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**Piston-operated volumetric
instruments — Determination of
uncertainty for volume measurements
made using the photometric method**

*Instruments volumétriques actionnés par piston — Détermination de
l'incertitude de mesure pour les mesurages volumétriques au moyen de
la méthode photométrique*



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Foreword

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Piston-operated volumetric instruments — Determination of uncertainty for volume measurements made using the photometric method

1 Scope

This Technical Report gives the detailed evaluation of uncertainty for volume measurements according to the *Guide to the expression of uncertainty in measurements* (GUM). ISO 8655-7 covers non-gravimetric methods of volume measurement. ISO 8655-7:—, Annex A, presents the details of a method that employs removable cells (vials) in a UV/VIS photometer. ISO 8655-7:—, Annex B, presents the details of a method that employs a flow cell fixed in place in a UV/VIS photometer. This Technical Report covers the uncertainty of measurement of both methods.

2 Uncertainty analysis for the replaceable cell photometric method as described in ISO 8655-7:—, Annex A

2.1 Uncertainties of each measurand

For purposes of creating an uncertainty budget, the uncertainty for each device used in the determination of unknown volume has been taken from manufacturer's literature. The uncertainty for the system linearity was measured using a reference UV/VIS photometer. UV/VIS photometric uncertainties are based on ISO 8655-7:—, Table A.1.

Table 1 — Uncertainties of the analytical devices used

Item	Laboratory grade UV/VIS-photometer	Reference grade UV/VIS-photometer	Type of uncertainty
Flask, Class A volumetric, 1 000 ml	0,4 ml		B
Pipette, Class A volumetric, 100 ml	0,08 ml		B
Pipette, Class A volumetric, 10 ml	0,02 ml		B
Pipette, Class A volumetric, 5 ml	0,015 ml		B
Pipette, Class A volumetric, 2 ml	0,010 ml		B
Photometric measurement at $A = 0$	0,001 0	0,000 3	A
Photometric measurement at $A = 0,5$	0,001 5	0,000 5	A
Photometric measurement at $A = 1,0$	0,001 5	0,000 5	A
Photometric measurement at $A = 1,5$	0,002 0	0,000 7	A
Temperature of sample	0,2 °C		B
Reproducibility of UV/VIS photometer wavelength	0,5 nm	0,2 nm	B
Non-linearity of photometric response with 2:1 dynamic range	—	0,14 %	A
Non-linearity of photometric response with 8:1 dynamic range	—	0,63 %	A

2.2 Mathematical model of method

The unknown volume is calculated based on the Beer-Lambert Law. The stock solution, if Ponceau S is used as chromophore, has extinction coefficient ε_1 at 520 nm, and zero absorbance at 730 nm. The concentration of the stock solution is C_S . The diluent has extinction coefficient ε_2 at its peak wavelength 730 nm and concentration C_D .

A standard is created by mixing stock and diluent very accurately using large volumes. An empty vial is placed in the cell holder; it is filled with buffer and the UV/VIS photometer is set to zero at both wavelengths. The buffer is removed without disturbing the vial and it is rinsed and filled with the standard solution. The absorbance of the standard at the two wavelengths, 520 nm and 730 nm respectively is:

$$A_{S1} = \varepsilon_1 C_S R l_{SV} \quad (1)$$

$$A_{S2} = \varepsilon_2 C_D (1 - R) l_{SV} \quad (2)$$

where R is the dilution ratio of stock to diluent used to create the standard, and l_{SV} is the path length of the vial containing the aliquot of standard solution.

Next, a fresh vial containing a measured amount V_D of diluent is put into the UV/VIS photometer and a reading is taken at both wavelengths. The reading at 520 nm, A_{D1} , serves the purpose of establishing zero, which may vary from that of a vial containing buffer because of vial imperfections. This reading at 520 nm is subtracted from the reading at 730 nm, A_{D2} , to make the zero correction:

$$\text{Absorbance of diluent} = A_{D2} - A_{D1} = \varepsilon_2 C_D l_S \quad (3)$$

The path length of this vial is l_S . Now the device under test is used to deliver an aliquot of stock solution into the diluent. The contents are mixed, and the absorbance is measured at 520 nm. The corrected absorbance of the unknown mixture at 520 nm is:

$$\text{Absorbance of unknown} = A_U - A_{D1} = \varepsilon_1 C_S \left(\frac{V_U}{V_U + V_D} \right) l_S \quad (4)$$

By dividing the last two equations, one obtains

$$\frac{A_U - A_{D1}}{A_{D2} - A_{D1}} = \left(\frac{\varepsilon_1 C_S}{\varepsilon_2 C_D} \right) \left(\frac{V_U}{V_U + V_D} \right) \quad (5)$$

The two equations from the standard can likewise be divided to give:

$$\frac{A_{S1}}{A_{S2}} = \left(\frac{\varepsilon_1 C_S}{\varepsilon_2 C_D} \right) \left(\frac{R}{1 - R} \right) \quad (6)$$

Between these two equations the extinction coefficients and concentrations can be eliminated, giving:

$$\frac{A_U - A_{D1}}{A_{D2} - A_{D1}} = \left(\frac{1 - R}{R} \right) \left(\frac{V_U}{V_U + V_D} \right) \frac{A_{S1}}{A_{S2}} \quad (7)$$

All of these quantities have been measured except for V_U . This equation can be solved for V_U with the following result:

$$V_U = V_D \left[\frac{\frac{A_U - A_{D1}}{A_{D2} - A_{D1}}}{\left(\frac{1-R}{R} \right) \frac{A_{S1}}{A_{S2}} - \left(\frac{A_U - A_{D1}}{A_{D2} - A_{D1}} \right)} \right] \quad (8)$$

This equation allows calculation of V_U based on measured quantities. Note that the path lengths of the vials do not enter into the result, even though two vials were used having different path lengths. Also, any variation in zero due to imperfections in the vials has been fully corrected. Measured absorbances appear as ratios, meaning that the UV/VIS photometer need not be accurate, only linear. No information is needed about extinction coefficients or concentrations of stock or diluent solutions.

2.3 Sensitivity coefficients

Sensitivity coefficients are obtained by taking partial derivatives of Equation (8) with respect to each of the measurands or other system parameters that can vary in a way to cause uncertainty in the results.

For the purpose of easily obtaining these partial derivatives, Equation (8) can be simplified by using the following approximations:

$$R \ll 1 \quad (9)$$

$$\frac{A_U - A_{D1}}{A_{D2} - A_{D1}} < 1 \quad (10)$$

$$A_{D1} \approx 0 \quad (11)$$

Concentrations of the stock solutions, dilution ratios and diluent have been created such as to make these approximations valid. With these approximations, Equation (8) becomes

$$V_U \approx V_D \frac{A_{S2} R}{A_{S1}} \left(\frac{A_U - A_{D1}}{A_{D2} - A_{D1}} \right) \quad (12)$$

This simplification creates an error no greater than 4 % in the sensitivity coefficients. They are in any case estimates, and this error is small compared to other possible sources of error. For purposes of illustrating the method of analysis, the values given in Table 2, which pertain to the calibration of a 0,5 µl pipette, are used.

Table 2 — Values for calibration of a 0,5 µl pipette using the replaceable cell method

$l = 1,8 \text{ cm}$	path length of vial used in making the photometric reading	
$V_S = 5 \text{ ml}$	volume of stock used to make the first dilution for the standard	
$V_{d1} = 1\ 000 \text{ ml}$	volume of diluent used to make the first dilution for the standard	
$V_{m1} = 5 \text{ ml}$	volume of the first mixture (dilution) used to make the second dilution	
$V_{d2} = 100 \text{ ml}$	volume of diluent used to make the second dilution	
$R = \left(\frac{5}{1\ 005}\right)\left(\frac{5}{105}\right) = 2,369 \times 10^{-4}$	dilution ratio of standard	
$A_{SK} = 1\ 111 \text{ abs/cm}$	absorbance per unit path length of the undiluted stock solution at λ_1	
$A_D = 0,600\ 0 \text{ abs/cm}$	absorbance per unit path length of the diluent at λ_2	
$A_{S1} = (111\ 1)(1,8)(2,369 \times 10^{-4}) = 0,473\ 8 \text{ abs}$	absorbance of standard at λ_1	
$A_{S2} = (1 - 2,369 \times 10^{-4})(0,600)(1,8) = 1,079\ 7 \text{ abs}$	absorbance of standard at λ_2	
$A_{D1} = 0$	absorbance of diluent at λ_1	
$A_{D2} = (0,600 \text{ abs/cm})(1,8 \text{ cm}) = 1,080 \text{ abs}$	absorbance of diluent at λ_2	
$V_D = 5\ 000 \text{ µl}$	volume of diluent used to dilute the unknown sample	
$A_U = (1\ 111 \text{ abs/cm})(1,8 \text{ cm}) \left(\frac{0,5}{5\ 000,5}\right) = 0,200 \text{ abs}$	absorbance of unknown at λ_1	
$V_U = 0,500 \text{ µl}$		

In this example, the standard is produced by first diluting 5 ml of stock solution with 1 000 ml of diluent, and then diluting 5 ml of that with 100 ml of diluent.

2.3.1 Uncertainty in reading of standard due to instrument imprecision at wavelength λ_1

In all of the following examples, the formulae are followed by a numeric evaluation using the parameters given above for a 0,5 µl pipette.

The partial derivative of Equation (12) with respect to A_{S1} is

$$\begin{aligned} \frac{\partial V_U}{\partial A_{S1}} &= - \frac{V_D A_{S2} R}{A_{S1}^2} \left(\frac{A_U - A_{D1}}{A_{D2} - A_{D1}} \right) \\ &= - 1,055 \text{ µl/abs} \end{aligned} \tag{13}$$

The sign is not relevant, since all sensitivity coefficients are squared to get the weighted variance.

