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Determination of Caffeine in Coffee Samples: Teaching Calibration Curve – Part II

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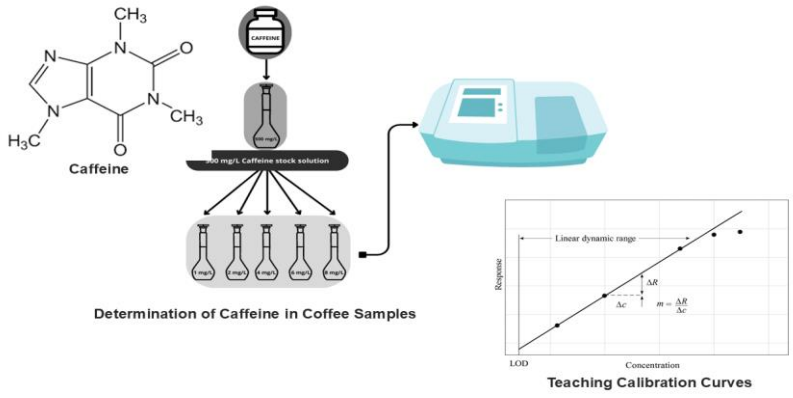
Analytical Chemistry involves the identification and quantification of chemical substances through the application of precise measurement techniques, quantitative reasoning, and analytical instrumentation. This study presents a clear and practical approach to introducing key quantitative concepts in Analytical Chemistry using UV–Vis spectrophotometry to determine caffeine in coffee. The method employs calibration curves to illustrate how instrumental signals can be related to analyte concentration, connecting fundamental analytical concepts to a real analytical problem. The proposed procedure involves liquid–liquid extraction of caffeine followed by UV–Vis measurements at its absorption maximum, and the construction of a calibration model in the range of 1–8 mg L^{-1} using duplicate measurements. The regression equation obtained was y = 0.0742x − 0.0016, with R^2 = 0.9987, indicating linear behavior in this range. Based on this model, the method presented a limit of detection (LOD) of 0.15 mg L^{-1} and a limit of quantification (LOQ) of 0.50 mg L^{-1}. When applied to commercial coffee samples, the analytical curve indicated caffeine concentrations ranging from 0.56 to 0.74% (w/w). These results allowed discussion of variability in caffeine content among the samples analyzed, as well as the comparison of the obtained values with current regulatory standards. This article corresponds to Part II of a teaching-oriented series in Analytical Chemistry. While Part I addressed method validation (precision and accuracy) through sodium determination in dietary supplements, the present work focuses on calibration, regression analysis, residual evaluation, and quantitative interpretation using a real food matrix.

Graphical abstract



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1. Introduction

The teaching of Chemistry can serve as either a motivating or a discouraging factor for students. Several studies indicate that traditional teaching approaches, heavily focused on rote memorization of formulas and limited contextualization of scientific knowledge, have led to low levels of engagement and attention among learners [1-3]. According to Finger and Bedin [2], high dropout and repetition rates in technical courses are partly linked to weaknesses in pedagogical practices, particularly in Analytical Chemistry. As Lima and Cunha [4] note, this subject is considered one of the most challenging in the curriculum due to its conceptual complexity and emphasis on quantitative relationships among chemical species.

Analytical Chemistry requires integrating mathematical concepts, which can be demanding for students with limited quantitative proficiency [5]. Araújo [6] emphasizes that well-structured didactic contextualization that integrates theory and practice in the teaching of Analytical Chemistry fosters more meaningful learning environments and promotes deeper conceptual understanding compared to traditional methods. Using familiar contexts to introduce complex topics such as linearity, calibration curves, UV-Vis spectrophotometry, and liquid–liquid extraction makes learning more accessible and engaging [7].

