Supplementary Information (SI)

Learning to Trust Experimental Data: A Validation-Based Approach to Experiment DeESIgn in Pre-Service Chemistry Teacher Education

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SI 1. Experimental system

Analytical measurements were carried out uESIng a UV–Vis spectrophotometer (PE-5400UF) equipped with quartz cuvettes possesESIng a 1 cm optical path length.

Spectral measurements for specificity assessment were conducted within the wavelength range of 350–500 nm. Quantitative measurements were taken at 415 nm, corresponding to the absorbance maximum of the flavonoid–$AlCl_3$ complex.

Reagents and Standards

The reagents listed below were used:

- rutin (purity ≥ 95%) used as the reference standard
- aluminium chloride solution (2%, prepared in 70% ethanol)
- ethanol solutions (40%, 70%, and 95% v/v)
- distilled water

Plant Material

Plant material of *Malva sylvestris* L. (leaves and flowers) was washed, air-dried, and further dried to a constant weight before analyESIs. The dried material was ground prior to extraction.

Extraction Conditions

Plant material extraction was carried out under the following conditions:

- Solvent volume: 100 mL
- Extraction temperature: 85 °C
- Extraction time: 45 minutes
- Extraction system: water bath fitted with a reflux condenser

The sample masses utilised for extraction are shown in Table S1.

Table S1. Sample portions utilised for extraction

Extract (EtOH, %)	Sample mass (g)
40	1.0012
70	1.0009
95	1.0124

Complexation Conditions

For both standards and samples, analytical solutions were prepared by adding 2.0 mL of 2% $AlCl_3$ solution to the respective aliquot and diluting to 25.0 mL with 70% ethanol. The solutions were incubated for 30 minutes before measurement.

Presentation of Results

Results were expressed as rutin equivalents (RE).

SI 2. Sample preparation

Preparation of plant extracts

Sample portions (Table S1) were extracted uESIng 100 mL of ethanol solutions (40%, 70%, and 95%, v/v) under the conditions described in Section SI 1. After extraction, the solutions were cooled to room temperature.

The parameters for extract preparation are summarised in Table S2.

Table S2. Parameters for preparing plant extracts

Ethanol concentration (% v/v)	Sample mass (g)	Final volume (mL)	Raw material concentration (mg/mL)
40	1.0012	100	10.012
70	1.0009	100	10.009
95	1.0124	100	10.124

Preparation of Standard Solutions

A primary standard solution of rutin was prepared by dissolving 0.0526 g of rutin (equivalent to 0.0500 g of pure substance) in 100 mL of 70% ethanol.

Standard solutions were prepared by transferring aliquots of the stock solution (0.5–3.0 mL) into 25.0 mL volumetric flasks and diluting to volume with 70% ethanol.

The preparation scheme is shown in Table S3.

Table S3. Preparation of rutin working standard solutions.

V_{stock} (mL)	C_{stock} (mg/mL)	m_i (mg)	C_i (mg/mL)
0.5	0.4997	0.24985	0.01
1.0	0.4997	0.49970	0.02
1.5	0.4997	0.74955	0.03
2.0	0.4997	0.99940	0.04
2.5	0.4997	1.24925	0.05
3.0	0.4997	1.49910	0.06

Preparation of Analytical Solutions

For calibration purposes:

- 1.0 mL aliquots of working standard solutions were transferred into 25.0 mL volumetric flasks.
- 2.0 mL of 2% $AlCl_3$ solution was added.
- Solutions were diluted to volume uESIng 70% ethanol.

For sample analyESIs:

- 3.0 mL aliquots of plant extracts were treated in the same way.

Blank Solution

The blank solution was prepared uESIng the same procedure without the addition of $AlCl_3$.

SI 3. Specificity of the analytical method

UV–Vis absorption spectra of the rutin–$AlCl_3$ complex and plant extracts (Malva sylvestris L.) were recorded within the wavelength range of 350–500 nm.

The spectra of both the reference standard and the plant extracts showed a characteristic absorption band in the range of 410–415 nm, which indicates the formation of flavonoid–$AlCl_3$ complexes. The maximum absorbance at 415 nm was chosen as the analytical wavelength for subsequent quantitative analyESIs.