The standard uncertainty in absorbance A_{S1} arises from two sources: the measurement at zero absorbance, which has an uncertainty of 0,000 3, and the measurement at an absorbance of 0,5 which has an uncertainty of 0,000 5. They combine as the square root of the sum of the squares:

$$U(A_{S1}) = \sqrt{0,000\ 3^2 + 0,000\ 5^2} \text{ abs} = 0,000\ 583 \text{ abs} \tag{14}$$

The standard uncertainty in volume measurement due to this uncertainty in A_{S1} is the product of the uncertainty in absorbance and the sensitivity factor:

$$U(V_{0,5})_{A_{S1}} = U(A_{S1}) \frac{\partial V_U}{\partial A_{S1}} = (0,000\ 583\ \text{abs})(1,055\ \mu\text{l/abs}) = 0,000\ 615\ \mu\text{l} = 6,15 \times 10^{-4}\ \mu\text{l} \quad (15)$$

2.3.2 Uncertainty in reading of standard due to instrument imprecision at wavelength λ_2

$$\begin{aligned} \frac{\partial V_U}{\partial A_{S2}} &= \frac{V_D R}{A_{S1}} \left(\frac{A_U - A_{D1}}{A_{D2} - A_{D1}} \right) \\ &= 0,463\ \mu\text{l/abs} \end{aligned} \quad (16)$$

The standard uncertainty in absorbance A_{S2} arises from two sources: the measurement at zero absorbance, which has an uncertainty of 0,000 3, and the measurement at 1,08 absorbance which has an uncertainty of 0,000 5. They combine as the square root of the sum of the squares:

$$U(A_{S1}) = \sqrt{0,000\ 3^2 + 0,000\ 5^2}\ \text{abs} = 0,000\ 583\ \text{abs} \quad (17)$$

The standard uncertainty in volume measurement due to this uncertainty in A_{S2} is the product of the uncertainty in absorbance and the sensitivity factor:

$$U(V_{0,5})_{A_{S2}} = U(A_{S2}) \frac{\partial V_U}{\partial A_{S2}} = (0,000\ 583\ \text{abs})(0,463\ \mu\text{l/abs}) = 2,70 \times 10^{-4}\ \mu\text{l} \quad (18)$$

2.3.3 Uncertainty in reading absorbance A_U due to instrument imprecision at λ_1 (520 nm)

The partial derivative of Equation (12) with respect to A_U is

$$\begin{aligned} \frac{\partial V_U}{\partial A_U} &= V_D \frac{A_{S2} R}{A_{S1}(A_{D2} - A_{D1})} \\ &= 2,50\ \mu\text{l/abs} \end{aligned} \quad (19)$$

The standard uncertainty in absorbance A_U at 0,2 absorbance units has an uncertainty of 0,000 5 (in this case the specification for the spectrophotometer does not include a specification at 0,2, so the assumption is made that the uncertainty is that specified at the next greatest absorbance). The measurement at zero absorbance is covered in 2.3.4. Thus,

$$U(A_U) = 0,000\ 5\ \text{abs} \quad (20)$$

The standard uncertainty in volume measurement due to this uncertainty in A_U is the product of the uncertainty in absorbance and the sensitivity factor:

$$U(V_{0,5})_{A_U} = U(A_U) \frac{\partial V_U}{\partial A_U} = (0,000\ 5\ \text{abs})(2,50\ \mu\text{l/abs}) = 1,25 \times 10^{-3}\ \mu\text{l} \quad (21)$$

2.3.4 Uncertainty in reading diluent absorbance A_{D1} due to instrument imprecision at λ_1 (520 nm)

$$\begin{aligned} \frac{\partial V_U}{\partial A_{D1}} &= \left(\frac{V_D A_{S2} R}{A_{S1}} \right) \left[\left(\frac{-1}{A_{D2} - A_{D1}} \right) + \left(\frac{A_U - A_{D1}}{(A_{D2} - A_{D1})^2} \right) \right] \\ &= 2,04\ \mu\text{l/abs} \end{aligned} \quad (22)$$

The absorbance of the diluent at 520 nm is nearly zero, so the imprecision in the reading of A_{D1} is 0,000 3. This is the only source of uncertainty in this initial determination of absorbance. It is used in two places, to provide a zero for the absorbance of the unknown A_U and the absorbance of the diluent at 730 nm, A_{D1} .

$$U(A_{D1}) = 0,000\ 3\ \text{abs} \quad (23)$$

The uncertainty in volume measurement due to this is:

$$U(V_{0,5})_{A_{D1}} = U(A_{D1}) \frac{\partial V_U}{\partial A_{D1}} = (0,000\ 3\ \text{abs})(2,04\ \mu\text{l/abs}) = 6,12 \times 10^{-4}\ \mu\text{l} \quad (24)$$

2.3.5 Uncertainty in reading diluent absorbance A_{D2} due to instrument imprecision at λ_2 (730 nm)

$$\begin{aligned} \frac{\partial V_U}{\partial A_{D2}} &= \left(\frac{V_D A_{S2} R}{A_{S1}} \right) \left\{ - \left[\frac{A_U - A_{D1}}{(A_{D2} - A_{D1})^2} \right] \right\} \\ &= 0,463\ \mu\text{l/abs} \end{aligned} \quad (25)$$

The absorbance of the diluent measured at 730 nm is about 1, so

$$U(A_{D2}) = 0,000\ 5\ \text{abs} \quad (26)$$

The uncertainty in V_U due to this is:

$$U(V_{0,5})_{A_{D2}} = U(A_{D2}) \frac{\partial V_U}{\partial A_{D2}} = (0,000\ 5\ \text{abs})(0,463\ \mu\text{l/abs}) = 2,32 \times 10^{-4}\ \mu\text{l} \quad (27)$$

2.3.6 Uncertainty in readings due to drift in wavelength λ_1 of photometer between the time that the standard is read and the unknown is read

The magnitude of the uncertainty depends on the slope of the absorbance versus wavelength curve at the wavelength chosen for making the readings. For the chromophore chosen in this case at 520 nm, the slope is no greater than 0,1 % nm⁻¹. The reading of the standard is taken as the correct wavelength, and it is the change from that which creates the uncertainty.

$$\begin{aligned} \frac{\partial V_U}{\partial \lambda_1} &= \left(\frac{\partial V_U}{\partial A_U} \right) \left(\frac{\partial A_U}{\partial \lambda_1} \right) \\ &= \left(\frac{V_D A_{S2} R}{A_{S1} A_{D2}} \right) (0,001)\ \mu\text{l/nm} \\ &= 2,50 \times 10^{-3}\ \mu\text{l/nm} \end{aligned} \quad (28)$$

The specification of the photometer is for a drift of no more than 0,2 nm. A rectangular probability is assumed, giving an uncertainty of:

$$U(\lambda_1) = 0,2\ \text{nm} / \sqrt{3} = 0,115\ \text{nm} \quad (29)$$

The square root of three is the conversion from a rectangular to standard uncertainty, as specified by the GUM. The resulting uncertainty in volume measurement due to uncertainty in wavelength is:

$$U(V_{0,5})_{\lambda_1} = U(\lambda_1) \frac{\partial V_U}{\partial \lambda_1} = (0,115\ \text{nm})(0,002\ 5\ \mu\text{l/nm}) = 2,89 \times 10^{-4}\ \mu\text{l} \quad (30)$$

2.3.7 Uncertainty in readings due to drift in wavelength λ_2 of photometer between the time that the standard is read and the unknown is read

The magnitude of the uncertainty depends on the slope of the absorbance versus wavelength curve at 730 nm for the chromophore in the diluent, in this case no greater than 0,05 % nm⁻¹. The reading of the standard is taken as the correct wavelength, and it is the change from that which creates the uncertainty.

$$\begin{aligned}\frac{\partial V_U}{\partial \lambda_2} &= \left(\frac{\partial V_U}{\partial A_{D2}} \right) \left(\frac{\partial A_{D2}}{\partial \lambda_2} \right) \\ &= \left(-\frac{V_D A_{S2} R A_U}{A_{S1} A_{D2}^2} \right) (0,000 5) \mu\text{l/nm} \\ &= 2,31 \times 10^{-4} \mu\text{l/nm}\end{aligned}\quad (31)$$

The specification of the spectrophotometer is for a drift of no more than 0,2 nm. A rectangular probability is assumed, giving an uncertainty of:

$$U(\lambda_2) = 0,2 \text{ nm} / \sqrt{3} = 0,115 \text{ nm} \quad (32)$$

The resulting uncertainty in volume measurement due to uncertainty in wavelength is:

$$U(V_{0,5})_{\lambda_2} = U(\lambda_2) \frac{\partial V_U}{\partial \lambda_2} = (0,115 \text{ nm})(0,000 23 \mu\text{l/nm}) = 2,67 \times 10^{-5} \mu\text{l} \quad (33)$$

2.3.8 Uncertainty in the volume of stock used in creating the standard

In this case there was a double dilution performed in making the standard. We first address the uncertainty in V_U due to uncertainty in the volume of stock used in creating the first dilution. An uncertainty in volume of stock results in an uncertainty in the ratio of dilution for the standard, which in turn results in an uncertainty in the determination of unknown volume. In the following analysis, R_1 is the ratio of dilution in the first mixture, and R_2 is the dilution of that mixture to create the final standard.

