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DNA quantification via nanodrop spectrophotometer: estimation of method uncertainty using DNA from standard reference materials, sprague dawley rats, and humans

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Abstract: Although the uncertainty calculation proposed by ISO was initially applied to physical quantities, it now also applies to chemical measurements. Therefore, uncertainty estimation contributes to the reliability of the results obtained in analytical chemical measurements, among other parameters. This work seeks to estimate the uncertainty of the analytical method for DNA quantification through a Nanodrop spectrophotometer, using DNA from certified reference materials (NIST 2372), Sprague Dawley rats, and humans. For these purposes, the sources of uncertainty were established and evaluated. Some of these sources are concentration uncertainty from the calibration curves, volumetric materials, dilution factors, analytical balance, repeatability, and reproducibility, as well as DNA concentrations used. The results obtained indicate that the expanded uncertainty was 1.189, 1.360, and 1.944 ng/µL of DNA for the reference material (NIST 2372), Sprague Dawley rats, and humans, thus representing 2.08%, 2.34%, and 2.12%, respectively, for the DNA concentrations from each source (57.0, 57.9, and 91.5 ng/µL DNA, respectively). The uncertainty source that contributes most to these calculations is the dilution factor uncertainty, although it should be noted that the dilution factor uncertainty also considers the volumetric material uncertainty, as well as the fact that five dilutions were used for the calibration curves. Hence, these results may be overestimated.

Keywords: DNA; nanodrop spectrophotometer; uncertainty.

1. Introduction

According to the guide for the expression of measurement uncertainty, it is defined as a "parameter associated with the result of a measurement, which characterizes the dispersion of the values that could reasonably be attributed to the measurand." In this sense, the associated parameters can be the standard

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deviation measured, the statistical distribution of the results, or information obtained from reference materials, among others [1]. On many occasions, in chemical analysis, the measurand is the concentration of the analyte or quantity subject to measurement, which includes the source of uncertainty that may contribute to possible errors during the measurement [2,3].

The uncertainty estimation contributes to the reliability of the results obtained after validating the analytical method used [4].

Gradually, in the published research works related to the measurement of measurement methods with DNA and/or molecular biology methods, uncertainty estimation has been included to give reliability to the results obtained [5]. Therefore, it is important to use certified reference materials to guarantee and compare the results obtained with DNA samples extracted and purified via the research laboratory itself [6]. Accurate quantification of the copy number of specific nucleic acid sequences is important for various applications both within the fields of red biotechnology (e.g., oncology and infectious diseases) and green biotechnology (e.g., testing in Genetically Modified Organisms) [7,8]. As sequencing becomes more routine in the clinic, it is important to consider the accuracy of these data and the validity of conclusions based on analytical DNA measurements [9,10]. In this regard, some work has been done evaluating the analytical uncertainty for DNA measurements by fluorescence in DNA microarray tests [11] or during PCR for physicochemical parameter measurements of the FRET (Förster Resonance Energy Transfer) system [12-14]. It is important to mention that an uncertainty principle has been established in genetics [15], and the analogy with Heisenberg's uncertainty principle in physics is discussed. The genetic information that drives living cells to function is better represented by a probabilistic model than as a fully defined object, the probabilistic model is related to the estimation of its uncertainty [16]. On the other hand, research has also been conducted to determine the analytical uncertainty in allelic DNA variants quantification in forensic genetics to avoid errors in obtaining genetic fingerprint profiles [17].

Additionally, research has been conducted to determine uncertainty in invasive aquatic organism measurements by analyzing methods for target DNA compared with DNA from native aquatic species [18-21].

Recently, our research group performed methodological validation for the quantification of DNA measured through a Nanodrop spectrophotometer using DNA from a certified reference material (NIST 2372), Sprague Dawley rats, and humans [22]. Complementary to our previous work, this research study seeks to estimate the expanded uncertainty of the newly validated method.

2. Experimental

2.1. Materials and Methods

The original methodology for DNA quantification using a Nanodrop spectrophotometer, related to this research work, can be found in García-Alegría et al. (2020) [22].

2.2. Sources of Uncertainty

The sources of uncertainty were established using the Ishikawa diagram (Figure 1), including the DNA concentration from the calibration curve, the volumetric material used, dilution factors, analytical balance, measurement repeatability, and the reference material used [23,24].

In this regard, our team explores the estimation processes of the analytical uncertainty of other measurands, such as glucose [25]. The mathematical models used to evaluate uncertainty are similar, changing only the properties of the measurand and the values obtained from the sources of uncertainty, in this paper, the measurand is the DNA. The mathematical models used to estimate analytical uncertainty are those proposed by international guidelines [1-4], which are adapted for any particular measurand.

