume that the dye molecules don't evaporate (an hypothesis justified in our experimental

conditions by the large molecular weight of the dye, Ponceau S, corresponding to $MW = 760.57$) we expect that, in the hypothesis that neither precipitation or staining would occur, the absorbance of the sample will be insensitive to the water evaporation over time. These hypotheses have been experimentally verified and documented in the next sections.

It is easy to highlight advantages and disadvantages of this specific experimental configuration of an absorbance procedure with respect to a gravimetric procedure. Primarily, minute and variable volume samples can be loaded into a buffer-containing array of wells, with reduced sensitivity to sample evaporation. This subsequently allows the possibility of a readout of all wells in parallel, something impossible by a gravimetric measurement, with a significant gain of time.

3. MATERIALS AND METHODS

We have designed and optimized a procedure for assessing the performances of pipettes in a normal laboratory environment, with the practical objective of a reproducible process without subjective interpretations. Differently from many present calibration methods that require highly skilled operators, we want to define a procedure that can be executed by anybody in the lab, automatically, and without narrow control of environmental conditions requiring expensive equipment and infrastructure. Also, we aim defining a procedure capable of measuring, unattended, a complete set of pipettes. This allows verifying the consistency of performances within the full liquid handling dynamic range (in our case, from 0.2 µL to 1000 µL) – excluding gaps and measuring the performances exactly in the same conditions of real experiments.

FIGURE 2. ANDREW, THE PIPETTING ROBOT USING MANUAL PIPETTES.

The experimental setup consists of the Andrew robot manipulating the Gilson Pipetman Classic pipettes P2, P20, P100, P200, and P1000. The choice of the pipette models and type, considered among the most performing among all manual pipettes, allows to fully cover the range 0.2…1000 µL mostly used in conventional biology.

Andrew is a compact, 10 kg robot that can be easily transported and installed onto a conventional laboratory bench, occupying as little as the size of a sheet of paper when folded but with the capacity of performing both small and significantly large experiments (from one to 11 microplates or from one to 165 microtubes, for example). Based on vision and sophisticated software algorithms, it can identify consumables and their location, and operate the pipettes as the best humans would do. Andrew can grab a pipette, set the volume, insert and eject tips, aspirate and dispense – precisely and reproducibly – without the intervention of the user and with precise timing and speed of operations. The Andrew robot utilized for this series of experiments is a commercial

unit (Andrew#132 when it is not otherwise indicated) without modifications to its hardware and software, and exploiting AndrewOS version 1.0.17.2.

The light-absorbing liquid samples we used consist of solutions containing Ponceau S (purchased from Sigma Aldrich) diluted into analytical grade water, but we also report results with the addition of detergents or other additives for a modified viscosity. It is well known, in fact, that the surface tension properties and the viscosity of liquids strongly affect the pipette performances, and the described setup allows to reproducibly measure the pipetting performances with any liquid, without the subjective component induced by a human operator manipulating the pipettes.

The samples produced by pipetting are measured either by an Andrew Alliance photometer or by a reference balance.

The choice of a custom photometric reader allows us to precisely fix the optical conditions and internal calibrations. The Andrew Alliance absorbance reader is a compact, LED based photometer designed for reading clear-bottom 96 wells microplates. It measures the optical absorbance of samples at the wavelength of 520 nm by a calibrated light beam that crosses the sample from bottom to top. The passing-through light beam intensity is measured by a photodetector capable of covering linearly the range of Optical Density from 0 OD to 4 OD.

Importantly, the reader also includes shaking of the microplate during readout (to avoid mixing issues and effects related to the meniscus shape) that complement mixing of the samples achieved by multiple aspiration and dispensing actions. The reader also integrates an internal calibration method to obtain reproducible results without the necessity of an external optical samples with known absorption properties.

The photometric method employed consists in dispensing dye-loaded samples with known concentration into a clear-bottom microplate, where the properties of the microplate, of the reader and of the liquid samples have been pre-calibrated by means of recording the samples absorbance in these optical conditions as a function of their weight. So, we indirectly exploit a gravimetric method to calibrate our photometry.

The main advantage of the method consists in achieving a calibration measurement in an unattended manner and with short turnaround that can be executed in the same laboratory environment effectively.

We exploit the same protocols for gravimetric and photometric measurements, and the same pipetting procedures. The specific volumes used for each pipette are reported in the table below, and we perform 10 repetitions for each volume – three different volumes for each pipette.

Volume (µL)	**Pipette**
300	P1000
200	P1000
200	P200
150	P200
100	P200
100	P100
50	P100
20	P100
20	P100
10	P20
5	P20
2	P20
2	P2
1	P2
0.5	P2

It should be noted that – due to the overlap of the pipette ranges - only the volume range actually used by the robot is measured. For better clarity, the pipette P200 is tested in the range 100 µL…200 µL since it is the range actually used during the Andrew robot operations. Presently, we do not probe the 1000 µL volume corresponding to the maximum volume of the P1000, as a consequence of the limitation of the maximum volume inside a clear-bottom microplate, of the order of 350 µL. However, this limitation will be removed by means of a clear-bottom 24-well microplate, compatible with our reader.

For the volumes below 200 µL, the sample is introduced into a buffer-containing well for an equalization of the optical conditions but also to prevent sample evaporation for the smallest volume range. All final samples contain therefore 200 µL of liquid, with the exception of the 300 µL calibration for the P1000 pipette. We have verified,

as described in details in the next section, that the amount of buffer and its intrinsic optical properties don't affect the measurement conclusions.

For each of the above-mentioned volumes, we have prepared 10 independent wells in the way described above, for a total of 150 sample readings over two microplates. Additional controls, in particular blanks, fill the remaining wells, using all 192 positions available in two microplates. The reading of the microplates is performed in few seconds, after thorough mixing achieved by aspiration and dispensing with a large volume tip – operation that's performed automatically and with high reproducibility by the robot itself according to the protocol description.

In terms of gravimetric methods, we exploit the conventional guidelines for the use of a semi-microbalance. We have chosen as reference balance a Mettler Toledo AX205DR (8). It should be noted that there's no commercial balance covering the full dynamic range of required volumes with the necessary precision and accuracy. In fact, the low-volume range (below 2 µL) puts extremely stringent requirements on the balance performances, requiring ultra-precision balances capable of measuring samples with a reproducibility that should be better than 10 micrograms. The balance can become very expensive, and also the environment conditions becomes challenging: constant temperature within 0.5°C, high humidity (above 50%), critical insulation from vibrations through marbles, a minimal distance from vibration sources like traffic roads, and control of the air drafts in the surroundings. We consider that the comparison between the two methods should be performed in conditions achievable in a normal laboratory, and therefore we adopted a single semi-microbalance with a nominal weight resolution that's economically equivalent to a photometer, and we use it with the precautions that are feasible in a normal laboratory environment, as described in the next sections in more details.

All pipetting operations performed by Andrew follow the pipetting guidelines indicated by the pipette manufacturer for correct use of the tools. Profiting of the Andrew reproducibility, we have explored and studied individually most critical aspects affecting liquid handling performances, confirming quantitatively the conclusions reported in the existing literature (see, for example, an interesting independent pipetting review in (9)). However, we have discovered some subtle clarifications about a specific procedure that allow to significantly enhance pipetting performances: tip pre-wetting.