V_{d1} is the volume of diluent used in preparing the first dilution.

$$R = R_1 R_2 \quad (34)$$

$$R_1 = \frac{V_S}{V_{d1} + V_S} \approx \frac{V_S}{V_{d1}} \quad (35)$$

$$\frac{\partial R}{\partial V_S} = R_2 \frac{\partial R_1}{\partial V_S} = R_2 \frac{1}{V_{d1}} \quad (36)$$

$$\begin{aligned}\frac{\partial V_U}{\partial V_S} &= \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_S} \\ &= \left(V_D \frac{A_{S2}}{A_{S1}} \frac{A_U}{A_{D2}} \right) \left(\frac{R_2}{V_{d1}} \right) \\ &= 1,00 \times 10^{-4} \mu\text{l}/\mu\text{l}\end{aligned}\quad (37)$$

The uncertainty in stock volume V_S when delivered by a 5 ml Class A pipette made from glass is found by dividing its tolerance of 0,015 ml by the factor $\sqrt{3}$ to convert from a rectangular probability distribution to a normal one:

$$U(V_S) = 0,015 \text{ ml} / \sqrt{3} = 8,66 \mu\text{l} \quad (38)$$

The resulting uncertainty in unknown volume V_U due to uncertainty in stock volume is:

$$U(V_{0,5})_{V_S} = U(V_S) \frac{\partial V_U}{\partial V_S} = (8,66 \mu\text{l})(0,000 100 \mu\text{l}/\mu\text{l}) = 8,66 \times 10^{-4} \mu\text{l} \quad (39)$$

2.3.9 Uncertainty in the volume of mixture 1 (V_{m1} , result of dilution 1) used in creating the second dilution

The analysis follows that given in 2.3.8.

The volume of diluent used in preparing the second dilution is V_{d2} .

$$R = R_1 R_2 \quad (40)$$

$$R_2 = \frac{V_{m1}}{V_{d2} + V_{m1}} \approx \frac{V_{m1}}{V_{d2}} \quad (41)$$

$$\frac{\partial R}{\partial V_{m1}} = R_1 \frac{\partial R_2}{\partial V_{m1}} = R_1 \frac{1}{V_{d2}} \quad (42)$$

$$\begin{aligned} \frac{\partial V_U}{\partial V_{m1}} &= \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{m1}} \\ &= \left(V_D \frac{A_{S2}}{A_{S1}} \frac{A_U}{A_{D2}} \right) \left(\frac{R_1}{V_{d2}} \right) \\ &= 1,05 \times 10^{-4} \mu\text{l}/\mu\text{l} \end{aligned} \quad (43)$$

A portion of the first mixture, V_{m1} , is measured and dispensed using a 5 ml Class A pipette. The uncertainty in this volume is

$$U(V_{m1}) = 0,015 \text{ ml} / \sqrt{3} = 8,66 \mu\text{l} \quad (44)$$

The resulting uncertainty in unknown volume V_U due to uncertainty in the volume of mixture 1 is:

$$U(V_{0,5})_{V_{m1}} = U(V_{m1}) \frac{\partial V_U}{\partial V_{m1}} = (8,66 \mu\text{l})(0,000 1 \mu\text{l}/\mu\text{l}) = 9,09 \times 10^{-4} \mu\text{l} \quad (45)$$

2.3.10 Uncertainty in volume of diluent V_{d1} used in creating the first dilution

$$\frac{\partial V_U}{\partial V_{d1}} = \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{d1}} \quad (46)$$

$$R = R_1 R_2 \quad (47)$$

$$\frac{\partial R}{\partial V_{d1}} = R_2 \frac{\partial R_1}{\partial V_{d1}} \quad (48)$$

$$R_1 = \frac{V_S}{V_S + V_{d1}} \approx \frac{V_S}{V_{d1}} \quad (49)$$

$$\frac{\partial R}{\partial V_{d1}} = -R_2 \frac{V_S}{(V_{d1})^2} \quad (50)$$

$$\begin{aligned} \frac{\partial V_U}{\partial V_{d1}} &= \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{d1}} \\ &= \left(V_D \frac{A_{S2}}{A_{S1}} \frac{A_U}{A_{D2}} \right) \left[\frac{-R_2 V_S}{(V_{d1})^2} \right] \\ &= -5,02 \times 10^{-7} \mu\text{l}/\mu\text{l} \end{aligned} \quad (51)$$

The first dilution is prepared using 1 000 ml of diluent measured using a Class A volumetric flask. The uncertainty in this diluent volume is

$$U(V_{d1}) = 400 \mu\text{l} / \sqrt{3} = 231 \mu\text{l} \quad (52)$$

The resulting uncertainty in unknown volume V_U due to uncertainty in the volume of diluent 1 is:

$$U(V_{0,5})_{V_{d1}} = U(V_{d1}) \frac{\partial V_U}{\partial V_{d1}} = (231 \mu\text{l}) (5,02 \times 10^{-7} \mu\text{l}/\mu\text{l}) = 1,16 \times 10^{-4} \mu\text{l} \quad (53)$$

2.3.11 Uncertainty in the volume of diluent V_{d2} used in making the second dilution

This analysis follows that of 2.3.10:

$$\frac{\partial V_U}{\partial V_{d2}} = \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{d2}} \quad (54)$$

$$R = R_1 R_2 \quad (55)$$

$$\frac{\partial R}{\partial V_{d2}} = R_1 \frac{\partial R_2}{\partial V_{d2}} \quad (56)$$

$$R_2 = \frac{V_{m1}}{V_{m1} + V_{d2}} \approx \frac{V_{m1}}{V_{d2}} \quad (57)$$

$$\frac{\partial R}{\partial V_{d2}} = -R_1 \frac{V_{m1}}{(V_{d2})^2} \quad (58)$$

$$\begin{aligned} \frac{\partial V_U}{\partial V_{d2}} &= \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{d2}} \\ &= \left(V_D \frac{A_{S2}}{A_{S1}} \frac{A_U}{A_{D2}} \right) \left(\frac{-R_1 V_{m1}}{(V_{d2})^2} \right) \\ &= -5,25 \times 10^{-6} \mu\text{l}/\mu\text{l} \end{aligned} \quad (59)$$

The second mixture is prepared using 100 ml of diluent measured using a glass Class A volumetric pipette. The uncertainty in volume is

$$U(V_{d2}) = 80 \mu\text{l} / \sqrt{3} = 46 \mu\text{l} \quad (60)$$

The resulting uncertainty in unknown volume V_U due to uncertainty in the volume of diluent 2 is:

$$U(V_{0,5})_{V_{d2}} = U(V_{d2}) \frac{\partial V_U}{\partial V_{d2}} = (46 \mu\text{l}) (5,25 \times 10^{-6} \mu\text{l}/\mu\text{l}) = 2,42 \times 10^{-4} \mu\text{l} \quad (61)$$

2.3.12 Uncertainty in the volume of diluent V_D used in the unknowns

$$\begin{aligned} \frac{\partial V_U}{\partial V_D} &= \frac{A_{S2} R}{A_{S1}} \left(\frac{A_U - A_{D1}}{A_{D2} - A_{D1}} \right) \\ &= 1,00 \times 10^{-4} \mu\text{l}/\mu\text{l} \end{aligned} \quad (62)$$

The dilution volume V_D is measured with a 5 ml glass Class A volumetric pipette. The uncertainty in volume is

$$U(V_D) = 15 \mu\text{l} / \sqrt{3} = 8,66 \mu\text{l} \quad (63)$$

The resulting uncertainty in unknown volume V_U due to uncertainty in the volume of diluent V_D is:

$$U(V_{0,5})_{V_D} = U(V_D) \frac{\partial V_U}{\partial V_D} = (8,66 \mu\text{l}) (1,00 \times 10^{-4} \mu\text{l}/\mu\text{l}) = 8,66 \times 10^{-4} \mu\text{l} \quad (64)$$

2.3.13 Uncertainty in temperature of standard versus unknowns

If the unknown samples are at a different temperature than the standard, then the temperature dependence of the absorptivity of the chromophore will cause an error in the results (the temperature coefficient of absorptivity, K_T , for this chromophore in this diluent is $0,05 \% \cdot ^\circ\text{C}^{-1}$). The method specifies that the temperature of the unknowns and control shall be the same within $\pm 0,2 ^\circ\text{C}$. The sensitivity of results to this temperature difference is given by the partial derivative of V_U with respect to the temperature of the unknown, assuming that the temperature of the standard is by definition correct:

$$\frac{\partial V_U}{\partial T} = \frac{\partial V_U}{\partial A_U} \frac{\partial A_U}{\partial T} \quad (65)$$

$$A_U(T) = A_U [1 - K_T (T - T_S)] \quad (66)$$

$$\begin{aligned} \frac{\partial V_U}{\partial T} &= - \left(V_D \frac{A_{S2}}{A_{S1}} \frac{R}{A_{D2}} \right) (A_U K_T) \\ &= 2,50 \times 10^{-4} \mu\text{l}/^\circ\text{C} \end{aligned} \quad (67)$$

The uncertainty in temperature arises from two measurements, one of the standard and one of the unknown. Each is uncertain by $0,2 ^\circ\text{C}$. The uncertainty in the difference will then be the $\sqrt{2}$ times $0,2 ^\circ\text{C}$ or $0,28 ^\circ\text{C}$.

Divide by $\sqrt{3}$ to convert from rectangular to normal distribution. The resulting uncertainty in unknown volume is:

$$U(V_{0,5})_T = U(T) \frac{\partial V_U}{\partial T} = (0,28 / \sqrt{3} ^\circ\text{C}) (2,50 \times 10^{-4} \mu\text{l}/^\circ\text{C}) = 4,04 \times 10^{-5} \mu\text{l} \quad (68)$$

2.3.14 Uncertainty due to incomplete mixing of standard

If the standard is incompletely mixed then its measured absorbance at λ_1 will be in error, leading to an error in the calculation of V_U . Because the dilution ratio R is so small, the measured absorbance at λ_2 will not be much affected by a lack of thorough mixing. The parameter M_S is defined as the fractional error in the reading of the standard's absorbance:

$$M_S = \frac{A_{S1}(O) - A_{S1}(I)}{A_{S1}(I)} \quad (69)$$

$$A_{S1}(O) = A_{S1}(I)(M_S + 1)$$

where $A_{S1}(I)$ is the absorbance of an ideally well-mixed standard at λ_1 , and $A_{S1}(O)$ is the observed absorbance.