2.3. Uncertainty of DNA Concentration from Calibration Curve

The standard uncertainty of DNA mass concentration by instrument was calculated by:

$$u_{\gamma_{CC}} = \frac{s}{b_1}\sqrt{\frac{1}{\rho} + \frac{1}{n} + \frac{\left(\gamma_{(x)i} - \bar{y}_{MR}\right)^2}{S_{xx}}} \quad (1)$$

where:

u_{γ_x} = uncertainty of the measurand (DNA) obtained by the calibrated instrument.
s = residual standard deviation from the linear regression calculation.
b_1 = calculated slope.
ρ = number of replicates of the study sample.
n = number of solutions (i) used in the calibration curve multiplied by the number of replicates (j) of each solution (total data) (i*j).
γ_x = concentration of DNA under study (ng/µL).
$\bar{y}_{MR}$ = average of the mass concentrations of the solutions used in the calibration curve.
S_{xx} = sum of squares of the residuals of the concentrations obtained.

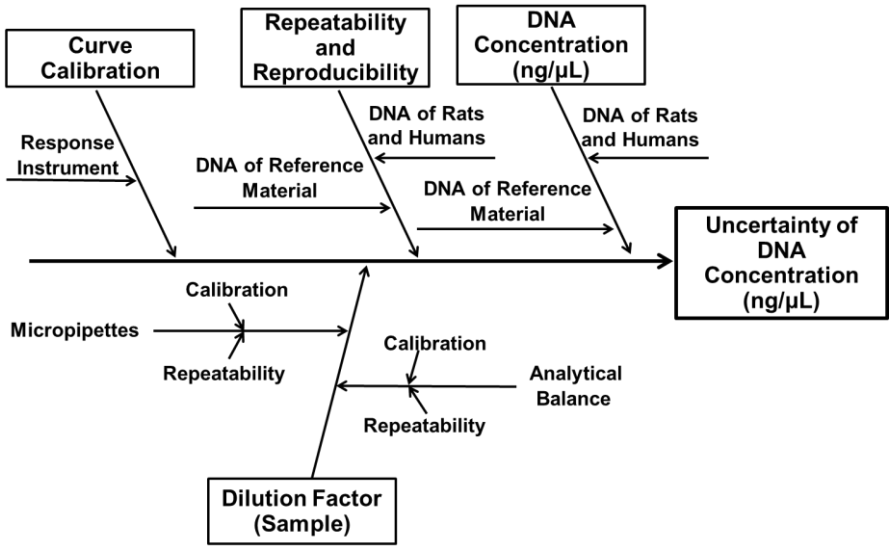

Figure 1. Ishikawa diagram with the sources of uncertainty evaluated.

The relative standard uncertainty for the DNA concentration obtained from the calibration curve produced by the instrument is determined by:

$$u_{r\gamma_{CC}} = \sqrt{\left(\frac{u_{\gamma_x}}{\gamma_x}\right)^2} \quad (2)$$

2.4. Volumetric Material Uncertainty

Regarding the estimation of the measurement uncertainty associated with the measurement of the aliquot volume (V_1) and gauging volume (V_2) in any volumetric material, there are three main sources of uncertainty [26]:

2.4.1. Volumetric Material Tolerance

$$u_{tol} = \frac{tol}{\sqrt{3}} \quad (3)$$

2.4.2. The Effect of the Variability or Repeatability of the Volume Measured by the Analyst

$$u_R = \frac{s_V}{\sqrt{n}} \quad (4)$$

2.4.3. The effect of temperature variation in relation to the calibration temperature:

$$u_{\Delta T} = \frac{[(T - T_{20}) \cdot \alpha \cdot V]}{\sqrt{3}} \quad \text{.....(5)}$$

where:
T = water temperature °C at the time of measurement.
T_{20} = volumetric material, calibration temperature: 20°C.
α = water volume expansion coefficient $°C^{-1}$.
V = volume of the pipette or flask.

2.5. Combined Uncertainty of Volumetric Material

The three uncertainties are combined, and their value is calculated using the following equation:

$$u_{comb\ vol\ mat} = \sqrt{(tol)^2 + (Rep)^2 + (\Delta T)^2} \quad (6)$$

While the relative uncertainty for the volumetric material is determined from the following equation:

$$u_{r_{comb\ vol\ mat}} = \sqrt{\left(\frac{u_{comb\ vol\ mat}}{vol}\right)^2} \quad (7)$$

2.6. Combined Standard Uncertainty for the Three Types of Volumetric Material

It is obtained from the following equation:

$$u_{comb\ vol\ mat} = \sqrt{(mat\ vol\ 1)^2 + (mat\ vol\ 2)^2 + (mat\ vol\ 3)^2} \quad (8)$$

The combined relative uncertainty for the three types of volumetric material used is obtained through the following equation:

$$u_{r_{comb\ vol\ mat}} = \sqrt{\left(\frac{u_{comb\ vol\ mat1}}{V_1}\right)^2 + \left(\frac{u_{comb\ vol\ mat2}}{V_2}\right)^2 + \left(\frac{u_{comb\ vol\ mat3}}{V_3}\right)^2} \quad (9)$$

2.7. Dilution Factor Uncertainty

Calculation of the standard uncertainty due to the dilution factors:

$$u_{fd_n=}\sqrt{\left(u_{V_1}\right)^2+\left(u_{V_2}\right)^2} \quad (10)$$

Calculation of the relative standard uncertainty due to dilution factors:

$$u_{rfd_n=}=\sqrt{\left(\frac{u_{V_1}}{V_1}\right)^2+\left(\frac{u_{V_2}}{V_2}\right)^2} \quad (11)$$