3.1. PRE-WETTING: THERE'S MORE ABOUT IT.

Most users are aware that, in order to dispense the correct volume, pipette tips have to be pre-wetted. However, this practice is often neglected since it implies a significant workload that somehow increases the experiments duration and consequently the probability of other mistakes, like an incorrect sequence of steps or wrong localization of wells into the microplate. Interestingly, the role of pre-wetting is usually emphasized in the context of liquid adhesion to the tip surface, improving and stabilizing the contact angle of the liquid inside the tip. While it is an evident effect, in particular with some liquids having a high affinity to polypropylene, it is not the only phenomenon occurring during the liquid aspiration. We have experimentally determined that there is a more important reason for performing pre-wetting, in particular when the environment has the humidity and temperature of a normal lab: vapor build-up. This understanding impacts the pre-wetting procedure and can improve the pipetting performances by avoiding systematic errors that can easily achieve 1 µL for a volume of 100 µL (1% systematic error): as it can be evinced by table 1, the magnitude of this systematic error induced by the method is by far larger than the admitted tolerances indicated in the ISO norms, and therefore we suggest following the procedure described below when pipetting accuracy is important.

We realized that most users perform pre-wetting by inserting the tip into the source, and aspirating various times the liquid inside the tip. After this step, the sample is normally aspirated inside the tip without extracting the tip from the liquid, as it would seem reasonable for the purpose of accomplishing surface wetting. However, we have measured quantitatively that this procedure doesn't allow to achieve the best pipetting performances. The pre-wetting of the tip has a second, more important role that's often ignored: the purpose of increasing the

humidity of air inside the tip and the piston cavity, to achieve a stable vapor pressure. Once a given volume of liquid is aspirated into the cavity of the tip and of the piston for the first time, the surface of the liquid in contact with the dry air keeps evaporating, and molecules of water go into gaseous form to reach equilibrium, shifting the liquid down by the equivalent volume occupied by the molecules that underwent phase change transition. As a reminder, at the boiling point 1 nL of water becomes 1.9 µL of vapor – so minutes amounts of evaporated water can have a significant effect on the volume of liquid retained inside the tip. Multiple aspiration and dispensing actions on the pipette plunger performed by keeping the tip inside the liquid bulk, are typically not capable to expel the extra volume of water vapor and air inside the tip, because of surface tension effects. So, the actual volume of liquid aspirated from the sample without extracting the tip from the liquid is correspondingly reduced. In order to deal with this phenomenon correctly, it suffices to extract the pipette tip from the source after pre-wetting, and re-inserting the tip into the liquid for the final sample aspiration before dispensing. In this way, the cavity of the piston and the tip is correctly saturated with vapor, and the amount of liquid aspirated into the tip corresponds to the nominal volume of gas (air and water vapor) displaced by the pipette piston. It should be noticed that the atmosphere inside the tip is not perturbed by the extraction in open air due to the impedance of the tip orifice. Performing this procedure correctly with Andrew, we have been able to achieve precisely and reproducibly the nominal performances of the pipettes used.

4. EXPERIMENTAL DATA

In photometric experiments, we have subtracted the instrument background by direct measurement from blanks (water); then, absorbance is converted into actual microliters according to a one-off gravimetric assessment. Random errors are therefore computed based on the standard deviation of the samples, irrespectively of the calibration scale; the systematic error of each set of volumes is determined on the basis of the nominal dispensed volume and the average value measured on all the samples. In all cases, the calibration scale is generated by a single reference absorbance measurement of a weighted sample read in the same conditions, allowing the conversion of absorbance data into microliters. In the tables below, we report typical data obtained by the photometric method:

	Expected Volume(µl)	Average Volume (µl)	Relative Inaccuracy (%)	CV (%)	Systematic Error (µL)	Random Error (µL)
P2-0.5	0.5	0.50	-0.5%	3.3%	0.00	0.02
P2-1	1	1.01	0.8%	0.9%	0.01	0.01
P2-2	2	1.95	-2.6%	0.7%	-0.05	0.01
P20-2	2	2.10	4.9%	0.7%	0.10	0.02
P20-5	5	5.12	2.4%	0.2%	0.12	0.01
P20-10	10	10.15	1.5%	0.7%	0.15	0.07
P20-20	20	20.04	0.2%	0.1%	0.04	0.03
P100-20	20	20.75	3.8%	0.5%	0.75	0.11
P100-50	50	50.48	1.0%	0.2%	0.48	0.13
P100-100	100	99.55	-0.5%	0.2%	-0.45	0.22
P200-100	100	100.86	0.9%	0.4%	0.86	0.46
P200-150	150	149.79	-0.1%	0.2%	-0.21	0.33
P200-200	200	199.34	-0.3%	0.3%	-0.66	0.51
P1000-200	200	201.62	0.8%	0.9%	1.62	1.83
P1000-300	300	301.04	0.3%	0.4%	1.04	1.19

TABLE 2. MEASURED PHOTOMETRIC PERFORMANCES FOR A SET OF 5 PIPETTES MEASURED BY ANDREW.

It is interesting to report these results into a chart normalized to the ISO-8655 upper limits, both for systematic and random errors for each individual measurement. In this plot, a systematic (random) error is equal to 100%

when it corresponds to the ISO systematic (random) error limit, and is proportionally below if the systematic (random) error is smaller. It can also be remarked that random errors are strictly positive, while systematic errors can also be negative. It is visible that all measurements stay within the required accuracy limits, but that the larger volume measurements approach the allowed upper values.