Using this definition,

$$\begin{aligned} \frac{\partial V_U}{\partial M_S} &= \frac{\partial V_U}{\partial A_{S1}(O)} \frac{\partial A_{S1}(O)}{\partial M_S} \\ &= - \left(\frac{V_D R A_U A_{S2}}{A_{S1}^2 A_{D2}} \right) \frac{\partial A_{S1}(O)}{\partial M_S} \\ &= - \left(\frac{V_D R A_U A_{S2}}{A_{S1}^2 A_{D2}} \right) A_{S1}(I) \\ &= -(1,054 \mu\text{l/abs})(0,474 \text{ abs}) \\ &= -0,50 \mu\text{l} \end{aligned} \quad (70)$$

The mixing process, 10 inversions, assures an uncertainty of less than 0,1 % in the absorbance of the standard, giving an uncertainty in the parameter M_S of:

$$U(M_S) = 0,001 \text{ abs/abs} \quad (71)$$

The corresponding uncertainty in unknown volume is

$$U(V_{0,5})_{M_S} = U(M_S) \frac{\partial V_U}{\partial M_S} = (0,001 \text{ abs/abs})(0,50 \mu\text{l}) = 5,00 \times 10^{-4} \mu\text{l} \quad (72)$$

2.3.15 Uncertainty due to incomplete mixing of unknowns

If the unknowns are incompletely mixed then their measured absorbance at λ_1 will be in error, leading to an error in the calculation of V_U . Following the explanation given above,

$$M_U = \frac{A_U(O) - A_U(I)}{A_U(I)} \quad (73)$$

$$A_U(O) = A_U(I)(M_U + 1)$$

Using this definition,

$$\begin{aligned}
 \frac{\partial V_U}{\partial M_U} &= \frac{\partial V_U}{\partial A_U(O)} \frac{\partial A_U(O)}{\partial M_U} \\
 &= \left(\frac{V_D R A_{S2}}{A_{S1} A_{D2}} \mu\text{l/abs} \right) \frac{\partial A_U(O)}{\partial M_U} \\
 &= \left(\frac{V_D R A_{S2}}{A_{S1} A_{D2}} \right) A_U(I) \\
 &= (2,50 \mu\text{l/abs}) (0,20 \text{ abs}) \\
 &= 0,50 \mu\text{l}
 \end{aligned} \tag{74}$$

Again, the mixing procedure of 10 inversions gives an uncertainty in absorbance of less than 0,1 %.

$$U(M_U) = 0,001 \text{ abs/abs} \tag{75}$$

The corresponding uncertainty in unknown volume is

$$U(V_{0,5})_{M_U} = U(M_U) \frac{\partial V_U}{\partial M_U} = (0,001 \text{ abs/abs})(0,50 \mu\text{l}) = 5,00 \times 10^{-4} \mu\text{l} \tag{76}$$

2.3.16 Uncertainty due to pH dependence of the chromophore used in the stock solution

If the unknown samples have a different pH than the standard, then a pH dependence of the absorptivity of the chromophore will cause an error in the results. The sensitivity of results to this pH difference is given by the partial derivative of V_U with respect to the pH of the unknown, assuming that the pH of the standard is by definition correct.

$$\begin{aligned}
 \frac{\partial V_U}{\partial(\text{pH})} &= \frac{\partial V_U}{\partial A_U} \frac{\partial A_U}{\partial(\text{pH})} \\
 &= \left(\frac{V_D R A_{S2}}{A_{S1} A_{D2}} \right) \frac{\partial A_U}{\partial(\text{pH})} \\
 &= \left(\frac{V_D R A_{S2}}{A_{S1} A_{D2}} \right) [0,01 \text{ abs}/(\text{abs} \cdot \text{pH})] (0,2 \text{ abs}) \\
 &= (2,50 \mu\text{l/abs}) [0,01 \text{ abs}/(\text{abs} \cdot \text{pH})] (0,2 \text{ abs}) \\
 &= 5,00 \times 10^{-3} \mu\text{l/pH}
 \end{aligned} \tag{77}$$

The pH dependence of the extinction coefficient of the chromophore (in this buffer at pH 6) is 1 % per pH unit, and the buffer is stable within 0,1 pH units.

The corresponding uncertainty in unknown volume is

$$U(V_{0,5})_{\text{pH}} = U(\text{pH}) \frac{\partial V_U}{\partial(\text{pH})} = (5,00 \times 10^{-3} \mu\text{l/pH})(0,1 \text{ pH}) = 5,0 \times 10^{-4} \mu\text{l} \tag{78}$$

2.3.17 Uncertainty due to evaporation loss of diluent while in storage

If the diluent is pre-dispensed into glass vials and capped, there will be some diffusion of water vapour through the cap liner during long-term storage. The resulting diluent concentration will be higher than expected and the volume of diluent lower than expected. The effect of higher diluent concentration is to increase the value of A_{D2} .

The effect of lower diluent volume will be to decrease the amount of dilution experienced by the unknown sample added to the diluent, so the resulting value of A_U will be higher than expected. The two effects cancel each other out whenever the condition $V_D \gg V_U$ is met, as it is in this example.

The parameter V_{ev} is defined as the amount of water lost to evaporation of diluent during storage.

Using this definition,

$$\begin{aligned} \frac{\partial V_U}{\partial V_{ev}} &= \frac{\partial V_U}{\partial A_U} \frac{\partial A_U}{\partial V_{ev}} + \frac{\partial V_U}{\partial A_{D2}} \frac{\partial A_{D2}}{\partial V_{ev}} \\ &= \left(\frac{V_D A_{S2} R}{A_{S1} A_{D2}} \mu\text{l/abs} \right) \left(\frac{A_U}{V_D} \text{abs}/\mu\text{l} \right) + \left(-\frac{V_D A_{S2} R A_U}{A_{S1} A_{D2}^2} \mu\text{l/abs} \right) \left(\frac{A_{D2}}{V_D} \text{abs}/\mu\text{l} \right) \\ &= \left(\frac{A_{S2} R A_U}{A_{S1} A_{D2}} \right) (1-1) \\ &= 0 \end{aligned} \quad (79)$$

2.3.18 Uncertainty due to evaporation of stock solution while in storage

An advantage of the photometric method described in ISO 8655-7:—, Annex A, is that it is amenable to preparing solutions ahead of time for later use, considerably streamlining the calibration procedure. If this is done, care shall be taken to minimize the concentration of the stock solutions due to diffusion of water vapour through the cap liners. The uncertainty introduced by the loss of water vapour through the cap of a 50 ml bottle is given by

$$\begin{aligned} \frac{\partial V_U}{\partial V_S} &= \frac{\partial V_U}{\partial A_U} \frac{\partial A_U}{\partial V_S} \\ &= \left(\frac{V_D A_{S2} R}{A_{S1} A_{D2}} \mu\text{l/abs} \right) \left(\frac{A_U}{V_S} \text{abs}/\mu\text{l} \right) \\ &= (2,50 \mu\text{l/abs}) \left(\frac{0,2 \text{ abs}}{5 \times 10^4 \mu\text{l}} \right) \\ &= 1 \times 10^{-5} \mu\text{l}/\mu\text{l} \end{aligned} \quad (80)$$

where V_S is the volume of stock solution in the bottle.

For conical polyethylene cap liners on a 50 ml bottle, the loss due to diffusion of water vapour is less than 50 μl per year at room temperature. Smaller-sized storage containers are not advisable because the uncertainty introduced by concentration changes will be proportionally greater.

The corresponding uncertainty in unknown volume V_U is:

$$U(V_{0,5})_{V_S} = U(V_S) \frac{\partial V_U}{\partial V_S} = \left(\frac{50 \mu\text{l}}{\sqrt{3}} \right) (1,00 \times 10^{-5} \mu\text{l}/\mu\text{l}) = 2,89 \times 10^{-4} \mu\text{l} \quad (81)$$

2.3.19 Uncertainty due to system non-linearity

The response of the complete system shall be characterized to determine the linearity of response at wavelength λ_1 . There is no need to characterize linearity of response at λ_2 because the standard and the unknown have nearly the same absorbance at λ_2 . The parameter L is defined as the fractional error in the reading of the unknown's absorbance A_U due to a lack of linearity:

$$L = \frac{A_U(O) - A_U(I)}{A_U(I)} \tag{82}$$

$$A_U(O) = A_U(I)(L + 1)$$

Using this definition,

$$\begin{aligned} \frac{\partial V_U}{\partial L} &= \frac{\partial V_U}{\partial A_U(O)} \frac{\partial A_U(O)}{\partial L} \\ &= \left(\frac{V_D R A_{S2}}{A_{S1} A_{D2}} \mu\text{l/abs} \right) \frac{\partial A_U(O)}{\partial L} \\ &= \left(\frac{V_D R A_{S2}}{A_{S1} A_{D2}} \right) A_U(I) \\ &= (2,50 \mu\text{l/abs})(0,20 \text{ abs}) \\ &= 0,50 \mu\text{l/(abs/abs)} \end{aligned} \tag{83}$$

In the case of a dynamic range of 2, the measured non-linearity was 0,14 %.

$$U(L) = 0,0014 \text{ abs/abs} \tag{84}$$

The corresponding uncertainty in unknown volume is

$$U(V_{0,5})_L = U(L) \frac{\partial V_U}{\partial L} = (0,0014 \text{ abs/abs}) [0,5 \mu\text{l/(abs/abs)}] = 7,00 \times 10^{-4} \mu\text{l} \tag{85}$$

2.3.20 Uncertainties too small for consideration in the uncertainty budget

There are several sources of uncertainty too small to warrant inclusion in the uncertainty budget. Among them are the following.