The dilution factor is given by:

$$fd_n=\frac{V_2}{V_1} \quad (12)$$

where:
fd_n = dilution factor n.
V_2 = volume capacity.
V_1 = aliquot volume.
The dilution factor uncertainty is calculated from the following equation:

$$u_{fd_n}=\sqrt{\left(u_{fd_1}\right)^2+\left(u_{fd_2}\right)^2+\left(u_{fd_3}\right)^2+\left(u_{fd_4}\right)^2+\left(u_{fd_5}\right)^2} \quad (13)$$

The relative dilution factor uncertainty is determined from the following equation:

$$u_{rfd_n}=\sqrt{\left(\frac{u_{fd_1}}{fd_1}\right)^2+\left(\frac{u_{fd_2}}{fd_2}\right)^2+\left(\frac{u_{fd_3}}{fd_3}\right)^2+\left(\frac{u_{fd_4}}{fd_4}\right)^2+\left(\frac{u_{fd_5}}{fd_5}\right)^2} \quad (14)$$

2.8. Repeatability Uncertainty of Analytical Balance

$$u_{balance}=\frac{s_{balance}}{\sqrt{n}} \quad (15)$$

Where *s* is the standard deviation and *n* is the number of replicates used.
The relative repeatability uncertainty of the analytical balance is determined by the following equation:

$$u_{r_{balance}}=\frac{u_{balance}}{m} \quad (16)$$

Where *m* is the standard mass (1 mg) used to verify the calibration of the analytical balance.

2.9. Repeatability and Reproducibility Uncertainty of DNA Measurements

According to the Uncertainty Guide [27,28], the uncertainty related to repeatability (*repea*) and reproducibility (*repro*) of DNA measurements must be included, and it is calculated according to the following equation:

$$u_{\gamma_{repea}}=\sqrt{\left(\frac{s_{\gamma repea}}{\sqrt{n}}\right)^2} \quad (17)$$

For the reproducibility uncertainty, "*repea"* is replaced by "*repro"* in the same equation. It is important to note that the precision under repeatability and reproducibility conditions for DNA was determined for the 7.6, 10.0, and 10.0 ng/µL DNA concentrations from certified reference material (NIST 2372), Sprague Dawley rats, and humans, respectively. Here, *s* represents the standard deviation and *n* = 20.

Meanwhile, the relative standard uncertainty for repeatability and reproducibility is calculated using the following equation:

$$u_{r_{repea}} = \sqrt{\left(\frac{u_{\gamma repea}}{y_x}\right)^2} \quad (18)$$

For the relative reproducibility uncertainty, "*repea"* is replaced by "*repro"* in the same equation.

2.10. Uncertainty of DNA concentration

a*) DNA of the reference material (NIST 2372)*

The reference material insert indicates that the DNA concentration is 57 ± 0.060 ng/µL. The value of ±0.060 represents the expanded uncertainty, which is why the expanded uncertainty (*U*) must be divided by the coverage factor ($k = 2$) to obtain the standard uncertainty, as per the following equation:

$$u_{NIST} = U_{exp}/k \quad (19)$$

Once the standard uncertainty is obtained, it is divided by $\sqrt{3}$, thus assuming a rectangular distribution, to obtain the estimate of the actual standard uncertainty of the reference material [20], as indicated in the following equation:

$$u_{\gamma_{NIST}} = \frac{U_{exp}/k}{\sqrt{3}} \quad (20)$$

As for the relative standard uncertainty for the reference material, it is obtained from the following equation:

$$u_{r\gamma_{NIST}} = \sqrt{\left(\frac{u_{\gamma_{NIST}}}{y\ NIST}\right)^2} \quad (21)$$

b) DNA from Sprague Dawley rats

$$u_{\gamma_{rats\ S-D}} = \frac{s}{\sqrt{n}} \quad (22)$$

s is the standard deviation and n is the number of replicates (n = 5).
While the relative standard uncertainty is obtained with the following equation:

$$u_{r_{rats\ S-D}} = \sqrt{\left(\frac{u_{\gamma_{rats\ S-D}}}{y\ rats\ S-D}\right)^2} \quad (23)$$

c) Human DNA

$$u_{\gamma_{human}} = \frac{s}{\sqrt{n}} \quad (24)$$

$$u_{r_{human}} = \sqrt{\left(\frac{u_{human}}{y\ human}\right)^2} \quad (25)$$

s is the standard deviation and n is the number of replicates (n = 5).