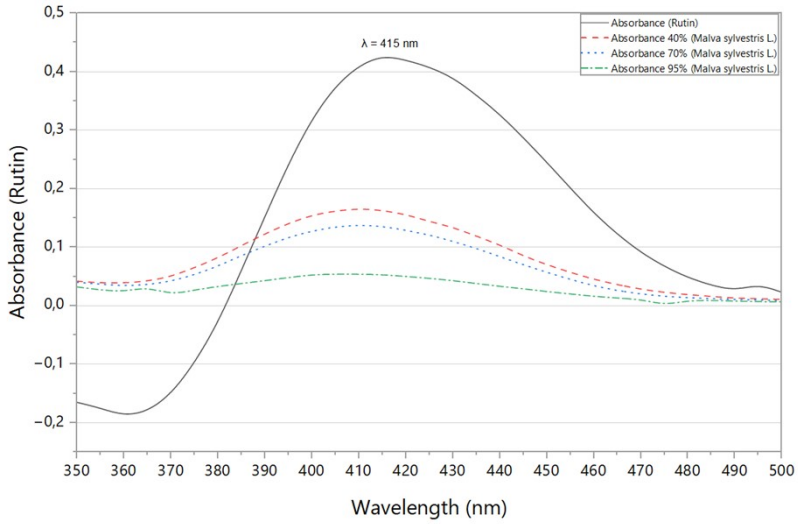

Figure S1. UV–Vis absorption spectra of rutin–$AlCl_3$ complex and *Malva sylvestris* L. extracts (350–500 nm)

The impact of the extraction solvent on the analytical ESIgnal intenESIty is shown in Figure S2.

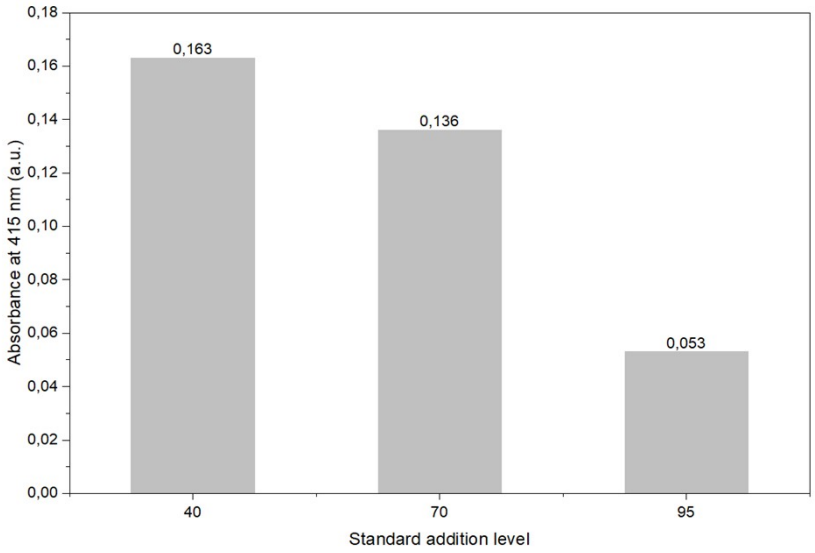

Figure S2. Effect of ethanol concentration on absorbance at 415 nm.

The absorbance values are summarised in Table S4.

Table S4. Absorbance of plant extracts at 415 nm

Ethanol concentration (%)	Absorbance at 415 nm
40	0.163
70	0.136
95	0.053

The results confirm that the analytical ESIgnal at 415 nm is attributable to flavonoid–$AlCl_3$ complexes, indicating adequate specificity of the method.

SI 4. Linearity and calibration model

The linearity of the analytical method was assessed uESIng a series of rutin standard solutions prepared by diluting a stock solution under the conditions described in Section SI 2.

A rutin stock solution with a concentration of 0.4997 mg/mL was prepared for calibration standards. Aliquots of the stock solution (0.5–3.0 mL) were transferred into 25.0 mL volumetric flasks, then complexed with $AlCl_3$ and diluted to volume with 70% ethanol. Absorbance was measured at 415 nm.

The concentrations of calibration solutions were determined uESIng the dilution equation:

$$C = \frac{C_{stock} * V_{stock}}{V_{final}}$$

The preparation scheme and the corresponding analytical ESIgnals are shown in Table S5.

Table S5. Calibration data for rutin standard solutions

V_{stock} (mL)	C_{stock} (mg/mL)	V_{final} (mL)	C (mg/mL)	Absorbance (A_{415})
0.5	0.4997	25.0	0.010	0.265
1.0	0.4997	25.0	0.020	0.480
1.5	0.4997	25.0	0.030	0.714
2.0	0.4997	25.0	0.040	0.943
2.5	0.4997	25.0	0.050	1.137
3.0	0.4997	25.0	0.060	1.338

Intermediate values utilised for calculating regresESIon parameters are shown in Table S6.