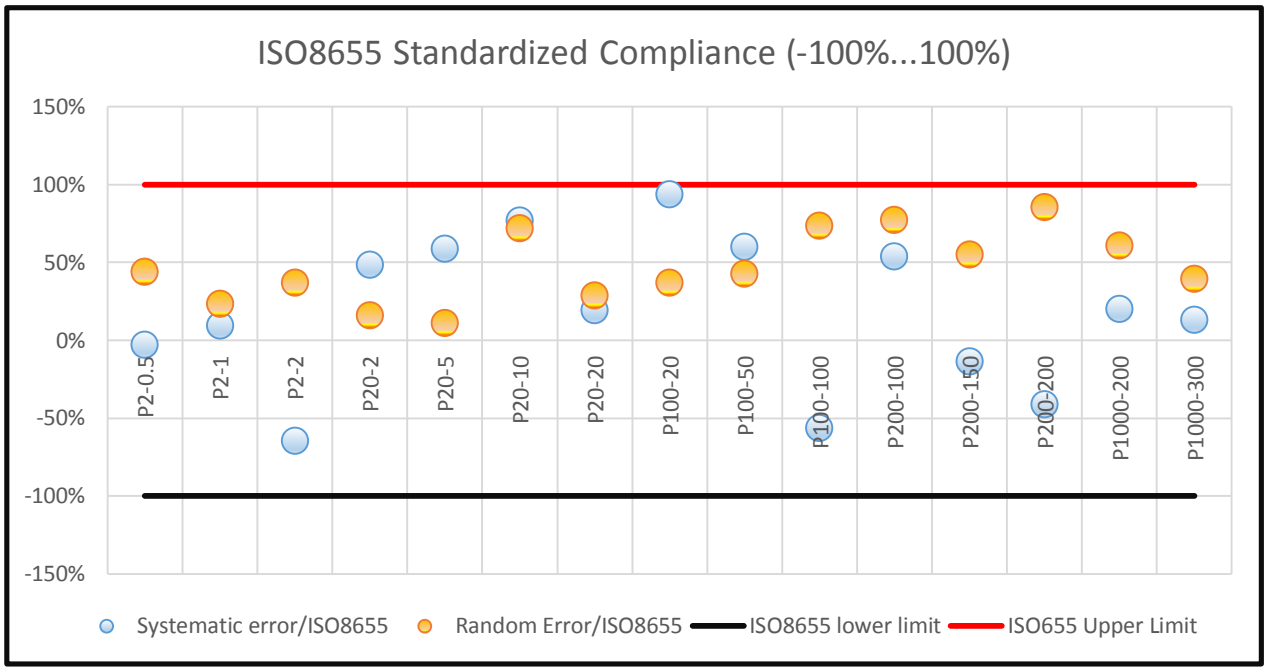

FIGURE 3: NORMALIZED REPRESENTATION OF PIPETTING PERFORMANCES (ANDREW)

We also generated data in the same experimental configuration by means of the gravimetric method. The protocol is essentially the same, but dispensing the liquids into a conventional Microtube of 1.5 mL volume. Andrew stops after every dispensing action, and the closed tube is put onto the balance. This procedure allows us to simultaneously measure the source and destination tube for each individual measurement, obtaining information about the correlation between aspirated and dispensed liquid volumes.

	Expected Volume(µl)	Average Volume (µl)	Relative Inaccuracy (%)	CV (%)	Systematic Error (µL)	Random Error (µL)
P2-0.5	0.50	0.42	-16.0%	0.09	-0.08	0.04
P2-1	1.00	0.96	-4.0%	0.03	-0.04	0.03
P2-2	2.00	1.93	-3.5%	0.03	-0.07	0.07
P20-2	2.00	1.98	-1.0%	0.05	-0.02	0.10
P20-10	10.00	9.80	-2.0%	0.01	-0.20	0.09
P20-20	20.00	19.83	-0.9%	0.01	-0.17	0.15
P100-20	20.00	20.24	1.2%	0.01	0.24	0.17
P100-50	50.00	50.08	0.2%	0.00	0.08	0.06
P100-100	100.00	100.09	0.1%	0.00	0.09	0.12
P200-100	100.00	100.19	0.2%	0.00	0.19	0.10
P200-150	200.00	200.41	0.2%	0.00	0.41	0.12
P200-200	200.00	199.57	-0.2%	0.00	-0.43	0.24
P1000-500	500.00	501.05	0.2%	0.00	1.05	0.57
P1000-1000	1000.00	1000.63	0.1%	0.00	0.63	0.38

TABLE 3. MEASURED GRAVIMETRIC PERFORMANCES FOR A SET OF 5 PIPETTES MEASURED BY ANDREW.

As expected qualitatively, the photometric and gravimetric method differ quantitatively in the ultimate performances determination of pipettes. We have identified precise explanations for this behavior that can be assigned to the following broad categories:

- Experimental resolution of the measurement instruments: reproducibility and calibration (linearity).
- An intrinsically different sensitivity to dye losses/mixing for Photometry vs. sensitivity to buffer losses/evaporation for the gravimetric method.

A critical review of the data is performed at the end of the final chapter, after the systematic review of possible random and systematic sources of errors, and in comparison with other liquid handling conditions and procedures.

5. Analysis of intrinsic measurement errors

In this section, we systematically review all intrinsic sources of systematic and random errors that could affect our final data. Useless to say, the quantitative conclusion of the analysis is specific to the method, materials and instrumentation involved.

5.1. Photometric method errors

5.1.1. Same well absorbance readings (statistical reproducibility)

The most obvious source of error comes from the absorbance reader itself: according to the light source technology, light detection technology, software algorithms and reading method, a number of potential uncertainties emerge. These uncertainties cannot be simply extrapolated from the optical reader manufacturer specifications since the reader specifications often refer to experimental conditions that are different from those exploited in the measurement we perform. For example, noise is strictly function of the absorbance, and varies across the measurement range. Therefore, we focused first on the most basic measurement: assessing the reproducibility for reading a single well – the same well with the same sample – multiple times. The result, in particular the relative standard deviation (CV) based on 30 consecutive readings, is reported in the plot below as a function of the absorbance:

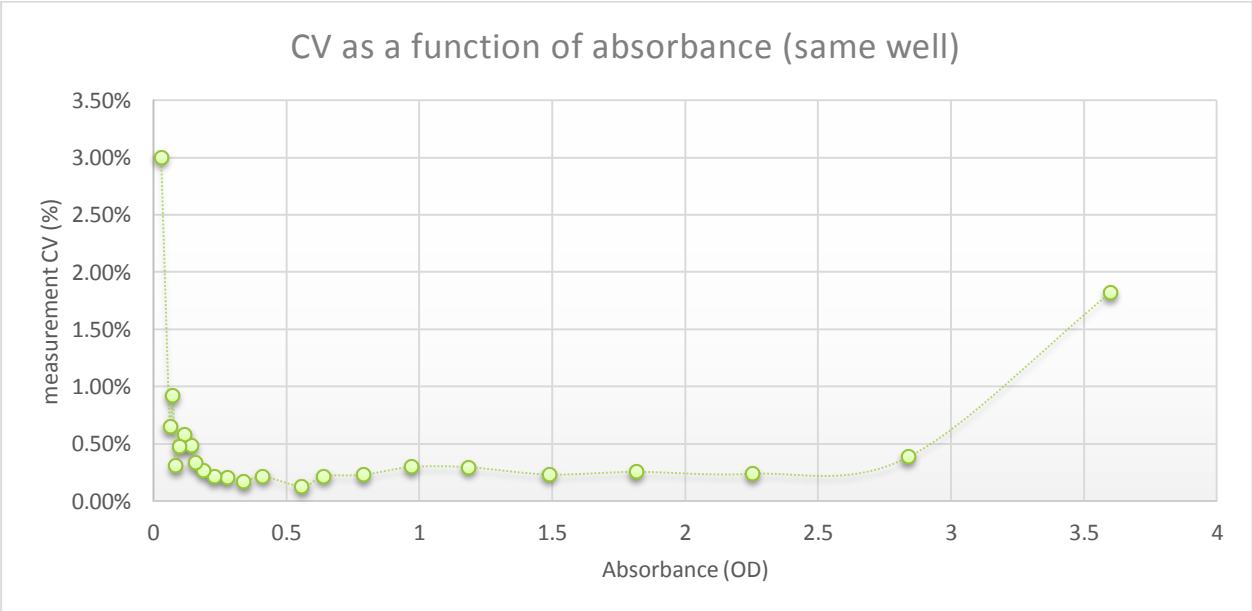

FIGURE 4: RELATIVE ERROR AS A FUNCTION OF ABSORBANCE (SAME WELL)