2.11. Combined Standard Uncertainty (CSU)

The sources of uncertainty considered in this study are combined in quadratic form by means of the following equation:

$$u_{c\gamma_{ADN}} = \sqrt{\left(u_{\gamma_{CC}}\right)^2 + (u_{volmat})^2 + \left(u_{fd_n}\right)^2 + (u_{balance})^2 + \left(u_{\gamma_{repea}}\right)^2 + \left(u_{\gamma_{repro}}\right)^2 \left(u_{\gamma_{ADN}}\right)^2} \quad (26)$$

2.12. Combined Relative Standard Uncertainty (CRSU)

Once the CSU is obtained, it is divided by the DNA concentration to obtain the combined relative uncertainty (CRSU), for which the following equation is used:

$$u_{rc\gamma_{ADN}} = \sqrt{\left(\frac{u_{C\gamma\ ADN}}{c\gamma_{ADN}}\right)^2} \quad (27)$$

On the other hand, the percentage of the combined relative uncertainty is determined by the following equation:

$$\%\ u_{rc\gamma_{ADN}} = u_{rc\gamma_{ADN}}\ x\ 100 \quad (28)$$

2.13. Expanded Uncertainty (U)

The CSU obtained is multiplied by a coverage factor κ = 2 to obtain *U*, whose value is equivalent to a 95% confidence interval [29]. The following equation is used for this purpose:

$$U_{exp_{\mathrm{ADN}}} = u_{c\gamma_{ADN}}\ x\ 2 \quad (29)$$

2.14. Graphical Contribution of Uncertainty Sources

The relative uncertainties obtained for DNA concentration from calibration curves (NIST 2372, Sprague Dawley rats, and humans), volumetric materials, dilution factor, analytical balance, repeatability, reproducibility, and DNA concentrations (NIST 2372, Sprague Dawley rats, and humans) were plotted to assess the degree of graphical contribution from each of these sources of uncertainty.

3. Results and Discussion

3.1. Uncertainty of the Mass Concentration of the Elements Measured in the Instrument from the Calibration Curve

To obtain the standard uncertainty of the concentration obtained from the calibration curve, it is substituted in Equation 1. To obtain the value of the relative uncertainty of the concentration from the calibration curves, it is substituted in Equation 2:

a) DNA from certified reference material (NIST 2372)

$$u_{yx} = \frac{31.8934\ x\ 10^{-4}}{0.0199}\ \mathrm{x}\ \sqrt{\frac{1}{5}+\frac{1}{15}+\frac{(20.5760-20.5300)^2}{3291.5158}} = 0.0827$$

$$u_{ryx} = \sqrt{\frac{0.0827}{20.576}} = 0.0634$$

b) DNA from Sprague Dawley rats

$$u_{yx} = \frac{7.0359\ x\ 10^{-4}}{0.0200}\ \mathrm{x}\ \sqrt{\frac{1}{5}+\frac{1}{15}+\frac{(26.1100-26.1160)^2}{5097.4100}} = 0.0181$$

$$u_{ryx} = \sqrt{\frac{0.0181}{26.610}} = 0.0261$$

c) Human DNA

$$u_{yx} = \frac{10.5463x\ 10^{-4}}{0.0199} \text{ x } \sqrt{\frac{1}{5} + \frac{1}{15} + \frac{(25.7760 - 25.7680)^2}{5012.1661}} = 0.0273$$

$$u_{ryx} = \sqrt{\frac{0.0273}{25.7760}} = 0.0325$$

3.2. Uncertainty of Volumetric Materials

3.2.1 Volumetric Material Tolerance

Substituting in equation 3:

$$u_{tol} = \frac{0.025}{\sqrt{6}} = 0.0102 \text{ (for 1 µL)}$$

$$u_{tol} = \frac{0.3}{\sqrt{6}} = 0.1224 \text{ (for 20 µL)}$$

$$u_{tol} = \frac{0.5}{\sqrt{6}} = 0.2041 \text{ (for 60 µL)}$$

3.2.2 Effect of the Variability or Repeatability of the Volume Measured by the Analyst

Substituting in Equation 4:

$$u_R = \frac{0.0110\ x\ 10^{-2}}{\sqrt{6}} = 0.0049 \text{ x } 10^{-2} \text{ (for 1 µL)}$$

$$u_R = \frac{0.1348\ x\ 10^{-2}}{\sqrt{6}} = 0.0550 \text{ x } 10^{-2} \text{ (for 20 µL)}$$

$$u_R = \frac{0.0191\ x\ 10^{-2}}{\sqrt{6}} = 0.0007 \text{ x } 10^{-2} \text{ (for 60 µL)}$$

3.2.3 Effect of Temperature Variation Relative to the Calibration Temperature

Substituting in equation 5:

$$u_{\Delta T} = \frac{[(24-20) \text{ x } 0.0021\ x\ 10^{-1} \text{ x } 1]}{\sqrt{3}} = 0.0485 \text{ x } 10^{-2} \text{ (for 1 µL)}$$

$$u_{\Delta T} = \frac{[(24-20) \text{ x } 0.0021\ x\ 10^{-1} \text{ x } 20]}{\sqrt{3}} = 0.9699 \text{ x } 10^{-2} \text{ (for 20 µL)}$$

$$u_{\Delta T} = \frac{[(24-20) \text{ x } 0.0021\ x\ 10^{-1} \text{ x } 60]}{\sqrt{3}} = 2.9099 \text{ x } 10^{-2} \text{ (for 60 µL)}$$