2.3.20.1 Uncertainty due to the temperature coefficient of the Class A glassware

Class A glassware has a temperature coefficient of approximately 0,002 5 %·°C⁻¹. If the unknown vials are filled with diluent using Class A glassware while at a temperature of 24 °C instead of the 20 °C that the glassware is adjusted at, an error of 0,01 % will occur in the results. This is much smaller than other sources of uncertainty.

2.3.20.2 Uncertainty due to degradation of the solutions in storage

The chromophores chosen are stable when stored in the dark in the buffer specified. If the reagents (stock, standards, diluent) are to be stored at room temperature, a preservative shall be added to inhibit microbiological growth. All reagents can be frozen to prevent microbiological growth.

2.4 Uncertainty budget

Table 3 shows the individual combined relative standard uncertainties as well as the combined relative standard uncertainty for the calibration of a 0,5 µl pipette using the replaceable cell photometric method in a reference grade UV/VIS photometer.

Table 3 — Uncertainty in the calibration of a 0,5 µl pipette using the replaceable cell method

Clause	Contribution to uncertainty	Probability distribution	Type of uncertainty	Range of values ±	Standard uncertainty	Unit	Sensitivity coefficient	Unit	Standard uncertainty µl
Instrument-related uncertainties									
2.3.1	Imprecision in reading absorbance of standard at λ_1 (A_{S1})	normal	A		$5,83 \times 10^{-4}$	abs	1,055	µl/abs	$6,15 \times 10^{-4}$
2.3.2	Imprecision in reading absorbance of standard at λ_2 (A_{S2})	normal	A		$5,83 \times 10^{-4}$	abs	$4,63 \times 10^{-1}$	µl/abs	$2,70 \times 10^{-4}$
2.3.3	Imprecision in reading absorbance of unknown at λ_1 (A_U)	normal	A		$5,00 \times 10^{-4}$	abs	2,50	µl/abs	$1,25 \times 10^{-3}$
2.3.4	Imprecision in reading absorbance of diluent at λ_1 (A_{D1})	normal	A		$3,00 \times 10^{-4}$	abs	2,04	µl/abs	$6,12 \times 10^{-4}$
2.3.5	Imprecision in reading absorbance of diluent at λ_2 (A_{D2})	normal	A		$5,00 \times 10^{-4}$	abs	$4,63 \times 10^{-1}$	µl/abs	$2,32 \times 10^{-4}$
2.3.6	Drift in λ_1 between reading standard and unknown	rectangle	B	0,2	$1,155 \times 10^{-1}$	nm	$2,50 \times 10^{-3}$	µl/nm	$2,89 \times 10^{-4}$
2.3.7	Drift in λ_2 between reading standard and diluent	rectangle	B	0,2	$1,155 \times 10^{-1}$	nm	$2,31 \times 10^{-4}$	µl/nm	$2,67 \times 10^{-5}$
Reagent-related uncertainties									
2.3.8	Volume of stock used in creating dilution 1 (V_S)	rectangle	B	15	8,66	µl	$1,00 \times 10^{-4}$	µl/µl	$8,66 \times 10^{-4}$
2.3.9	Volume of mixture 1 used in creating dilution 2 (V_{m1})	rectangle	B	15	8,66	µl	$1,05 \times 10^{-4}$	µl/µl	$9,09 \times 10^{-4}$
2.3.10	Volume of diluent used in creating dilution 1 (V_{d1})	rectangle	B	400	$2,31 \times 10^2$	µl	$5,02 \times 10^{-7}$	µl/µl	$1,16 \times 10^{-4}$
2.3.11	Volume of diluent used in creating dilution 2 (V_{d2})	rectangle	B	80	$4,619 \times 10^1$	µl	$5,25 \times 10^{-6}$	µl/µl	$2,42 \times 10^{-4}$
2.3.12	Volume of diluent used in unknowns (V_D)	rectangle	B	15	8,66	µl	$1,00 \times 10^{-4}$	µl/µl	$8,66 \times 10^{-4}$
2.3.13	Temperature of unknowns versus standards	rectangle	B	$2,8 \times 10^{-4}$	$1,617 \times 10^{-1}$	°C	$2,50 \times 10^{-4}$	µl/°C	$4,04 \times 10^{-5}$
2.3.14	Incomplete mixing of standard	normal	A		$1,00 \times 10^{-3}$	abs/abs	$5,00 \times 10^{-1}$	(µl/abs)/abs	$5,00 \times 10^{-4}$
2.3.15	Incomplete mixing of unknowns	normal	A		$1,00 \times 10^{-3}$	abs/abs	$5,00 \times 10^{-1}$	(µl/abs)/abs	$5,00 \times 10^{-4}$
2.3.16	pH dependence of the chromophore	normal	A		$1,00 \times 10^{-1}$	pH	$5,00 \times 10^{-3}$	µl/pH	$5,00 \times 10^{-4}$
2.3.17	Evaporation of diluent while in storage	rectangle	B	5	2,89	µl	0	µl/µl	0
2.3.18	Evaporation of stock while in storage	rectangle	B	50	$2,89 \times 10^1$	µl	$1,00 \times 10^{-5}$	µl/µl	$2,89 \times 10^{-4}$
System-related uncertainties									
2.3.19	System non-linearity	normal	A		$1,40 \times 10^{-3}$	abs/abs	$5,00 \times 10^{-1}$	(µl/abs)/abs	$7,00 \times 10^{-4}$
Combined standard uncertainty									
	Absolute							µl	$2,50 \times 10^{-3}$
	Relative							%	0,500

3 Uncertainty analysis for the flow cell photometric method as given in ISO 8655-7:—, Annex B

3.1 Uncertainties of each measurand

See Table 1.

3.2 Mathematical model of method

The unknown volumes are calculated using the Lambert-Beer law, which relates the absorbance of a solution to the concentration of the chromophore C , the extinction coefficient of the chromophore ε and the path length of the measuring cell l . For stock solution of concentration C_S , the absorbance A_{SK} is given by:

$$A_{SK} = \varepsilon_1 C_S l \quad (86)$$

In this instance, ε_1 is the extinction coefficient as measured at wavelength 1, i.e. 520 nm. Once the stock is diluted in making the standard, the absorbance of the standard is

$$A_S = \varepsilon_1 C_S R l \quad (87)$$

where R is the dilution ratio of stock by diluent.

When the unknown volume V_U of stock is added to a known volume V_D of diluent, the absorbance of the solution is:

$$A_U = \varepsilon_1 C_S \left(\frac{V_U}{V_U + V_D} \right) l \quad (88)$$

By combining this equation with the one for the absorbance of the standard, the terms, ε_1 , C_S and l can be eliminated, leaving

$$A_U = \frac{A_S}{R} \left(\frac{V_U}{V_U + V_D} \right) \quad (89)$$

This equation can be solved for V_U , giving:

$$V_U = V_D \left(\frac{A_U R}{A_S - A_U R} \right) \quad (90)$$

3.3 Sensitivity coefficients

Sensitivity coefficients are obtained by taking partial derivatives of Equation (90) with respect to each of the measurands or other system parameters that can vary in a way to cause uncertainty in the results.

For the purpose of easily obtaining these partial derivatives, Equation (90) is simplified to

$$V_U \approx V_D \frac{A_U R}{A_S} \quad (91)$$

For the example illustrated in Table 4, an error of 0,01 % is introduced in the calculation of V_U by using this simplification. This is much smaller than other sources of error in the uncertainty determination.

This simplification creates an error no greater than 4 % in the sensitivity coefficients. They are in any case estimates, and this error is small compared to other possible sources of error. For purposes of illustrating the method of analysis, the following values, which pertain to the calibration of a 0,5 μ l pipette, are used.

Table 4 — Values for calibration of a 0,5 µl pipette using the flow cell method

$l = 1,000 \text{ cm}$	path length of flow cell used in making the photometric reading	
$V_S = 5 \text{ ml}$	volume of stock solution used to make the first dilution for the standard	
$V_{d1} = 1\,000 \text{ ml}$	volume of diluent used to make the first dilution for the standard	
$V_{m1} = 5 \text{ ml}$	volume of the first mixture (dilution) used to make the second dilution	
$V_{d2} = 100 \text{ ml}$	volume of diluent used to make the second dilution	
$R = \left(\frac{5}{1005}\right)\left(\frac{5}{105}\right) = 2,369 \times 10^{-4}$	dilution ratio of standard	
$A_{SK} = 2\,000 \text{ abs/cm}$	absorbance per unit path length of the undiluted stock solution at λ_1	
$A_S = (2\,000)(1,000)(2,369 \times 10^{-4}) = 0,473\,8 \text{ abs}$	absorbance of standard at λ_1	
$V_D = 5\,000 \text{ µl}$	volume of diluent used to dilute the unknown sample	
$A_U = (2\,000 \text{ abs/cm})(1,000 \text{ cm})\left(\frac{0,5}{5\,000,5}\right) = 0,200 \text{ abs}$ absorbance of unknown at λ_1 when $V_U = 0,500 \text{ µl}$		

3.3.1 Uncertainty in reading of standards due to instrument imprecision

In all of the following examples, the formulae are followed by a numeric evaluation using the parameters given above for a 0,5 µl pipette.

The partial derivative of Equation (93) with respect to A_S is

$$\begin{aligned} \frac{\partial V_U}{\partial A_S} &= -\frac{V_D A_U R}{A_S^2} \\ &= -1,06 \text{ µl/abs} \end{aligned} \quad (92)$$

The sign is not relevant, since all sensitivity coefficients are squared to get the weighted variance.