It is clearly visible that there're two regimes to avoid: the case of samples with low concentration/low absorbance (left part, where the error rises steeply for absorbance <0.1 OD) and those where the sample absorbs most of the light (right part, smooth error increase beyond ~3 OD). In order to contain random errors introduced by the reader, all experiments needs to be designed in order to generate data that are limited to the useful range. All data reported in this document have been **limited to the range 0.3-3.0 OD** in absorbance, and in this range the statistical error contribution is substantially flat and corresponds to a **0.24%** contribution (with a Gaussian-shaped distribution). It should be noted that, being the measurement performed on the same physical sample, no contribution from pipetting is present. It is extremely critical to compare this instrument reproducibility with the required reproducibility on ISO-8655-2 pipetting random errors reported in Table 1. It can be noted that all volumes above 100 µL require to test random pipetting errors to a reproducibility of 0.3%, so if the measurement instrument has an intrinsic reproducibility of 0.24% the measured distribution for a pipette that performs according to the norms can be as large as 0.38%. According to our internal investigation, there's no commercially available photometer performing in these experimental regime with reproducibility that's negligible with respect to the norms limit. We conclude that, in order to assess the pipettes' norm compatibility by photometry, the photometric method will simply require including the intrinsic measurement errors in the analysis, with the practical consequence of a limited sensitivity to assess the pipette compliance for those pipettes that are marginally performing within the norms upper limit.

5.1.2. WELL TO WELL VARIATIONS (SYSTEMATIC REPRODUCIBILITY)

According to the well position within the microplate, it is reasonable to expect that the same sample could provide different absorbance measurements. There are multiple potential reasons: the different optical configuration of a well at the edges of a microplate or at the center; simple irregularities or defects of the clear bottom and of the well plate walls; the internal design of the optical reader, in particular the number of light sources and detection heads utilized in a microplate scan. Ultimately, such a test includes also the contribution from the pipetting itself, since pipetting samples will be affected by the unknown pipetting random error at the specific volume. We have performed experiments by loading a constant amount of 150 µL of dye – by means of Andrew – in the various location and verified the uniformity of the reading. To minimize the random error generated by the reader itself, already described in the previous section, the wells have been measured five consecutive times and the average result has been used in the subsequent analysis.

	A	B	C	D	E	F	G	H	I	J	K	L
1	-0.8%	-0.5%	-0.6%	-0.7%	-0.5%	-0.6%	-0.9%	-0.2%	-0.3%	0.3%	0.1%	0.6%
2	-0.1%	-0.4%	-0.2%	-0.4%	-0.1%	-0.6%	-0.5%	-0.2%	0.6%	-0.2%	0.0%	-1.1%
3	-0.1%	0.1%	0.2%	0.0%	0.0%	-0.2%	-0.4%	-0.4%	-0.2%	-0.9%	0.5%	0.5%
4	0.2%	-0.3%	0.2%	-0.1%	-0.2%	-0.1%	-0.2%	-0.2%	0.2%	0.1%	0.3%	0.0%
5	0.1%	-0.1%	0.2%	-0.2%	-0.1%	0.3%	-0.4%	0.1%	0.5%	0.0%	0.1%	0.4%
6	-0.3%	0.0%	0.1%	0.2%	-0.1%	0.2%	0.3%	0.6%	0.5%	0.2%	0.4%	0.1%
7	0.2%	0.0%	-1.1%	0.4%	0.2%	0.2%	0.4%	0.4%	0.5%	0.7%	0.5%	0.3%
8	0.1%	0.2%	0.1%	0.0%	0.0%	0.2%	0.0%	0.4%	0.7%	-0.1%	0.6%	0.6%

TABLE 5: 2D MAP OF A MICROPLATE (% VARIATIONS WITH RESPECT TO AVERAGE SIGNAL)

The data shows a global 0.51% variation among the wells, and there's no specific row or column dependence of the data, nor evidence that the edges of the microplate would lead to different results.

We can conclude that the combination of pipetting random errors and the global photometer systematic effects account for **0.51%.** In consideration that the nominal random error for the pipetting itself (at this volume) is of the order of 0.2%, the present analysis indicates that the main error contribution with this dispensing volume comes from the absorbance reader itself. However, the performances of our microplate-based absorbance reader are very representative of the products that are commercially available, and that can be practically used

for the purpose. So, we confirm the previous conclusion indicating that the present performances of microplate-based absorbance readers constitute the main limitation for the random error assessment in the range of 100…200 µL where the pipette performances have random errors below their accuracy (see table 1).

5.1.3. BUFFER LIGHT ABSORPTION AND VERIFICATION OF THE METHOD

We have experimentally verified the hypothesis of the buffer-volume independence and the statement that the measurements depend only on the absolute amount of dye loaded into a well. This statement had some theoretical assumptions that require experimental verification: in particular, the hypothesis of a negligible light absorption from the buffer, that the conical shape of the wells can be approximated as a cylinder (it is known that, for manufacturing-related reasons, wells sides have a draft angle of 1-2% to improve demolding), and that no unidentified effects (like a modulation of the refraction index by the dye concentration modifying the light reflections at the surfaces) affect our method.