Table S6. Intermediate values for regresESIon analyESIs

x (mg/mL)	y (A_{415})	x^2	xy
0.01	0.265	0.0001	0.00265

0.02	0.480	0.0004	0.00960
0.03	0.714	0.0009	0.02142
0.04	0.943	0.0016	0.03772
0.05	1.137	0.0025	0.05685
0.06	1.338	0.0036	0.08028
Σ	4.877	0.0091	0.20852

The slope (b) and intercept (a) of the calibration curve were determined uESIng standard least-squares equations.

$$b = \frac{n\sum xy - \sum x \sum y}{n\sum x^2 - (\sum x)^2}$$

$$a = \frac{\sum y - b\sum x}{n}$$

The values obtained were:

b = 21.6273

a = 0.0563

The coefficient of determination (R^2) was determined uESIng the sum of squares method.

$$R^2 = 1 - \frac{SS_{res}}{SS_{tot}}$$

where:

$$SS_{res} = \sum (y_i - \hat{y}_i)^2$$

$$SS_{tot} = \sum (y_i - \bar{y}_i)^2$$

Predicted values ($\hat{y}$) and reESIduals are shown in Table S7.

Table S7. Calculation of R^2

x (mg/mL)	**y (A_{415})**	**$\hat{y}$**	**$(y - \hat{y})^2$**
0.01	0.265	0.2726	5.79×10^{-5}
0.02	0.480	0.4889	7.88×10^{-5}
0.03	0.714	0.7052	7.83×10^{-5}
0.04	0.943	0.9214	4.66×10^{-4}
0.05	1.137	1.1377	4.85×10^{-7}
0.06	1.338	1.3540	2.55×10^{-4}

The following values were recorded:

$\bar{y} = 0.812833$

$SS_{res} = 0.000936$

$SS_{tot} = 0.81849$

$R^2 = 0.9989$

Calibration Equation

The calibration curve was characterised by the linear equation:

$$A = 21.63 \cdot C + 0.0563$$

with a coefficient of determination:

$$R^2 = 0.9989$$

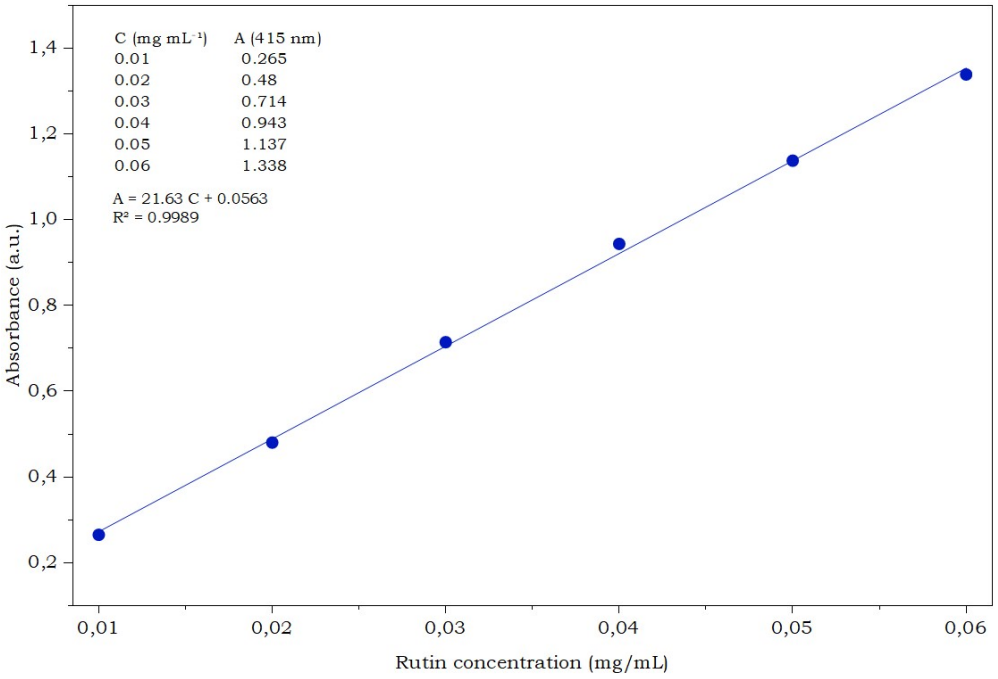

Figure S3. Calibration curve for rutin obtained uESIng the $AlCl_3$ spectrophotometric method at 415 nm within the concentration range of 0.01–0.06 mg/mL

SI 5. PreciESIon (repeatability)

Repeatability of the analytical method was assessed by conducting ESIx independent measurements (n = 6) of the same plant extract under identical experimental conditions, including extraction, complexation with $AlCl_3$, and spectrophotometric measurement at 415 nm.