The standard uncertainty given in the budget arises from two sources: when the photometer is zeroed, and when the reading is made at 0,50 abs. These two sources of uncertainty are combined using the square root of the sum of the squares.

$$U(A_S) = \sqrt{0,000\,3^2 + 0,000\,5^2} \text{ abs} = 0,000\,583 \text{ abs} \quad (93)$$

The standard uncertainty in volume measurement due to this uncertainty in A_S is

$$U(V_{0,5})_{A_S} = (1,05 \text{ µl/abs})\left(5,83 \times 10^{-4} \text{ abs}\right) = 6,15 \times 10^{-4} \text{ µl} \quad (94)$$

3.3.2 Uncertainty in reading unknown due to instrument imprecision

The partial derivative of A_U [Equation (90)] with respect to A_U is

$$\begin{aligned} \frac{\partial V_U}{\partial A_U} &= V_D \frac{R}{A_S} \\ &= 2,50 \text{ µl/abs} \end{aligned} \quad (95)$$

As with the uncertainty in A_S , the uncertainty in A_U is composed of two parts, one of them due to photometer noise at zero, and one due to photometer noise at A_U .

$$U(A_U) = \sqrt{0,000\ 3^2 + 0,000\ 5^2} \text{ abs} = 0,000\ 583 \text{ abs} \quad (96)$$

The standard uncertainty in volume measurement due to this uncertainty in A_U is

$$U(V_{0,5})_{A_U} = (2,5 \mu\text{l/abs})(5,83 \times 10^{-4} \text{ abs}) = 1,46 \times 10^{-3} \mu\text{l} \quad (97)$$

3.3.3 Uncertainty in readings due to drift in wavelength of photometer between the time that the standard is read and the unknown is read

The magnitude of the uncertainty depends on the slope of the absorbance versus wavelength curve at the wavelength chosen for making the readings. For the chromophore chosen in this case at 520 nm, the slope is no greater than 0,1 % nm⁻¹. The reading of the standard is taken as the correct wavelength, and it is the change from that which creates the uncertainty.

$$\begin{aligned} \frac{\partial V_U}{\partial \lambda} &= \left(\frac{\partial V_U}{\partial A_U} \right) \left(\frac{\partial A_U}{\partial \lambda} \right) \\ &= \left(\frac{V_D R}{A_S} \right) (0,001) \mu\text{l/nm} \\ &= 2,50 \times 10^{-3} \mu\text{l/nm} \end{aligned} \quad (98)$$

The specification for the spectrophotometer is for a drift of no more than 0,2 nm. A rectangular probability is assumed, giving an uncertainty of

$$U(\lambda_1) = 0,2 \text{ nm} / \sqrt{3} = 0,115 \text{ nm} \quad (99)$$

The uncertainty in volume measurement due to wavelength drift is

$$U(V_{0,5})_{\lambda_1} = U(\lambda_1) \frac{\partial V_U}{\partial \lambda_1} = (0,115 \text{ nm})(2,50 \times 10^{-3} \mu\text{l/nm}) = 2,88 \times 10^{-4} \mu\text{l} \quad (100)$$

3.3.4 Uncertainty in the volume of stock used in creating the dilution 1

In this case there was a double dilution performed in making the standard. We first address the uncertainty in V_U due to uncertainty in the volume of stock used in creating the first dilution. An uncertainty in volume of stock results in an uncertainty in the ratio of dilution for the standard, which in turn results in an uncertainty in the determination of unknown volume. In the analysis below, R_1 is the ratio of dilution in the first mixture, and R_2 is the dilution of that mixture to create the final standard.

$$R = R_1 R_2 \quad (101)$$

$$R_1 = \frac{V_S}{V_{d1} + V_S} \approx \frac{V_S}{V_{d1}} \quad (102)$$

$$\frac{\partial R}{\partial V_S} = R_2 \frac{\partial R_1}{\partial V_S} = R_2 \frac{1}{V_{d1}} \quad (103)$$

$$\begin{aligned}\frac{\partial V_U}{\partial V_S} &= \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_S} \\ &= \left(V_D \frac{A_U}{A_S} \right) \left(\frac{R_2}{V_{d1}} \right) \\ &= 1 \times 10^{-4} \mu\text{l}/\mu\text{l}\end{aligned}\quad (104)$$

The volume of stock used in making the first dilution was 5 ml, and the uncertainty in it is:

$$U(V_S) = 15 \mu\text{l} / \sqrt{3} = 8,66 \mu\text{l} \quad (105)$$

and the standard uncertainty in the unknown volume due to uncertainty in the volume of stock is

$$U(V_{0,5})_{V_S} = U(V_S) \frac{\partial V_U}{\partial V_S} = (8,66 \mu\text{l}) (1,00 \times 10^{-4} \mu\text{l}/\mu\text{l}) = 8,66 \times 10^{-4} \mu\text{l} \quad (106)$$

3.3.5 Uncertainty in the volume of mixture 1 (V_{m1} , result of dilution 1), used in creating the second dilution

The analysis follows that given in 3.3.4.

$$R = R_1 R_2 \quad (107)$$

$$R_2 = \frac{V_{m1}}{V_{d2} + V_{m1}} \approx \frac{V_{m1}}{V_{d2}} \quad (108)$$

$$\frac{\partial R}{\partial V_{m1}} = R_1 \frac{\partial R_2}{\partial V_{m1}} = R_1 \frac{1}{V_{d2}} \quad (109)$$

$$\begin{aligned}\frac{\partial V_U}{\partial V_{m1}} &= \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{m1}} \\ &= \left(V_D \frac{A_U}{A_S} \right) \left(\frac{R_1}{V_{d2}} \right) \\ &= 1,05 \times 10^{-4} \mu\text{l}/\mu\text{l}\end{aligned}\quad (110)$$

The uncertainty in volume of the first mixture that goes into the second dilution is 8,66 μl , and the resulting uncertainty in unknown volume is:

$$U(V_{0,5})_{V_{m1}} = U(V_{m1}) \frac{\partial V_U}{\partial V_{m1}} = (8,66 \mu\text{l}) (1,05 \times 10^{-4} \mu\text{l}/\mu\text{l}) = 9,09 \times 10^{-4} \mu\text{l} \quad (111)$$

3.3.6 Uncertainty in volume of diluent used in creating the first dilution

$$\frac{\partial V_U}{\partial V_{d1}} = \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{d1}} \quad (112)$$

$$R = R_1 R_2 \quad (113)$$

$$\frac{\partial R}{\partial V_{d1}} = R_2 \frac{\partial R_1}{\partial V_{d1}} \quad (114)$$

$$R_1 = \frac{V_S}{V_S + V_{d1}} \approx \frac{V_S}{V_{d1}} \quad (115)$$

$$\frac{\partial R}{\partial V_{d1}} = -R_2 \frac{V_S}{(V_{d1})^2} \quad (116)$$

$$\begin{aligned} \frac{\partial V_U}{\partial V_{d1}} &= \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{d1}} \\ &= \left(V_D \frac{A_U}{A_S} \right) \left[\frac{-R_2 V_S}{(V_{d1})^2} \right] \\ &= \left(V_D \frac{A_U}{A_S} \right) \left[\frac{V_{m1} V_S}{V_{d2} (V_{d1})^2} \right] \\ &= -5,28 \times 10^{-7} \mu\text{l}/\mu\text{l} \end{aligned} \quad (117)$$

The standard uncertainty in volume delivered by a 1 000 ml Class A volumetric flask is

$$U(V_{d1}) = 400 \mu\text{l} / \sqrt{3} = 231 \mu\text{l} \quad (118)$$

and the uncertainty in unknown volume is

$$U(V_{0,5})_{V_{d1}} = U(V_{d1}) \frac{\partial V_U}{\partial V_{d1}} = (231 \mu\text{l}) (5,28 \times 10^{-7} \mu\text{l}/\mu\text{l}) = 1,22 \times 10^{-4} \mu\text{l} \quad (119)$$

3.3.7 Uncertainty in the volume of diluent used in the second dilution

This analysis follows that given in 3.3.6:

$$\frac{\partial V_U}{\partial V_{d2}} = \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{d2}} \quad (120)$$

$$R = R_1 R_2 \quad (121)$$

$$\frac{\partial R}{\partial V_{d2}} = R_1 \frac{\partial R_2}{\partial V_{d2}} \quad (122)$$

$$R_2 = \frac{V_{m1}}{V_{m1} + V_{d2}} \approx \frac{V_{m1}}{V_{d2}} \quad (123)$$

$$\frac{\partial R}{\partial V_{d2}} = -R_1 \frac{V_{m1}}{(V_{d2})^2} \quad (124)$$

$$\begin{aligned}
 \frac{\partial V_U}{\partial V_{d2}} &= \frac{\partial V_U}{\partial R} \frac{\partial R}{\partial V_{d2}} \\
 &= \left(V_D \frac{A_U}{A_S} \right) \left[\frac{-R_1 V_{m1}}{(V_{d2})^2} \right] \\
 &= \left(V_D \frac{A_U}{A_S} \right) \left[\frac{V_{m1} V_S}{V_{d1} (V_{d2})^2} \right] \\
 &= -5,28 \times 10^{-6} \mu\text{l}/\mu\text{l}
 \end{aligned}
 \tag{125}$$

The standard uncertainty in volume delivered by a 100 ml Class A volumetric pipette is

$$U(V_{d2}) = 80 \mu\text{l} / \sqrt{3} = 46,2 \mu\text{l} \tag{126}$$

and the uncertainty in unknown volume is

$$U(V_{0,5})_{V_{d2}} = U(V_{d2}) \frac{\partial V_U}{\partial V_{d2}} = (46,2 \mu\text{l}) (5,28 \times 10^{-6} \mu\text{l}/\mu\text{l}) = 2,44 \times 10^{-4} \mu\text{l} \tag{127}$$