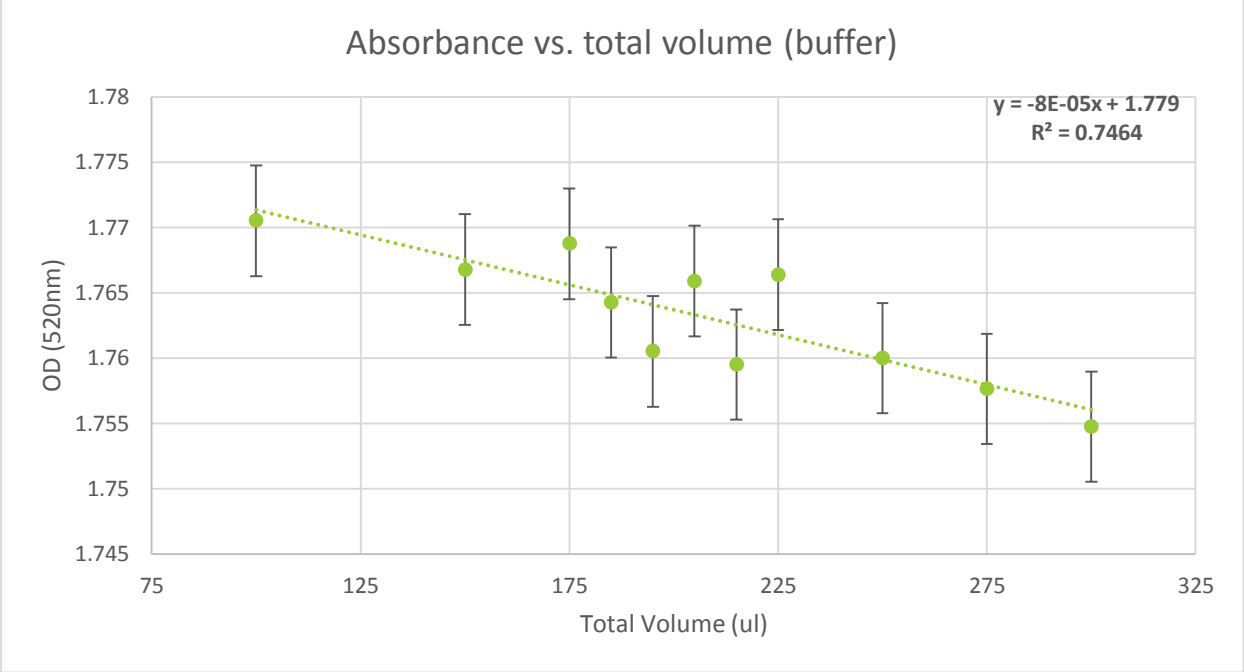

FIGURE 6: SIGNAL VARIATIONS FOR WELLS ADDITIONALLY LOADED WITH DIFFERENT AMOUNTS OF BUFFER

Twelve samples have been positioned on the same microplate row to minimize the intrinsic reader systematical error in the comparison, and 100 µL of dye have been loaded by an additional amount of water comprised between 0 and 200 µL, to achieve a total volume of the samples between 100 µL and 300 µL. The measurement is reported in the figure above, and the random error of 0.25% - expected in consideration of the previous measurements – is also reported. We can surely state that buffer volume inaccuracies, introduced by pipetting, don't affect our measurements. However, we do observe a certain dependence on the buffer volume that becomes experimentally significant for differences of hundreds microliters: for example , the same amount of dye diluted in a total volume of 300 µL produces a signal that's 0.45% lower than the same dye contained in a volume of 200 µL. We suspect this difference to be introduced by the conical shape of the microplate wells, and we simply correct for the systematic difference whenever required (e.g. when the nominal total volume of the samples doesn't correspond). Indirectly, this measurement validates also our mixing procedure, since inaccuracies in mixing could indeed result into well-to-well differences that we don't observe. We have experimentally verified that not only the average absorbance is identical with varying amounts of buffer, but that the random error of the measurements are not affected by the amount of buffer. This is an important verification since any mixing inaccuracy will immediately impact the quality of data, and possibly introduce time-dependent effects.

5.1.4. PHOTOMETER LINEARITY

In order to use the calibration factor described above, we need to assess that the photometer behaves linearly - like a calibrated weighting scale should. We perform this verification by the addition of a constant amount of sample into buffer containing wells - in consideration of the fact that any systematic pipetting error would cancel, being the linearity essentially based on the *digital* counting of the number of subsequent additions. Limiting ourselves to the data within the dynamic range of the photometer 0.3…3.0 OD, we know from an experiment described previously that the expected accuracy of the experimental configuration of microplate, pipetting and readout random errors, and the distribution of a constant volume of sample corresponds to 0.52% deviations. The data and a linear fit is shown in the following plot of absorbance vs. the number of fixed volumes of sample that has been added to the well:

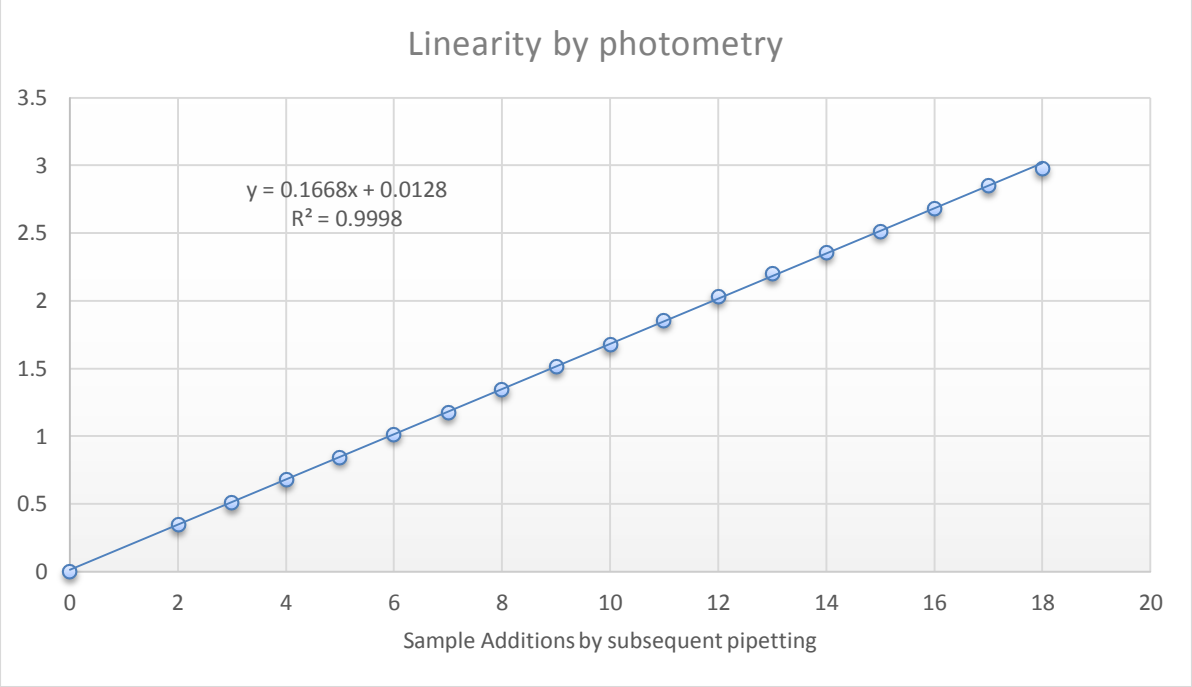

FIGURE 7: LINEAR FIT TO DATA GENERATED BY DISCRETE ADDITIONS OF DYE

It is important to note that data is background subtracted and that we have previously demonstrated that the measurements are independent on the total volume of the liquid contained in the readout well. It is interesting to analyze the relative deviations of the individual measurements with respect to the straight line fit: the relative deviations are reported in the plot below, highlighting for each individual measurement the systematic and random error assessed for samples with uniform light absorption (reported previously). It can be noted from the present plot that some systematic errors compete on random errors, being the points 2...10 in row A and the points 11…18 in row B. This is a confirmation of the evidence emerged during the analysis of the difference of signals from wells localized in different positions of the microplate, larger than the intrinsic same-well reproducibility of the reader.