3.3. Combined Volumetric Material Uncertainty

Substituting in equation 6:

$$u_{comb\ mat\ vol} = \sqrt{(0.0102)^2 + (0.0049\ x\ 10^{-2})^2 + (0.000485)^2} = 0.0102 \text{ (for 1 µL)}$$

$$u_{comb\ mat\ vol} = \sqrt{(0.1224)^2 + (0.0550\ x\ 10^{-2})^2 + (0.009699)^2} = 0.1228 \text{ (for 20 µL)}$$

$$u_{comb\ mat\ vol} = \sqrt{(0.2041)^2 + (0.0007\ x\ 10^{-2})^2 + (0.029099)^2} = 0.2061 \text{ (for 60 µL)}$$

The total combined uncertainty for the three types of volumetric material used is substituted into Equation 7:

$$u_{comb\ mat\ vol} = \sqrt{(0.0102)^2 + (0.1228)^2 + (0.2061)^2} = 0.2399$$

The relative uncertainty for the volumetric material is determined by substituting into Equation 8:

$$u_{r_{comb\ mat\ vol}} = \sqrt{\left(\frac{0.0102}{1}\right)^2} = 0.0120 \text{ (for 1 µL)}$$

$$u_{r_{comb\ mat\ vol}} = \sqrt{\left(\frac{0.1228}{20}\right)^2} = 0.0061 \text{ (for 20 µL)}$$

$$u_{r_{comb\ mat\ vol}} = \sqrt{\left(\frac{0.2061}{60}\right)^2} = 0.0586 \text{ (for 60 µL)}$$

The relative uncertainty for the three types of volumetric materials used, substituting into Equation 9:

$$u_{r_{comb\ mat\ vol}} = \sqrt{\left(\frac{0.0102}{1}\right)^2 + \left(\frac{0.1228}{20}\right)^2 + \left(\frac{0.2061}{60}\right)^2} = 0.0767$$

3.4. Dilution Factor Uncertainty

To obtain the standard dilution factor uncertainty (20:60 (1:3) = 3), it is substituted into Equation 10:

$$u_{fd_n} = \sqrt{(0.1228)^2 + (0.2061)^2} = 0.2399$$

To obtain the standard dilution factor uncertainties that were used to obtain the calibration curve, it is substituted into Equation 11:

$$u_{rfd_n} = \sqrt{\left(\frac{0.1228}{20}\right)^2 + \left(\frac{0.2061}{60}\right)^2} = 0.0070$$

To obtain the combined standard uncertainty of the dilution factors that were used to derive the calibration curve, substitute into Equation 13:

$$u_{fd_n} = \sqrt{(0.2399)^2 + (0.2399)^2 + (0.2399)^2 + (0.2399)^2 + (0.2399)^2} = 0.5363$$

To obtain the combined relative uncertainty of the dilution factors, substitute into Equation 14:

$$u_{rfd_n} = \sqrt{\left(\frac{1.8464}{3}\right)^2 + \left(\frac{1.8464}{3}\right)^2 + \left(\frac{1.8464}{3}\right)^2 + \left(\frac{1.8464}{3}\right)^2 + \left(\frac{1.8464}{3}\right)^2} = 1.8939$$

3.5. Analytical Balance Uncertainty

To obtain the standard repeatability uncertainty of the analytical balance with a standard mass of 1 mg, it is substituted into Equation 15:

$$u_{balance} = \frac{0.2291\ x\ 10^{-4}}{\sqrt{30}} = 4.1829 \text{ x } 10^{-6} \text{ mg}$$

To obtain the relative repeatability uncertainty of the analytical balance, it is substituted into Equation 16:

$$u_{r_{balance}} = \sqrt{\left(\frac{4.1829\ x\ 10^{-6}}{1}\right)^2} = 4.1829 \text{ x } 10^{-6}$$

3.6. Repeatability uncertainty of DNA measurements

To obtain the standard repeatability uncertainty (*repea*), it is substituted into Equation 17. To obtain the relative repeatability uncertainty of reference material measurements, it is substituted into Equation 18:

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a) DNA of certified reference material (NIST 2372), at the concentration of 7.6 ng/μL

$$u_{\gamma_{repea}} = \sqrt{\left(\frac{1.5522\ x\ 10^{-1}}{\sqrt{30}}\right)^2} = 0.0283$$

$$u_{r_{\gamma_{repea}}} = \sqrt{\left(\frac{0.0283}{7.6}\right)^2} = 0.0037$$

b) DNA from Sprague Dawley rats, at the concentration of 10 ng/μL

$$u_{\gamma_{repea}} = \sqrt{\left(\frac{3.9869\ x\ 10^{-1}}{\sqrt{30}}\right)^2} = 0.0727$$

$$u_{r_{\gamma_{repea}}} = \sqrt{\left(\frac{0.0727}{10}\right)^2} = 0.0073$$

c) Human DNA, at the concentration of 10 ng/μL

$$u_{\gamma_{repea}} = \sqrt{\left(\frac{2.8959\ x\ 10^{-1}}{\sqrt{30}}\right)^2} = 0.0528$$