3.3.8 Uncertainty in the volume of diluent used in the unknowns

$$\begin{aligned}
 \frac{\partial V_U}{\partial V_D} &= \frac{A_U}{A_S} R \\
 &= 1,00 \times 10^{-4} \mu\text{l}/\mu\text{l}
 \end{aligned}
 \tag{128}$$

The standard uncertainty in volume delivered by a 5 ml Class A volumetric pipette is

$$U(V_D) = 15 \mu\text{l} / \sqrt{3} = 8,66 \mu\text{l} \tag{129}$$

and the uncertainty in unknown volume is

$$U(V_{0,5})_{V_D} = U(V_D) \frac{\partial V_U}{\partial V_D} = (8,66 \mu\text{l}) (1,00 \times 10^{-4} \mu\text{l}/\mu\text{l}) = 8,66 \times 10^{-4} \mu\text{l} \tag{130}$$

3.3.9 Uncertainty in temperature of standard versus unknowns

If the unknown samples are at a different temperature than that at which the standard was when it was read in the photometer, then the temperature dependence of the absorptivity of the chromophore will cause an error in the results (the temperature coefficient of absorptivity, K_T , for this chromophore in this diluent is $0,05 \text{ \%} \cdot \text{°C}^{-1}$). The method specifies that the temperature of the unknowns and control shall be the same within $0,2 \text{ °C}$. The sensitivity of results to this temperature difference is given by the partial derivative of V_U with respect to the temperature of the unknown, assuming that the temperature of the standard is by definition correct:

$$\frac{\partial V_U}{\partial T} = \frac{\partial V_U}{\partial A_U} \frac{\partial A_U}{\partial T} \tag{131}$$

$$A_U(T) = A_U [1 - K_T (T - T_S)] \tag{132}$$

$$\begin{aligned}\frac{\partial V_U}{\partial T} &= \left(V_D \frac{R}{A_S} \right) (A_U K_T) \\ &= 2,50 \times 10^{-4} \mu\text{l}/^\circ\text{C}\end{aligned}\quad (133)$$

The uncertainty in temperature arises from two measurements, one of the standard and one of the unknown. Each is uncertain by 0,2 °C. The uncertainty in the difference will then be the $\sqrt{2}$ times 0,2 °C or 0,28 °C.

Divide by $\sqrt{3}$ to obtain the standard uncertainty of the rectangular distribution. The resulting uncertainty in unknown volume is:

$$U(V_{0,5})_T = U(T) \frac{\partial V_U}{\partial T} = (0,28/\sqrt{3} \text{ }^\circ\text{C}) (2,50 \times 10^{-4} \mu\text{l}/^\circ\text{C}) = 4,04 \times 10^{-5} \mu\text{l} \quad (134)$$

3.3.10 Uncertainty due to incomplete mixing of standard

If the standard is incompletely mixed, then its measured absorbance at λ_1 will be in error, leading to an error in the calculation of V_U . The parameter M_S is defined as the fractional error in the reading of the standard's absorbance:

$$\begin{aligned}M_S &= \frac{A_S(O) - A_S(I)}{A_S(I)} \\ A_S(O) &= A_S(I)(M_S + 1)\end{aligned}\quad (135)$$

where $A_S(I)$ is the absorbance of a perfectly mixed standard, and $A_{S1}(O)$ is the observed absorbance.

Using this definition,

$$\begin{aligned}\frac{\partial V_U}{\partial M_S} &= \frac{\partial V_U}{\partial A_S} \frac{\partial A_S}{\partial M_S} \\ &= - \left(\frac{V_D R A_U}{A_S^2} \right) \frac{\partial A_S}{\partial M_S} \\ &= - \left(\frac{V_D R A_U}{A_S^2} \right) A_S(I) \\ &= -(1,054 \mu\text{l}/\text{abs})(0,474 \text{ abs}) \\ &= -0,500 \mu\text{l}\end{aligned}\quad (136)$$

The mixing process, 10 inversions, assures an uncertainty of less than 0,1 % in the absorbance of the standard, giving an uncertainty in the parameter M_S of:

$$U(M_S) = 0,001 \text{ abs}/\text{abs} \quad (137)$$

The corresponding uncertainty in unknown volume is

$$U(V_{0,5})_{M_S} = U(M_S) \frac{\partial V_U}{\partial M_S} = (0,001 \text{ abs}/\text{abs})(0,500 \mu\text{l}) = 5,00 \times 10^{-4} \mu\text{l} \quad (138)$$

3.3.11 Uncertainty in unknown volume V_U due to incomplete mixing of unknowns

If the unknowns are incompletely mixed, then their measured absorbance at λ_1 will be in error, leading to an error in the calculation of V_U . Following the explanation given in 3.3.10,

$$M_U = \frac{A_U(O) - A_U(I)}{A_U(I)} \quad (139)$$

$$A_U(O) = A_U(I)(M_U + 1)$$

Using this definition,

$$\begin{aligned} \frac{\partial V_U}{\partial M_U} &= \frac{\partial V_U}{\partial A_U} \frac{\partial A_U}{\partial M_U} \\ &= \left(\frac{V_D R}{A_S} \mu\text{l/abs} \right) \frac{\partial A_U}{\partial M_U} \\ &= \left(\frac{V_D R}{A_S} \right) A_U(I) \\ &= (2,50 \mu\text{l/abs})(0,20 \text{ abs}) \\ &= 0,50 \mu\text{l} \end{aligned} \quad (140)$$

Again the mixing procedure of 10 inversions gives an uncertainty in absorbance of less than 0,1 %:

$$U(M_U) = 0,001 \text{ abs/abs} \quad (141)$$

The corresponding uncertainty in unknown volume is

$$U(V_{0,5})_{M_U} = U(M_U) \frac{\partial V_U}{\partial M_U} = (0,001 \text{ abs/abs})(0,50 \mu\text{l}) = 5,00 \times 10^{-4} \mu\text{l} \quad (142)$$

3.3.12 Uncertainty due to pH dependence of the chromophore used in the stock solution

If the unknown samples have a different pH than the standard, then a pH dependence of the absorptivity of the chromophore will cause an error in the results. The sensitivity of results to this pH difference is given by the partial derivative of V_U with respect to the pH of the unknown, assuming that the pH of the standard is by definition correct.

$$\begin{aligned} \frac{\partial V_U}{\partial(\text{pH})} &= \frac{\partial V_U}{\partial A_U} \frac{\partial A_U}{\partial(\text{pH})} \\ &= \left(\frac{V_D R}{A_S} \right) \frac{\partial A_U}{\partial(\text{pH})} \\ &= \left(\frac{V_D R}{A_S} \right) [0,01 \text{ abs}/(\text{abs} \cdot \text{pH})] (0,2 \text{ abs}) \\ &= (2,50 \mu\text{l/abs}) [0,01 \text{ abs}/(\text{abs} \cdot \text{pH})] (0,2 \text{ abs}) \\ &= 5,00 \times 10^{-3} \mu\text{l/pH} \end{aligned} \quad (143)$$

The pH dependence of the extinction coefficient of the chromophore (in this buffer at pH 6) is 1 % per pH unit, and the buffer is stable within $\pm 0,1$ pH units.

The corresponding uncertainty in unknown volume is

$$U(V_{0,5})_{\text{pH}} = U(\text{pH}) \frac{\partial V_{\text{U}}}{\partial(\text{pH})} = (5,00 \times 10^{-3} \mu\text{l/pH})(0,1 \text{ pH}) = 5,0 \times 10^{-4} \mu\text{l} \quad (144)$$

3.3.13 Uncertainty in unknown volume V_{U} due to system non-linearity

The response of the complete system shall be characterized to determine the linearity of response at the wavelength λ_1 . The parameter L is defined as the fractional error in the reading of the unknown's absorbance A_{U} due to a lack of linearity:

$$L = \frac{A_{\text{U}}(O) - A_{\text{U}}(I)}{A_{\text{U}}(I)} \quad (145)$$

$$A_{\text{U}}(O) = A_{\text{U}}(I)(L + 1)$$

Using this definition,

$$\begin{aligned} \frac{\partial V_{\text{U}}}{\partial L} &= \frac{\partial V_{\text{U}}}{\partial A_{\text{U}}} \frac{\partial A_{\text{U}}}{\partial L} \\ &= \left(\frac{V_{\text{D}}R}{A_{\text{S}}} \mu\text{l/abs} \right) \frac{\partial A_{\text{U}}}{\partial L} \\ &= \left(\frac{V_{\text{D}}R}{A_{\text{S}}} \right) A_{\text{U}}(I) \\ &= (2,50 \mu\text{l/abs})(0,20 \text{ abs}) \\ &= 0,50 \mu\text{l/(abs/abs)} \end{aligned} \quad (146)$$

In the case of a dynamic range of 2, the measured non-linearity was 0,14 %.

$$U(L) = 0,0014 \text{ abs/abs} \quad (147)$$

The corresponding uncertainty in unknown volume is

$$U(V_{0,5})_L = U(L) \frac{\partial V_{\text{U}}}{\partial L} = (0,0014 \text{ abs/abs})[0,50 \mu\text{l/(abs/abs)}] = 7,00 \times 10^{-4} \mu\text{l} \quad (148)$$

3.3.14 Uncertainties too small for consideration in the uncertainty budget

There are several sources of uncertainty which are not large enough to warrant inclusion in the uncertainty budget. Among them are the following.

3.3.14.1 Uncertainty due to the temperature coefficient of the Class A glassware

Class A glassware has a temperature coefficient of approximately $0,0025 \text{ \%} \cdot \text{°C}^{-1}$. If the unknown vials are filled with diluent while at a temperature of 24 °C , an uncertainty of $0,01 \text{ \%}$ will occur in the results. This is much smaller than other sources of uncertainty.