This paper continues the didactic sequence presented in Part I, which introduced an interdisciplinary experimental proposal to teach analytical validation (precision and accuracy) through sodium determination in dietary supplements [8]. Part II extends this approach to quantitative instrumental analysis, focusing on linear calibration curves and regression tools for UV–Vis determination of caffeine in coffee samples, reinforcing the connection between mathematical data treatment and real-world analytical challenges.

1.1 CALIBRATION CURVES

Calibration curves, or analytical curves, are essential tools in chemical analysis, enabling the quantification of an analyte in unknown samples. They are constructed by measuring the response of an analytical instrument (e.g., absorbance, chromatographic peak area, or signal intensity) for standard solutions of known concentration. From these data, a mathematical relationship (typically via linear regression) models the dependence between the analytical response and the analyte concentration [9-11].

External standards are used when matrix interference is negligible. The analytical curve is built by plotting concentration against instrumental response and performing regression analysis (See Figure 6 in the Results section).

A linear equation of the form $y = a + bx$ is fitted, where y is the instrumental response, x is the analyte concentration, a is the intercept, and b is the slope. The fit is performed using the least-squares method, which minimizes the sum of squared differences between observed and predicted values [9]. The curve can then be used to estimate analyte concentrations in unknown samples. The quality of the regression fit is evaluated by statistical parameters such as the coefficient of determination (R^2) and analysis of variance (ANOVA) [11,12]. Ideally, the curve approximates a straight line, and R^2 approaches unity, ensuring accurate predictions [11].

1.2 CAFFEINE IN COFFEE: UV–VIS ABSORPTION AND MAXIMUM WAVELENGTH

Coffee seeds contain between 1% and 2% w/w caffeine, the primary alkaloid responsible for the stimulant effects of the beverage. They also contain lower concentrations of other methylxanthine alkaloids, such as theophylline and theobromine, with relevant pharmacological properties [13]. The molecular structure of caffeine contains a heterogeneous unsaturated carbon chain with nitrogen heteroatoms and two fused heterocyclic rings, as well as three methyl groups (-CH_3) (Figure 1).

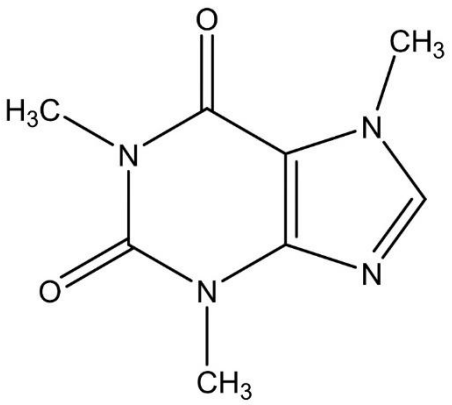

Fig. 1. Molecular structure of caffeine. Source: Dewick (2002).

Caffeine belongs to the xanthine family and stimulates the central nervous system and skeletal muscles, producing physiological effects such as increased alertness, reduced drowsiness, and enhanced cognitive performance [14]. Due to its widespread consumption and pharmacological relevance, caffeine is often analyzed in coffee to determine variations in compound concentration, discriminate coffee varieties or types, and assess geographical origin.

For this purpose, spectrometric techniques, particularly UV-Vis spectroscopy, are commonly used. These compounds absorb energy in the visible (400–800 nm) or ultraviolet (200–400 nm) regions [15] (Figure 2).

UV-Vis spectroscopy is employed in food analysis due to its accessibility and relatively low cost compared with other analytical techniques. To quantify caffeine in coffee samples, an analytical calibration curve must be constructed using external standard, as mentioned previously [7].

2. Material and Methods

2.1 Instrumentation

A UV/Vis molecular absorption spectrophotometer (BioMate 3S, Thermo Fisher Scientific, USA) equipped with a quartz cuvette was used to determine caffeine in coffee samples. Calibration curve solutions were prepared from pure solid caffeine standard (Sigma-Aldrich, Brazil) dissolved in analytical-grade dichloromethane (Vetec, Brazil). All standard solutions were prepared with appropriate dilutions in analytical-grade dichloromethane (Vetec, Brazil). For caffeine extraction, a heating plate (TMA20R, Thelga, Brazil) and borosilicate glassware cleaned with distilled water and dried were used. Dichloromethane P.A. (Vetec, Brazil) was employed for the extraction of caffeine from the aqueous

phase.