$$u_{r_{\gamma_{repea}}} = \sqrt{\left(\frac{0.0528}{10}\right)^2} = 0.0052$$

3.7. Reproducibility Uncertainty of DNA Measurements

To obtain the standard reproducibility uncertainty (*repro*), it is substituted into Equation 17. To obtain the relative reproducibility uncertainty of the measurements of the reference material, it is substituted into Equation 18:

a) DNA of certified reference material (NIST 2372), at the concentration of 7.6 ng/μL

$$u_{\gamma_{repro}} = \sqrt{\left(\frac{1.3047\ x\ 10^{-1}}{\sqrt{30}}\right)^2} = 0.0238$$

$$u_{r_{\gamma_{repro}}} = \sqrt{\left(\frac{0.0238}{7.6}\right)^2} = 0.0031$$

b) *DNA from Sprague Dawley rats*, at the concentration of 10 ng/μL

$$u_{\gamma_{repro}} = \sqrt{\left(\frac{2.3947\ x\ 10^{-1}}{\sqrt{30}}\right)^2} = 0.0437$$

$$u_{r_{\gamma_{repro}}} = \sqrt{\left(\frac{0.0437}{10}\right)^2} = 0.0043$$

c) *Human DNA*, at the concentration of 10 ng/μL

$$u_{\gamma_{repro}} = \sqrt{\left(\frac{2.0457\ x\ 10^{-1}}{\sqrt{30}}\right)^2} = 0.0372$$

$$u_{r_{\gamma_{repro}}} = \sqrt{\left(\frac{0.0372}{10}\right)^2} = 0.0037$$

Despite obtaining low relative repeatability and reproducibility uncertainty values (which is one way to estimate analytical precision), others recommend doing the same to estimate the trueness uncertainty due to bias [30].

3.8. DNA Concentration Uncertainty

a) DNA of certified reference material (NIST 2372)

To obtain the standard uncertainty of the reference material, it is substituted into Equations 19 and 20:

$$u_{\gamma_{NIST}} = \frac{57/2}{\sqrt{3}} = 0.0164$$

To obtain the relative uncertainty of the reference material, substitute in Equation 21:

$$u_{r_{NIST}} \sqrt{\left(\frac{0.0164}{57}\right)^2} = 2.87\text{x } 10^{-4}$$

b) DNA from Sprague Dawley rats

To obtain the standard uncertainty of DNA from Sprague Dawley rats, substitute into equation 22:

$$u_{\gamma_{rats\ S-D}} = \frac{0.7416}{\sqrt{5}} = 0.3316$$

To obtain the relative uncertainty of DNA from Sprague Dawley rats, substitute into Equation 23:

$$u_{r_{rats\ S-D}} \sqrt{\left(\frac{0.3316}{57.9}\right)^2} = 5.72 \text{ x } 10^{-3}$$

c) Human DNA

To obtain the standard uncertainty of human DNA, substitute into Equation 24:

$$u_{\gamma_{human}} = \frac{1.7111}{\sqrt{5}} = 0.7652$$

To obtain the relative uncertainty of human DNA, substitute into Equation 25:

$$u_{r_{human}} \sqrt{\left(\frac{0.7652}{91.55}\right)^2} = 8.35 \text{ x } 10^{-3}$$

3.9. Combined Standard Uncertainty (CSU)

To obtain the standard CSU, it is substituted into Equation 26:

a) DNA of certified reference material (NIST 2372)

$$u_{c\gamma_{DNA}} = \sqrt{(0.0827)^2 + (0.2399)^2 + (0.5363)^2 + (0.0418\ x\ 10^{-4})^2 + (0.0283)^2 + (0.0238)^2 + (0.0164)^2} = 0.5943$$

b) DNA from Sprague Dawley rats

$$u_{c\gamma_{DNA}} = \sqrt{(0.0181)^2 + (0.2399)^2 + (0.5363)^2 + (0.0418\ x\ 10^{-4})^2 + (0.0727)^2 + (0.0437)^2 + (0.3316)^2} = 0.6801$$

c) Human DNA

$$u_{c\gamma_{DNA}} = \sqrt{(0.0273)^2 + (0.2399)^2 + (0.5363)^2 + (0.0418\ x\ 10^{-4})^2 + (0.0528)^2 + (0.0372)^2 + (0.7652)^2} = 0.9672$$

3.10. Combined Relative Uncertainty (CRU)

To obtain the CRU, it is substituted into Equation 27 and to obtain the combined relative uncertainty percentage, substituted into Equation 28:

a) DNA of certified reference material (NIST 2372)

$$u_{rc\gamma_{DNA}} = \sqrt{\left(\frac{0.5943}{57}\right)^2} = 0.0104$$

$$\%\ u_{rc\gamma_{DNA}} = 0.0104\ \text{x}\ 100 = 1.04\%$$

b) DNA from Sprague Dawley rats

$$u_{rc\gamma_{DNA}} = \sqrt{\left(\frac{0.6801}{57.9}\right)^2} = 0.0117$$

$$\%\ u_{rc\gamma_{DNA}} = 0.0117\ \text{x}\ 100 = 1.17\%$$

c) Human DNA

$$u_{rc\gamma_{DNA}} = \sqrt{\left(\frac{0.9672}{91.55}\right)^2} = 0.0105$$

$$\%\ u_{rc\gamma_{DNA}} = 0.0105\ \text{x}\ 100 = 1.05\%$$

Linko et al., (2001) [31], obtained a percentage uncertainty of 1.2% for methods to quantify calcium and glucose. Here our group reports a similar value using DNA from different sources.