The calibration equation derived in Section SI 4 was utilised for all calculations.

$$A = 21.63 \cdot C + 0.0563$$

$$C = \frac{A - 0.0563}{21.63}$$

where:

- A — absorbance
- a — intercept (0.0563)
- b — slope (21.63)

For each replicate, the absorbance value was converted to concentration in the analytical solution, then to the mass of rutin equivalent in the 25.0 mL volumetric flask, and ultimately to flavonoid content expressed as mg RE/g of plant material.

The following constants were used for the calculations:

- Final volume of the analytical solution: V=25.0 mL
- Aliquot of plant extract: 3.0 mL.
- concentration of plant material in the extract: 10.009 mg/mL
- mass of plant material for the 3.0 mL aliquot:

$$m_{plant,\ aliquot} = 10.009 \times 3.0 = 30.027 \text{ mg}$$

5.1. Repeatability of absorbance measurements

The absorbance values from ESIx replicate measurements are shown in Table S8.

Table S8. Repeatability of absorbance measurements (λ = 415 nm)

№	**Absorbance (A)**	$A_i - \bar{A}$	$(A_i - \bar{A})^2$
1	0.386	0.0015	2.25×10^{-6}
2	0.385	0.0005	2.50×10^{-7}
3	0.384	−0.0005	2.50×10^{-7}
4	0.387	0.0025	6.25×10^{-6}
5	0.379	−0.0055	3.03×10^{-5}
6	0.386	0.0015	2.25×10^{-6}
Sum	2.307	-	0.00004150

The average absorbance was:

$$\bar{A} = \frac{\sum A_i}{n} = \frac{2.307}{6} = 0.3845$$

The standard deviation was determined as:

$$s_A = \sqrt{\frac{\sum (A_i - \bar{A})^2}{n-1}} = \sqrt{\frac{0.00004150}{5}} = 0.00288$$

The relative standard deviation was determined as follows:

$$RSD_A = \frac{s}{\bar{A}} \cdot 100\% = \frac{0.00288}{0.3845} \cdot 100\% = 0.75\%$$

5.2. Repeatability of Calculated Concentration

The concentration of rutin equivalents in the analytical solution was calculated uESIng the calibration equation from Section SI 4:

$$C = \frac{A - 0.0563}{21.63}$$

The concentrations calculated are shown in Table S9.

Table S9. Repeatability of calculated concentrations

№	Absorbance (A)	C (mg/mL)	$C_i - \bar{C}$	$(C_i - \bar{C})^2$
1	0.386	0.0152	0.00003	9.0×10^{-10}
2	0.385	0.0152	0.00003	9.0×10^{-10}
3	0.384	0.0152	0.00003	9.0×10^{-10}
4	0.387	0.0153	0.00013	1.69×10^{-8}
5	0.379	0.0149	−0.00027	7.29×10^{-8}
6	0.386	0.0152	0.00003	9.0×10^{-10}
Sum	—	0.0910	—	9.34×10^{-8}

$$\bar{C} = \frac{\sum C_i}{n} = \frac{0.0910}{6} = 0.01517\ mg/mL$$

$$s_C = \sqrt{\frac{\sum (C_i - \bar{C})^2}{n-1}} = \sqrt{\frac{9.34 \cdot 10^{-8}}{5}} = 0.000133$$

$$RSD_C = \frac{s}{\bar{C}} \cdot 100\% = \frac{0.000133}{0.01517} \cdot 100\% = 0.88\%$$

5.3. Repeatability of rutin-equivalent mass

The mass of rutin equivalent in the 25 mL volumetric flask was calculated as:

$$m = C \cdot V_m$$

where:

m — mass of rutin equivalent in the analytical solution (mg),

C — concentration of rutin equivalent in the analytical solution (mg/mL).

$$V = 25\ mL$$

The calculated values are shown in Table S10.

Table S10. Repeatability of rutin-equivalent mass in the 25.0 mL volumetric flask.