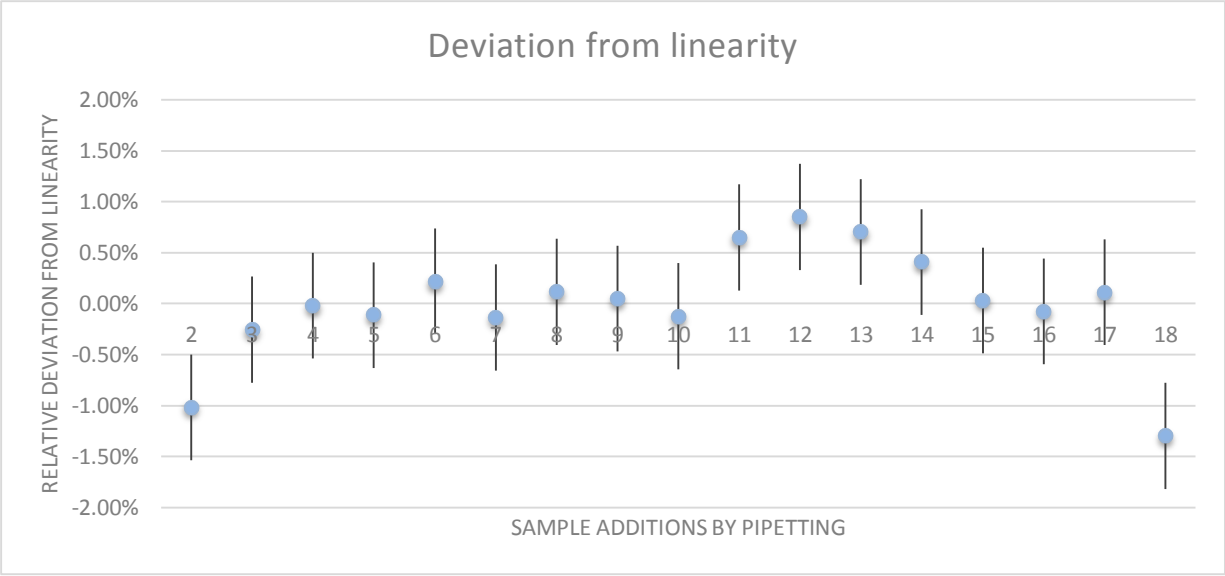

FIGURE 8: DEVIATION FROM LINEARITY, IN VIEW OF EXPECTED ERRORS

In consideration of the fact that the relative standard deviation of the individual measurements from the straight line fit corresponds to **0.54%**, with an expected standard deviation of 0.51%, we conclude that the photometer is linear within the identified dynamic range and within the previously identified systematic errors already present in the analysis of samples at constant light absorbance. This linearity measurement will affect the systematic error assessment, and in particular the comparison with the gravimetric method. It should be noted that in order to stay within the dynamic range where the photometer measurements have the required reproducibility, there is no linear relation between the volume and the absorbance of the sample. In other words, the use of multiple dilution factors for the absorbance dye can indeed create the situation where identical volumes are measured in different absorbance regimes, making the non-linearity discontinuities more visible. This is indeed the case in the present experimental configuration for the measurements P2-2 and P20-2, P20-20 and P100-20, P100-100 and P200-100 where the same liquid volume is measured by optical densities that differ by more than one order of magnitude. In this conditions, differences below 0.54% can have an instrumental origin.

5.2. GRAVIMETRIC METHOD ERRORS

We have previously shown that the photometer errors somehow affect the resolution on the pipette performances. However, the choice of a classical semi-microbalance impacts the performances of the measurements too. Our balance has a nominal readability of 0.01 mg and a nominal reproducibility between 0.04 mg and 0.05mg dependent on the weighting conditions, and this reproducibility limitation introduces some constraints in the weighting of the small volume samples. In particular, Table 1. Indicates that below 20 µL volumes the balance reproducibility limits the possibility of verifying strictly the compliance to the ISO-8655 norm, since the balance measurement error has the same magnitude of the maximum random error of the data. We have experimentally measured the reproducibility of our balance, in different days and in a normal laboratory environment, with the conclusion that the balance reproducibility corresponds indeed to 0.05 mg. We consider this factor to be the dominant measurement error for gravimetric measurements, since the Andrew operations have a very constant speed, allowing to statistically compensate for the water evaporation losses during the measurements (subtracted statistically). Also, a balance can be affected by a non-linear scale that could compromise the systematic error measurement. Without entering into aspects that are beyond the present study (reported, for example, in (10)) we highlight that these effects are modest in the small dynamic range of our measurements, and typically compensated by the substantially equally weight of our samples (dominated by the weight of the micro-tube and the support).

5.3. COMMON SOURCES OF ERRORS

5.3.1. CHOICE OF TIPS.

In the real world, most users profit of a large commercial offer of tips compatible with their pipettes in order to better adapt to their needs and minimize costs. Without entering into absorption and biocompatibility aspects, are the liquid handling performances equivalent and independent from the tips that are used? There are speculations from different manufacturer about the tightness and mechanical compliance with the pipette end, and also considerations that the different pipette tip shapes affect the hydrostatic forces acting on the liquid (11). All these parameters are difficult to quantify experimentally in a reproducible manner once a human manipulates a pipette. However, Andrew operates reproducibly and in constant conditions independently from the pipette tip involved. So we have performed a comparative experiment reported below, where we have repeated in sequence calibration experiments performed by Andrew without changing the pipettes, using first Gilson Diamond tips (suggested tips for the pipettes used, data reported in Table 2) and tips from a different brand (Eppendorf epTips). As visible from the data, no observable effect is visible within the measurement errors. Other tests and data for different brands are available on request.

	Expected Volume(µl)	Average Volume (µl)	Relative Inaccuracy (%)	CV (%)	Systematic Error (µL)	Random Error (µL)
P2-0.5	0.5	0.47	-5.7%	2.1%	-0.03	0.01
P2-1	1	0.97	-2.8%	0.8%	-0.03	0.01
P2-2	2	1.94	-2.9%	0.4%	-0.06	0.01
P20-2	2	2.09	4.4%	2.0%	0.09	0.05
P20-5	5	5.12	2.5%	0.6%	0.12	0.03
P20-10	10	10.12	1.2%	0.4%	0.12	0.05
P20-20	20	20.12	0.6%	0.3%	0.12	0.07
P100-20	20	20.71	3.5%	0.4%	0.71	0.09
P100-50	50	50.42	0.8%	0.3%	0.42	0.15
P100-100	100	99.70	-0.3%	0.3%	-0.30	0.27
P200-100	100	101.09	1.1%	0.2%	1.09	0.22
P200-150	150	150.58	0.4%	0.4%	0.58	0.56
P200-200	200	199.82	-0.1%	0.2%	-0.18	0.33
P1000-200	200	199.81	-0.1%	0.3%	-0.19	0.53
P1000-300	300	297.62	-0.8%	0.5%	-2.38	1.49

TABLE 4: CALIBRATION DATA OBTAINED WITH EPPENDORF EPTIPS TIPS.

5.3.2. PERFORMANCES WITH DIFFERENT LIQUID TYPES

It is very rare that biological samples have the same physical properties of distilled water, and it is even more improbable with industrial and food-related samples. The addition of reagents and chemicals modifies the surface tension, pH, viscosity and many other parameters, not to speak of suspensions, emulsions or heterogeneous samples. We have loaded our dye-containing water samples with 0.01% of Tween™ 20 (a common registered trade name for polysorbate 20)– a minute amount of a well-known detergent typically used for improving proteins' stability – or 10% glycerol (v/v), often used to store enzymes at low temperature - and we have repeated the same calibration protocols described above with these modified liquids. The results are reported in the tables below:

	Expected Volume(µl)	Average Volume (µl)	Relative Inaccuracy (%)	CV (%)	Systematic Error (µl)	Random Error (µl)
P2-0.5	0.5	0.68	35.2%	11.6%	0.18	0.08
P2-1	1	1.23	23.2%	5.8%	0.23	0.07
P2-2	2	2.17	8.4%	3.7%	0.17	0.08
P20-2	2	2.47	23.7%	6.5%	0.47	0.19
P20-5	5	5.82	16.4%	1.0%	0.82	0.06
P20-10	10	10.98	9.8%	0.6%	0.98	0.07
P20-20	20	21.08	5.4%	0.6%	1.08	0.12
P100-20	20	22.53	12.7%	0.5%	2.53	0.13
P100-50	50	52.35	4.7%	0.4%	2.35	0.19
P100-100	100	102.23	2.2%	0.4%	2.23	0.28
P200-100	100	103.32	3.3%	1.3%	3.32	1.35
P200-150	150	153.87	2.6%	0.4%	3.87	0.57
P200-200	200	201.17	0.6%	1.8%	1.17	3.65
P1000-200	200	206.09	3.0%	0.5%	6.09	1.04
P1000-300	300	314.95	5.0%	1.7%	14.95	5.51

TABLE 5: CALIBRATION DATA OBTAINED WITH LIQUID CONTAINING 0.01% OF TWEEN™ 20

	Expected Volume(µl)	Average Volume (µl)	Relative Inaccuracy (%)	CV (%)	Systematic Error (µl)	Random Error (µl)
P2-0.5	0.5	0.55	9.1%	4.2%	0.05	0.02
P2-1	1	1.05	5.5%	0.7%	0.05	0.01
P2-2	2	2.02	0.9%	0.9%	0.02	0.02
P20-2	2	2.16	8.0%	0.8%	0.16	0.02
P20-5	5	5.24	4.9%	0.9%	0.24	0.05
P20-10	10	10.34	3.4%	0.6%	0.34	0.06
P20-20	20	20.52	2.6%	0.3%	0.52	0.07
P100-20	20	21.32	6.6%	0.4%	1.32	0.09
P100-50	50	51.68	3.4%	0.3%	1.68	0.16
P100-100	100	102.18	2.2%	0.3%	2.18	0.29
P200-100	100	103.82	3.8%	0.5%	3.82	0.51
P200-150	150	154.34	2.9%	0.1%	4.34	0.19
P200-200	200	205.45	2.7%	0.2%	5.45	0.45
P1000-200	200	203.62	1.8%	1.4%	3.62	2.98
P1000-300	300	301.38	0.5%	0.7%	1.38	2.27

TABLE 6: CALIBRATION DATA OBTAINED WITH LIQUID CONTAINING 10% GLYCEROL

The presence of Tween™ 20 in the solution creates bubbles affecting both the systematic and the random errors: The Tween™ 20 reduces the surface tension of solutions but it also creates microscopic bubbles that can be trapped inside the tip and cannot being ejected when the second stop is pressed. When a tip is used several times, the bubbles trapped inside the tip, can also be retained for a long time and ejected randomly at any dispensing action. These results show that in the presence of Tween™ 20, the dispensed volume is systematically larger when compared to the nominal volume set. This is most probably induced by the different contact angle of the modified solution against the polypropylene material of the tip.

The results obtained for the 10% glycerol show a very different scenario, since the presence of glycerol affects only the systematic errors and not the random errors.

The volumes dispensed are slightly bigger that the nominal volumes but in a very reproducible manner. However, the density of the glycerol-based solution is 1.021 g/cm^3 at our working temperature (approaching 25°C), while distilled water has a density of 0.997 g/cm^3: a difference of 2.4%, that surely affects the pipetting systematic performances since the additional liquid weight modifies the internal air pressure through a higher Bernoulli force.

We conclude that, also for water-based samples, the liquid physical properties can have significant and practically unpredictable consequences on the volumetric performances, exceeding easily the limits of the ISO-8655 norms.

5.3.3. EXPERIMENT REPEATABILITY

We have performed a large series of experiments, both consecutively and in different days. One clear advantage of photometry is related to the substantially reduced dependence from environmental factors, validated by the good reproducibility of the data obtained for the same setup. The dispersion of three consecutive experiments by the same Andrew system and the same pipette set is shown in the table below for the full volume range. This data is ultimately representative of the repeatability of one Andrew system in the same laboratory. We can quantitatively conclude that, within our measurement errors, the system systematically performs within 1% tolerance with respect to the nominal set volume, with an exception in the region below 2 µL where pipette performances degrade to about 5% (but corresponding to the minute amount of a <40 nL reproducibility).

	Experiment repeatability (%)	**Experiment repeatability (µl)**
P2-0.5	6.80%	0.03
P2-1	2.08%	0.02
P2-2	2.07%	0.04
P20-2	0.72%	0.02
P20-5	0.70%	0.04
P20-10	0.53%	0.05
P20-20	0.38%	0.08
P100-20	0.45%	0.09
P100-50	0.41%	0.20
P100-100	0.77%	0.77
P200-100	0.62%	0.63
P200-150	0.60%	0.90
P200-200	0.22%	0.44
P1000-200	0.83%	1.66
P1000-300	0.42%	1.24

TABLE 7: EXPERIMENT REPEATABILITY (SAME ANDREW, SAME PIPETTES)

5.4. SUMMARY OF MEASUREMENT ERRORS

Essentially, photometric and gravimetric methods allow assessing pipetting performances with different limitations and advantages. Photometry has a limited best reproducibility of about 0.3% making the assessment of pipette performances challenging in the region of 100-200 µL where pipettes have a reproducibility of the same order of magnitude. However, its little sensitivity to evaporation makes it very practical for the small volumes region, also in consideration of the convenience of the reader. On the contrary, a semi-microbalance excels in the large volume region, being limited by an absolute weighting error of 0.05 mg corresponding to 0.05 µL, totally negligible above 100 µL. However, the method becomes unpractical in normal laboratory conditions when it comes to measurements below few microliters. Ultimately, a weight measurement is the only method that can provide an absolute calibration scale – an aspect that justifies its mainstream use in calibration laboratories and maintenance services.

6. OTHER SOURCES OF ERRORS

Being pipettes conceived for being used by humans, it is unavoidable to assess the errors induced by an operator.

6.1. SKILLED PIPETTING OPERATOR VS. ANDREW

We have performed manual experiments reproducing in full details the operations performed by Andrew, and we have measured the pipetting performances by photometric and by weighting. Our operator, fully trained in conventional laboratory procedures and GLPs, explicitly applied all precision enhancing techniques suggested by the pipette manufacturers for calibration purposes, including tip pre-wetting. The results by photometry are reported below:

	Expected Volume(µl)	Average Volume (µl)	Relative Inaccuracy (%)	CV (%)	Systematic Error (µL)	Random Error (µL)
P2-0.5	0.5	0.52	3.2%	3.4%	0.02	0.02
P2-1	1	1.05	4.9%	1.5%	0.05	0.02
P2-2	2	1.99	-0.7%	0.6%	-0.01	0.01
P20-2	2	2.12	5.9%	0.8%	0.12	0.02
P20-5	5	5.16	3.1%	0.6%	0.16	0.03
P20-10	10	10.19	1.9%	1.1%	0.19	0.11
P20-20	20	20.20	1.0%	0.3%	0.20	0.05
P100-20	20	20.63	3.2%	1.4%	0.63	0.30
P100-50	50	50.18	0.4%	0.3%	0.18	0.16
P100-100	100	99.36	-0.6%	0.3%	-0.64	0.26
P200-100	100	100.87	0.9%	0.4%	0.87	0.37
P200-150	150	149.43	-0.4%	0.2%	-0.57	0.37
P200-200	200	199.39	-0.3%	0.3%	-0.61	0.56
P1000-200	200	202.66	1.3%	0.3%	2.66	0.53
P1000-300	300	297.57	-0.8%	0.3%	-2.43	0.82

TABLE 8: PHOTOMETRIC DATA FROM A SKILLED OPERATOR (SAME PROTOCOL)

It is interesting to compare, graphically, the data obtained by a skilled human operator and Andrew itself, by reporting all data in the same plot (Fig. 9): it is evident that in the majority of cases random errors by Andrew (green dots) are below the random errors from the operator (Blue dots). Consistently, systematic errors are correlated (yellow and red dots), meaning that we have been successful in tuning Andrew to behave like a skilled

operator. We conclude that Andrew behaves like a skilled operator would do, exploiting at the limit the ideal pipetting performances with high reproducibility.