3.11. Expanded Uncertainty (U)

Substitute into Equation 29:

a) DNA of certified reference material (NIST 2372)

$$U_{exp_{\text{DNA}}} = 0.5943\ \text{x}\ 2 = 1.1886\ \text{ng/µL}$$

b) DNA from Sprague Dawley rats

$$U_{exp_{\text{DNA}}} = 0.6801\ \text{x}\ 2 = 1.3602\ \text{ng/µL}$$

c) Human DNA

$$U_{exp_{\mathrm{DNA}}} = 0.9672 \text{ x } 2 = 1.9544 \text{ ng/µL}$$

These *U* values represent 2.08%, 2.34%, and 2.12% of the DNA concentration if we consider that the concentrations for DNA from NIST 2372, Sprague Dawley rats, and humans are 57.0, 57.9, and 91.5 ng/µL, respectively.

The values obtained are considered acceptable since in other investigations, analytical uncertainty values of up to 10% have been reported [32]. Furthermore, we must understand that we can validate our measurement methods based on the estimation of analytical uncertainty; through the Law of Propagation of Uncertainty, and thus we generate greater reliability of the results obtained because the validation process is more truthful [33]. That is, it is not enough to validate the analytical method with the pre-established parameters through the theory of errors, but also during the validation, the analytical uncertainty must be estimated [34]. On the other hand, the result obtained from the expanded uncertainty (*U*), is considered acceptable since seven sources of uncertainty were taken into account, which is greater than the number of sources of uncertainty used in other investigations [35,36]. Deprez et al., (2016) [8] mention that relative uncertainty percentages of 1.1 to 3.2% have been determined when assessing a reference standard material (SRM AD623) by digital polymerase chain reaction (dPCR). This work includes data from the previously validated methods, as well as sources of uncertainty, such as the limits of detection and quantification (LOD and LOQ), robustness, selectivity, trueness, precision, and linearity range, among others.

Furthermore, Griffiths et al., (2011) [37] have obtained a relative uncertainty of 0.075 (7.5%) for a standard reference material of lambda phage DNA measured using dPCR and ssDNA, which they also consider a high value of relative uncertainty percentage.

Nevertheless, Petrovykh et al., (2003) [38], have determined a relative uncertainty percentage between 15% and 20% when evaluating the quantitative characterization of DNA Films by X-ray Photoelectron Spectroscopy (Quantitative Characterization of DNA Films by X-ray).

In addition, Ki et al., (2021) [39] have estimated EU values of 1.70 to 1.93 for a standard reference material (6205-a) measured by real-time qPCR.

Please note that, in this case, the uncertainty estimation procedure used was the uncertainty propagation law [1]. Recently, some researchers have begun to recommend the use of specialized software such as the Monte Carlo simulation method [40], in order to avoid complex mathematical operations and obtain results in a practical way with the use of this type of software.

In addition to the aforementioned, the Bayesian approach defined in other studies estimated DNA concentrations from environmental samples using absolute standard curves generated by real-time qPCR. The approach account for uncertainty from multiple sources, such as experiment-to-experiment variation, variability between repeated measurements, as well as uncertainty when using calibration curves generated from absolute plasmid DNA standards [41]; analytical uncertainty has even been determined in a novel variant of PCR called digital droplet PCR (ddPCR) with promising results as *U* estimates, much lower than with conventional real-time PCR methods, have been obtained [42].

Love et al. Broeders et al., and Shehata et al. [5,43,44] point out that without the prior validation of the analytical method to determine or quantify DNA, neither reliable results nor a reliable estimate of uncertainty may be obtained.

Table 1 includes the concentrated summary of the calculations for each of the sources of uncertainty evaluated and considered as such in the Ishikawa diagram.

Figure 2 shows that the source of uncertainty that provides the greatest relative uncertainty is that of the dilution factor, as has been described in other investigations by our research team [45,46]. In particular, we had already commented on this in a previous publication [25], in which it is recommended that the estimation of the uncertainty for the dilution factors should be carefully studied, since when preparing the calibration curves five dilutions are used, and the five factors of dilution are considered together when estimating their contribution to analytical uncertainty, which is not the case with the other sources of uncertainty [47,48].

This will have to be studied carefully because when the calibration curves are developed. Here, five dilutions are used, and five dilution factors are considered together when estimating their contribution to analytical uncertainty, which is not the case with the other sources of uncertainty.

Table 1. Concentration of the calculations of standard and relative uncertainty for each one of the sources of uncertainty. It includes the percent relative uncertainty and the expanded uncertainty for each DNA source.