№	C (mg/mL)	m (mg)	$m_i - \bar{m}$	$(m_i - \bar{m})^2$

1	0.0152	0.3811	0.0018	0.000003
2	0.0152	0.3799	0.0006	0.000000
3	0.0152	0.3788	-0.0005	0.000000
4	0.0153	0.3822	0.0029	0.000008
5	0.0149	0.3730	-0.0063	0.000040
6	0.0152	0.3811	0.0018	0.000003
Sum	—	2.2761	—	0.000055

$$\bar{m} = \frac{\sum m_i}{n} = \frac{2.2761}{6} = 0.3793\ mg$$

$$s_m = \sqrt{\frac{\sum (m_i - \bar{m})^2}{n-1}} = \sqrt{\frac{0.000055}{5}} = 0.00333$$

$$RSD_m = \frac{s}{\bar{m}} \cdot 100\% = \frac{0.00333}{0.3793} \cdot 100\% = 0.88\%$$

5.4. Repeatability of flavonoid levels in plant material

Flavonoid content was determined as:

$$X = \frac{m}{m_{plant,\,aliquot}}$$

where:

- X — flavonoid content in plant material (mg RE/g)
- m — mass of rutin equivalent (mg)
- $m_{plant,aliquot}$ — mass of plant material corresponding to the analysed aliquot.

The results are shown in Table S11.

Table S11. Flavonoid content in plant material

№	**m (mg)**	**X (mg RE/g)**	$X_i - \bar{X}$	$(X_i - \bar{X})^2$
1	0.3811	12.687	0.057	0.003249
2	0.3799	12.649	0.019	0.000361
3	0.3788	12.610	-0.020	0.000400
4	0.3822	12.726	0.096	0.009216
5	0.3730	12.418	-0.212	0.044944
6	0.3811	12.687	0.057	0.003249
Sum	—	75.777	—	0.061419

$$\bar{X} = \frac{\sum X_i}{n} = \frac{75.777}{6} = 12.63\ mg\ RE/g$$

$$s_X = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n-1}} = \sqrt{\frac{0.061419}{5}} = 0.111$$

$$RSD_X = \frac{s}{\bar{X}} \cdot 100\% = \frac{0.111}{12.63} \cdot 100\% = 0.88\%$$

5.5. Summary of Repeatability

The repeatability parameters for absorbance, concentration, rutin-equivalent mass in the analytical solution, and flavonoid content in plant material are summarised in Table S12.

Table S12. Repeatability parameters for ESIx replicate measurements of Malva sylvestris L. extract (λ = 415 nm)

Parameter	A_{415}	C (mg/mL, RE)	m (mg)	X (mg RE/g)
Number of replicates. n	6	6	6	6
Mean value	0.3845	0.01517	0.3793	12.63
Standard deviation. s	0.00288	0.000133	0.00333	0.111
Relative standard deviation. RSD. %	0.75	0.88	0.88	0.88