2.2 Preparation of the analytical curve

Standard solutions were prepared by successive dilutions of a stock solution of 500 mg L^{-1} caffeine (Figure 3), obtained by dissolving a pure caffeine standard (Sigma-Aldrich, Brazil). The resulting standard solutions had concentrations of 1, 2, 4, 6, and 8 mg L^{-1} in analytical-grade dichloromethane (Vetec, Brazil). The standard solutions were prepared in duplicate. Dilutions were carried out in 25 mL volumetric flasks and completed to the calibration mark with analytical-grade dichloromethane (Vetec, Brazil).

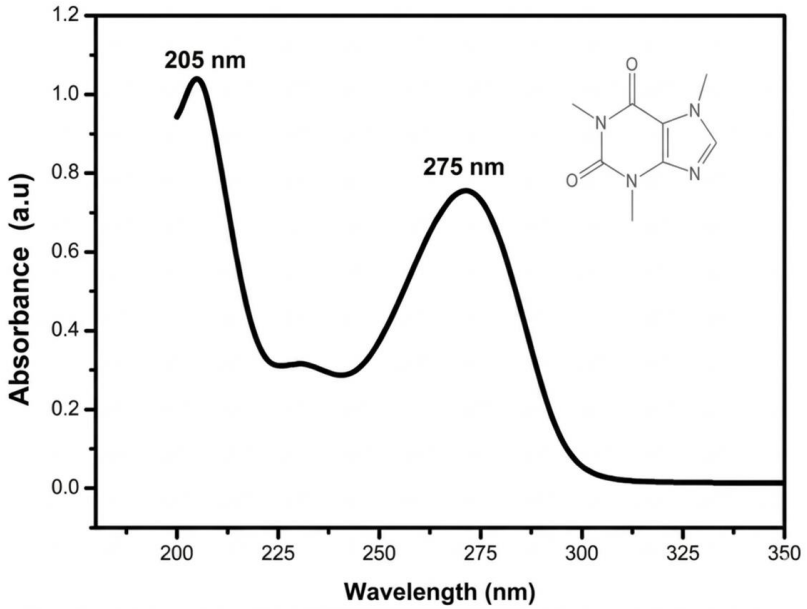

Fig. 2. UV-Vis absorption spectrum of caffeine. The maximum absorbance (λ_{max}) is observed at 275 nm, which was used for quantitative determination in calibration and sample analysis. Source: Dado adapted (2020) [16].