Sources of uncertainty	NIST (2372)	S-D rats	Humans
Standard and relative uncertainty of concentration from the calibration curve.	0.0827 ng/µL 0.0634	0.0181 ng/µL 0.0261	0.0263 ng/µL 0.0325
Standard and relative uncertainty of the volumetric material.	0.2399 µL 0.0767	0.2399 µL 0.0767	0.2399 µL 0.0767
Standard and relative uncertainty of the dilution factor.	0.5363 1.8939	0.5363 1.8939	0.5363 1.8939
Standard and relative uncertainty of the analytical balance.	4.1829×10^{-6} mg 4.1829×10^{-6}	4.1829×10^{-6} mg 4.1829×10^{-6}	4.1829×10^{-6} mg 4.1829×10^{-6}
Standard and relative uncertainty of repeatability.	0.0283 ng/µL 0.0037	0.0727 ng/µL 0.0073	0.0528 ng/µL 0.0052
Standard and relative uncertainty of reproducibility.	00238 ng/µL 0.0031	0.0437 ng/µL 0.0043	0.0372 ng/µL 0.0037
Concentration uncertainty of each DNA source.	0.0164 ng/µL 0.0287×10^{-2}	0.3316 ng/µL 0.0572×10^{-1}	0.7652 ng/µL 0.0835×10^{-1}
Combined Standard Uncertainty (CSU), Combined Relative Uncertainty (CRU) and percent relative uncertainty.	0.5943 0.0104 1.04%	0.6801 0.0117 1.17%	0.9672 0.0105 1.05%
Expanded uncertainty (*U*)	1.189 ng/µL	1.360 ng/µL	1.944 ng/µL
Percentage of the concentration in relation to the expanded uncertainty.	2.08%	2.34%	2.12%

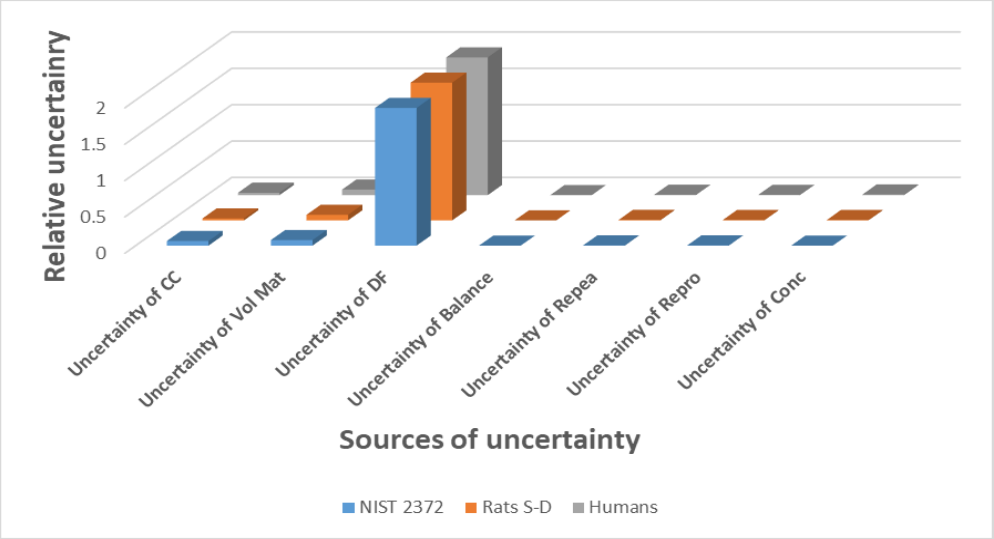

Figure 2. Graphical contribution of the sources of uncertainty evaluated

Although the value obtained for the uncertainty of the dilution factor is high, the other sources of uncertainty studied contribute with a lower relative uncertainty [47].

On the other hand, some research groups report differences in the estimation of uncertainty between the pre-analytical and analytical phases [48], to such an extent that this has begun to be handled as a variable of interest to consider when making analytical determinations in Clinical Chemistry [49,50].

In order that the above is not considered as an analytical problem similar to "the tip of the iceberg"; Our research team suggests ensuring the calibration processes of the materials and equipment used from the pre-analytical phase, prior to the determinations that will be made in the analytical phase [5,22,43,44,46,51].

Finally, it is important to establish "cutoff points" or magnitude values of the uncertainties to know if we are doing our analytical work well. Reference values have not yet been established for acceptance or rejection of the result obtained, nor values for the evaluation of conformity. Establishing the aforementioned parameters is still a pending issue.

4. Conclusions

The expanded uncertainty (*U*) was estimated from DNA concentrations measured from a certified reference material (NIST 2372), from Sprague Dawley rats and humans, measured through a Nanodrop spectrophotometer. The *U* values obtained were 1.189, 1.360, and 1.944 ng/µL DNA, respectively. These results represent 2.08%, 2.34%, and 2.12% for the concentrations of each DNA source (57.0, 57.9, and 91.5 ng/µL DNA, respectively). The source of uncertainty that contributes most to these calculations dilution factor uncertainty, although it should be noted that the dilution factor uncertainty also considers the volumetric material uncertainty and five dilutions were used for the calibration curves. Hence, these results may be overestimated.

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