3.3.14.2 Degradation of the chromophore

If the stock solutions degrade while in storage, this will not affect the results because of the ratiometric nature of the method, as long as standards are freshly prepared.

3.4 Combined standard uncertainty

A detailed uncertainty budget for the calibration of a 0,5 µl pipette using this photometric method with a flow cell in a reference grade UV/VIS spectrophotometer is given in Table 5.

Table 5 — Uncertainty in the calibration of a 0,5 µl pipette using the flow cell method

Clause	Contribution to uncertainty	Probability distribution	Type of uncertainty	Range of value ±	Standard uncertainty	Unit	Sensitivity coefficient	Unit	Standard uncertainty µl
Instrument-related uncertainties									
3.3.1	Imprecision in reading absorbance of standard (A_S)	normal	A		$5,83 \times 10^{-4}$	abs	1,055	µl/abs	$6,15 \times 10^{-4}$
3.3.2	Imprecision in reading absorbance of unknown (A_U)	normal	A		$5,83 \times 10^{-4}$	abs	2,50	µl/abs	$1,46 \times 10^{-3}$
3.3.3	Drift in wavelength between reading standard and unknown	rectangle	B	0,2	$1,15 \times 10^{-1}$	nm	$2,50 \times 10^{-3}$	µl/nm	$2,88 \times 10^{-4}$
Reagent-related uncertainties									
3.3.4	Volume of stock used in creating dilution 1 (V_S)	rectangle	B	15	8,66	µl	$1,00 \times 10^{-4}$	µl/µl	$8,66 \times 10^{-4}$
3.3.5	Volume of mixture 1 used in creating dilution 2 (V_{m1})	rectangle	B	15	8,66	µl	$1,05 \times 10^{-4}$	µl/µl	$9,09 \times 10^{-4}$
3.3.6	Volume of diluent used in creating dilution 1 (V_{d1})	rectangle	B	400	$2,31 \times 10^2$	µl	$5,28 \times 10^{-7}$	µl/µl	$1,22 \times 10^{-4}$
3.3.7	Volume of diluent used in creating dilution 2 (V_{d2})	rectangle	B	80	$4,62 \times 10^1$	µl	$5,28 \times 10^{-6}$	µl/µl	$2,44 \times 10^{-4}$
3.3.8	Volume of diluent used in unknowns (V_D)	rectangle	B	15	8,66	µl	$1,00 \times 10^{-4}$	µl/µl	$8,66 \times 10^{-4}$
3.3.9	Temperature of unknowns versus standards	rectangle	B	0,28	$1,617 \times 10^{-1}$	°C	$2,50 \times 10^{-4}$	µl/°C	$4,04 \times 10^{-5}$
3.3.10	Incomplete mixing of standard	normal	A		$1,00 \times 10^{-3}$	abs/abs	$5,00 \times 10^{-1}$	(µl/abs)/abs	$5,00 \times 10^{-4}$
3.3.11	Incomplete mixing of unknowns	normal	A		$1,00 \times 10^{-3}$	abs/abs	$5,00 \times 10^{-1}$	(µl/abs)/abs	$5,00 \times 10^{-4}$
3.3.12	pH dependence of the chromophore	normal	A		$1,00 \times 10^{-1}$	pH	$5,00 \times 10^{-3}$	µl/pH	$5,00 \times 10^{-4}$
System-related uncertainties									
3.3.13	System non-linearity	normal	A		$1,40 \times 10^{-3}$	abs/abs	$5,00 \times 10^{-1}$	(µl/abs)/abs	$7,00 \times 10^{-4}$
Combined standard uncertainty									
	Absolute							µl	$2,50 \times 10^{-3}$
	Relative							%	0,50

4 Traceability of the method

Because of the ratiometric nature of this method, traceability is entirely through the Class A volumetric pipettes. They can be purchased from several suppliers with certificates of individual traceability, to tolerances that are adequate for this purpose. When used according to the manufacturer's directions they are suitable for use as transfer standards within the uncertainties specified by the manufacturer.

For this method neither the dye material nor the UV/VIS photometer's accuracy or wavelength need be traceable, since the final result depends only on the linearity of response of the combination. Linearity of response is established by using volumetric pipettes to make a series of dilutions that are tested with the UV/VIS photometer at the wavelength setting to be used for the measurements of the unknowns. Thus, the overall performance of the combined system is verified without reference to standards for the particular UV/VIS photometer, wavelength setting, dye lot, diluent, etc.

5 Validation of photometric method

5.1 Principle of validation

To validate this photometric method, a comparison of results using this method can be made to a gravimetric method.

Since the uncertainty in delivery volume that is inherent in air displacement pipettes is dependent on many factors^[3] ^[4], the validation method is designed to make a comparison of results without relying on the accuracy or precision of the liquid delivery device being used to make the transfer. Further details of this validation method and results are given in reference [5].

5.2 Equipment for validation

The method of comparison is to put a small (2 ml) aliquot of sample solution on the pan of a suitably sensitive balance (in this case the readability of the balance was 1 µg). A humidity shield should be placed over the pan area to minimize evaporation from the aliquot container (~ 5 nl per sample delivery). The pipette being tested is used to aspirate sample from the container and deliver it to the sample vial, where the tip is washed with diluent to assure complete delivery of sample. Thus, for each individual delivery, the amount taken from the balance as measured gravimetrically can be compared to the amount delivered to the vial as determined photometrically. The loss to evaporation (about 0,5 % at a 1 µl sample size) shall be estimated and applied to correct the gravimetric determination. Uncertainty in evaporation (standard deviation ≈ 4 nl) is generally the leading source of uncertainty in the gravimetric determination at small volumes. Comparison of a photometric result to the corresponding gravimetric result is made for each delivery on a one-for-one basis; thus the amount of the delivery is not of importance and the pipette is not being used as a transfer standard.

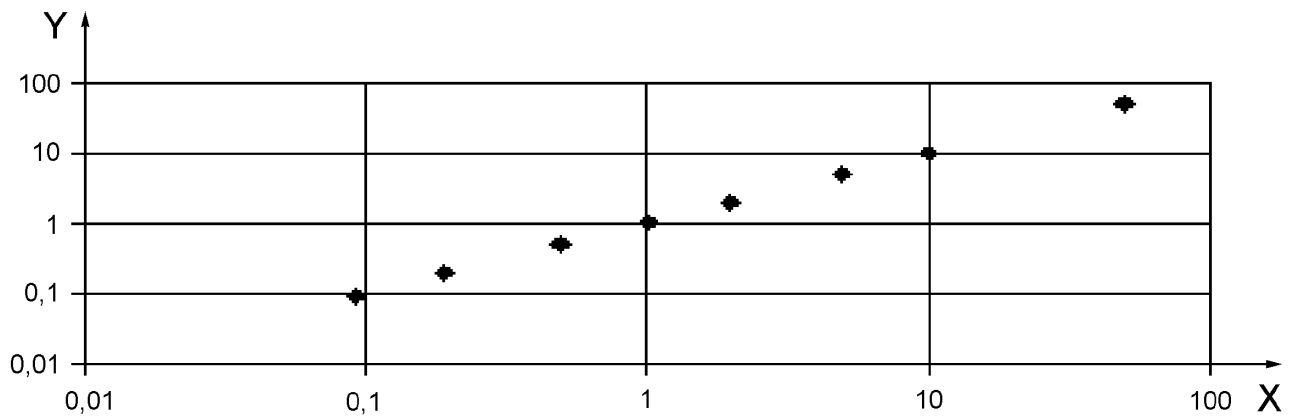
5.3 Density of solutions

In order to make the gravimetric determination, it is necessary to know the densities of the sample solutions and diluent versus degassed deionized water. These can be determined by use of a pycnometer and balance or with an oscillation-type density meter. Weighing values shall be corrected for air buoyancy and temperature.

5.4 Experimental results

The following results were obtained when using this validation method to calibrate pipettes ranging from 0,1 µl to 50 µl in size.

Figure 1 and Table 6 show the relationship between the gravimetric measurements and photometric measurements for eight different volumes between 0,1 µl and 50 µl. The data demonstrate correlation between the methods well within the estimated uncertainties. A linear regression yields $R^2 = 0,999\ 999$ and $y = 0,999x + 0,004$.

**Key**

X gravimetric results, μl

Y photometric results, μl

Figure 1 — Relationship between the gravimetric measurements and photometric measurements**Table 6 — Comparison of photometric and gravimetric results**

Nominal volume μl	Number of data points	Gravimetric results		Photometric results		Differences between results		
		Mean volume μl	CV %	Mean volume μl	CV %	Difference in means μl	Difference in means %	CV of difference %
0,1	20	0,092 8	13,23	0,091 3	13,53	-0,001 5	1,57	2,03
0,2	20	0,191 4	8,11	0,190 7	7,81	-0,000 7	0,37	1,38
0,5	20	0,491	1,86	0,49	1,66	-0,000 5	-0,11	1,12
1	18	1,019	1,17	1,021	1,05	0,002	0,19	0,67
2	20	1,969	0,58	1,968	0,55	-0,001	-0,07	0,08
5	20	4,923	0,29	4,922	0,27	-0,002	-0,04	0,11
10	10	10,025	0,4	10,05	0,38	0,025	0,25	0,13
50	10	49,75	0,19	49,71	0,27	-0,037	-0,08	0,18

CV = coefficient of variation

5.5 Additional validation studies of the photometric method

There have been several published studies (see references [6] and [7]) comparing the photometric method of POVA volume measurement to the gravimetric method; in all cases the results of the two methods agreed within the calculated uncertainties of the methods.

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