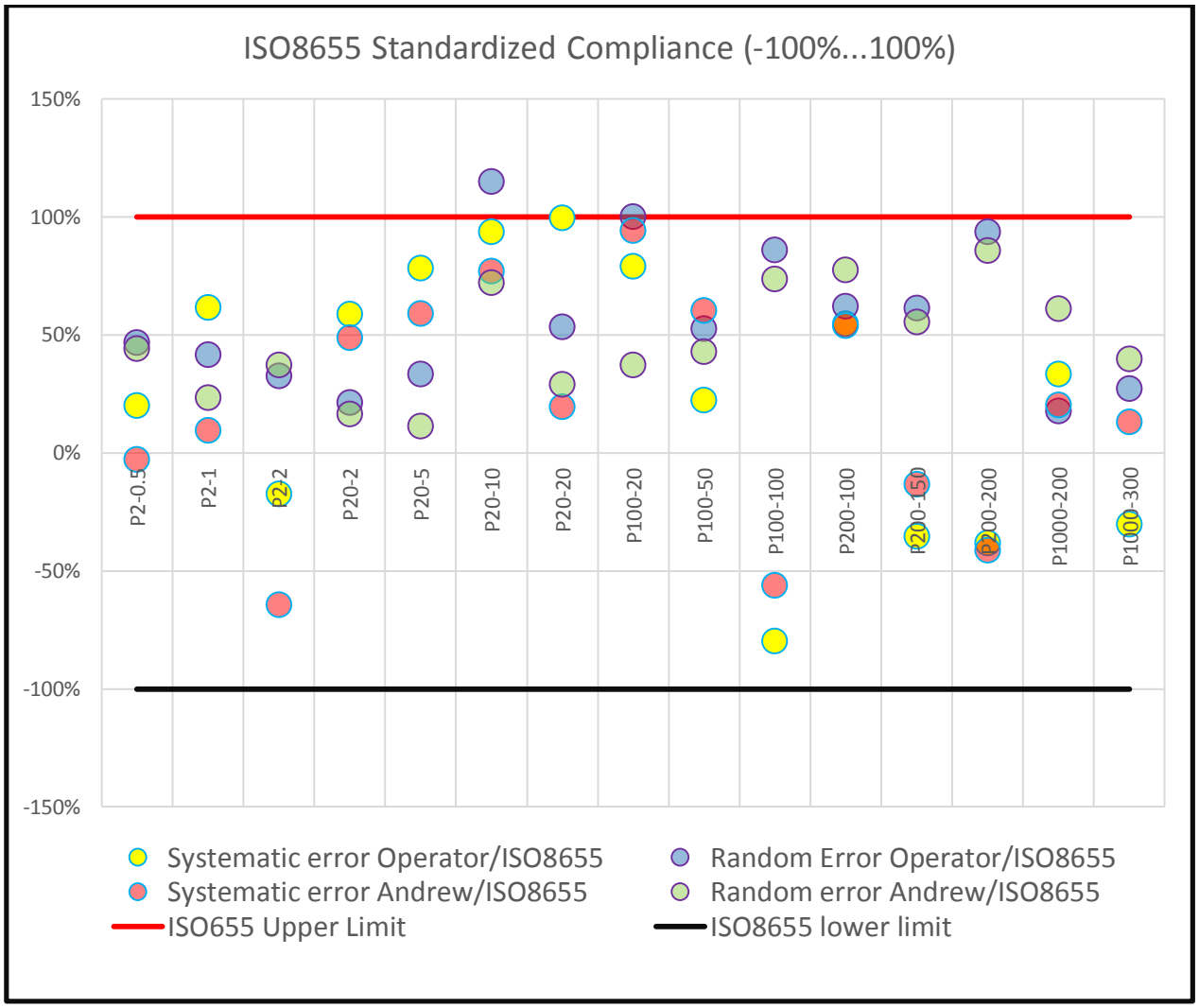

FIGURE 9: NORMALIZED REPRESENTATION OF PIPETTING PERFORMANCES (OPERATOR VS. ANDREW)

6.2. DAILY OPERATIONS IN THE LAB: A DIFFERENT STORY.

Even if it is not technically connected with intrinsic pipetting performances, we have to remind that there is a plethora of aspects that affect pipetting performances in the lab, most of them being simply related to daily practices.

- Not performing pre-wetting account for errors up to 2% (12);
- Uneven rhythm and timing during pipetting can increase errors by 1.5%;
- Non-consistent and incorrect tip immersion depth into the liquid can account for up to 1% variations;
- A deviation from verticality of the piston axis of 20% can induce a 1% error (13)
- Approximate sensing of the piston “first stop” can introduce systematic errors of 0.5%;
- Parallax errors and incorrect volume setting can account to errors up to 0.5%. It suffices to keep the pipette in front of the eyes’ field of view, and always set the volume by exceeding the desired target by three units, and moving back the counter to the nominal volume to avoid hysteresis;
- Low quality tips can induce leaks accounting for 0.5…50% losses.

7. CONCLUSIONS

We have defined, performed and analyzed various experimental procedures for assessing pipettes performances, and exploited the Andrew robot to perform pipetting in a precisely reproducible manner – removing the uncertainty induced by the operator. We have compared the gravimetric and photometric methods, to conclude that in normal laboratory conditions and using commercial equipment the two methods have different limitations and performances. The photometric method is efficiently automatized and – by means of Andrew - allows performing pipette calibration in a fully unattended manner. It excels in testing low volume pipettes effectively in comparison to the ISO standard, but it is marginal in the compliance assessment of large volume pipettes due to the limited reproducibility of commercial photometers. The gravimetric method provides the best data for large volume pipettes and provides an absolute calibration scale, but it is very challenging to apply for the low-volume range. Overall, it is less prone to automation and in the low-volume range requires expensive and delicate equipment. Furthermore, its sensitivity to evaporation make it unpractical in the humidity and temperature conditions of a typical laboratory. After a careful review of errors and performances in the conditions of a normal laboratory, we conclude that photometry is a valuable practical method to validate volumetric performances in the range 0.5 µL-1000 µL, and that this procedure can be fully achieved and automatized by means of the Andrew robot complemented by a compact, low cost photometer. Independently of the preferred assessment method, we conclude that the Andrew robot performs, in standard laboratory conditions, as the best pipetting operator would do.

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