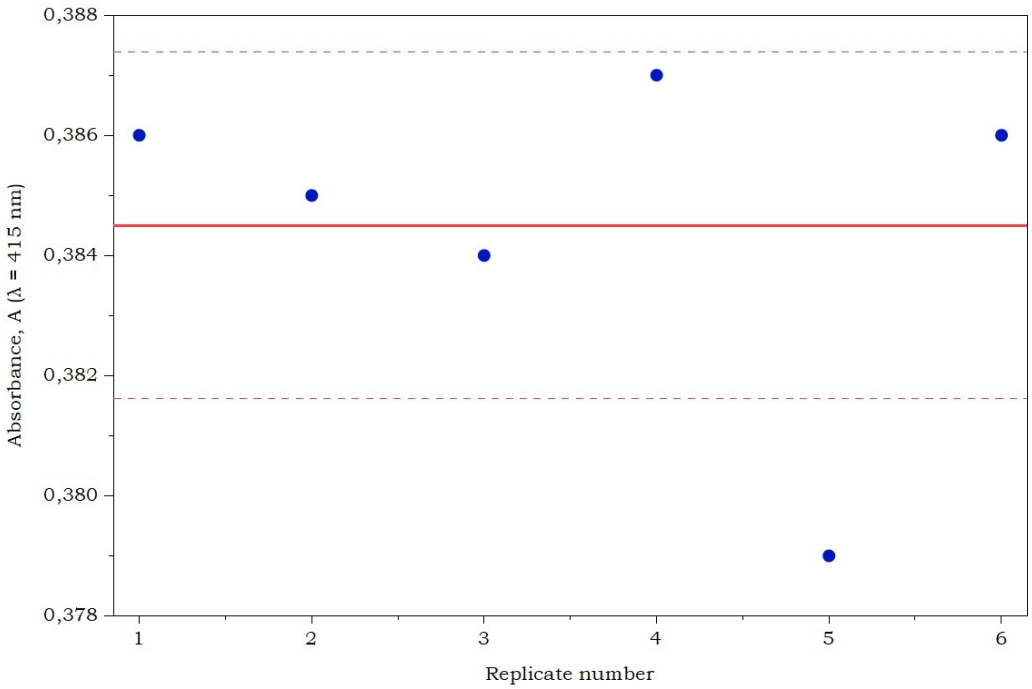

Figure S4. Repeatability of the $AlCl_3$ spectrophotometric method assessed from ESIx replicate measurements of a Malva sylvestris L. extract at 415 nm (solid line represents the mean; dashed lines show ±1 SD).

SI 6. Trueness

The trueness of the analytical method was assessed uESIng the standard addition approach at three levels: 80%, 100%, and 120% of the initial analyte amount in the unspiked sample aliquot. Each level was analysed in triplicate under identical experimental conditions.

6.1. Calculation Principles

The total mass of rutin equivalent in the 25.0 mL volumetric flask after standard addition was calculated as:

$m_{found,total} = C \cdot V$

where:

V = 25.0 mL

ESInce the sample already contained flavonoids before the standard addition, the mass corresponding solely to the added analyte was calculated as:

$m_{found,add} = m_{found,total} - m_0$

where:
m_0 = 0.379344 mg — initial analyte amount in the unspiked sample.

Recovery was determined as:

$$Recovery = \frac{m_{found,add}}{m_{add}} \cdot 100\%$$

6.2. Experimental constants

- Wavelength: 415 nm
- Final volume: 25.0 mL
- Initial analyte amount: m_0 = 0.379344 mg
- Addition levels: 80%, 100%, 120%

6.3. Raw Data and Calculations

Table S13. Results of standard addition and recovery calculations

Addition level	**Replicate**	**A_{415}**	**C (mg/mL)**	**$m_{found,total}$ (mg)**	**$m_{found,add}$ (mg)**	**m_{add} (mg)**	**Recovery (%)**
80%	1	0.635	0.026756	0.668909	0.289565	0.303475	95.416
80%	2	0.635	0.026756	0.668909	0.289565	0.303475	95.416
80%	3	0.632	0.026618	0.665441	0.286097	0.303475	94.274
80% mean ± SD	—	0.6340 ± 0.0017	0.026710 ± 0.000080	0.667753 ± 0.002002	0.288409 ± 0.002002	—	**95.035 ± 0.660**
100%	1	0.690	0.029299	0.732486	0.353142	0.379344	93.093
100%	2	0.691	0.029346	0.733642	0.354298	0.379344	93.398
100%	3	0.694	0.029484	0.737110	0.357766	0.379344	94.312
100% mean ± SD	—	0.6917 ± 0.0021	0.029377 ± 0.000096	0.734413 ± 0.002406	0.355069 ± 0.002406	—	**93.601 ± 0.634**
120%	1	0.760	0.032536	0.813403	0.434059	0.455213	95.353

120%	2	0.751	0.032120	0.802999	0.423655	0.455213	93.067
120%	3	0.752	0.032166	0.804155	0.424811	0.455213	93.321
120% mean ± SD	—	0.7543 ± 0.0049	0.032274 ± 0.000228	0.806852 ± 0.005702	0.427508 ± 0.005702	—	**93.914 ± 1.253**

6.4. Example of Calculation

For the initial replicate at the 80% addition level:

$$C = \frac{0.635 - 0.0563}{21.63} = 0.026756\ mg/mL$$

$$m_{found,total} = 0.026756 \times 25 = 0.668909\ mg$$

$$m_{found,add} = 0.668909 - 0.379344 = 0.289565\ mg$$

$$Recovery = \frac{0.289565}{0.303475} \times 100\ \% = 95.416\ \%$$

6.5. Summary of accuracy

The recovery values observed at the three levels of addition were:

- 80%: 95.035 ± 0.660 %
- 100%: 93.601 ± 0.634 %
- 120%: 93.914 ± 1.253 %

The results show conESIstent recovery across the examined concentration range, indicating no ESIgnificant systematic error and confirming the method's accuracy.