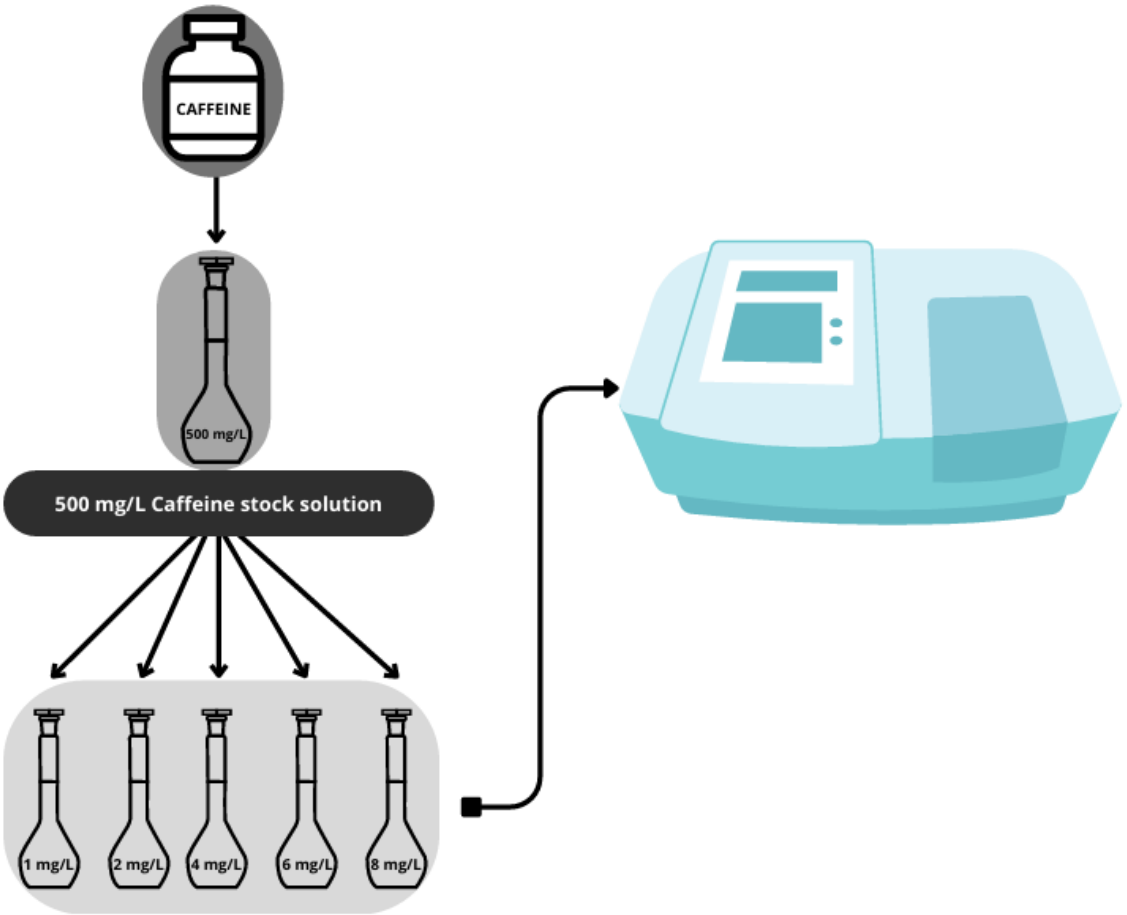

Fig. 3. Preparation of caffeine standard solutions for the calibration curve. Source: Authors (2025).

With the sequence of standard solutions prepared, absorbance measurements were carried out using the UV/Vis molecular absorption spectrophotometer. The standard solutions were analyzed at the maximum absorption wavelength of caffeine (275 nm) in dichloromethane solution [7].

2.2.1 Calculating the analytical curve

Using the concentrations of the standard caffeine solutions and the corresponding absorbance values obtained from the UV-Vis equipment, Microsoft Excel was employed to construct the calibration curve. A linear regression analysis was performed using the least-squares method to correlate the concentrations of the standard solutions with the absorbances measured by the spectrophotometer, yielding a regression equation. To calculate the slope (**a**) of the calibration curve, Eq. 1 was applied:

$$a = \frac{n\sum(x.y) - \sum x.\sum y}{n\sum(x^2) - (\sum x)^2} \quad \text{Eq. 1}$$

The intercept (**b**) was calculated as:

$$b = \frac{\sum y - a.\sum x}{n}. \quad \text{Eq. 2}$$

Where ***y*** represents the instrumental response (absorbance), ***x*** corresponds to caffeine concentration, ***a*** is the slope, ***b*** is the intercept where the line crosses the y-axis, and ***n*** is the number of readings performed. The regression fitting was performed by the least squares method, in which the line is adjusted to minimize the sum of squared errors (residuals) between the experimental data and the predicted values. The residuals (e_i) were calculated as:

$$e_i = y_i - \hat{y}_i \quad \text{Eq. 3}$$

Where e_i is the residual, y_i the observed absorbance, and $\hat{y}_i$ the estimated absorbance given by the regression equation. The Sum of Squared Residuals (**SSr**) was calculated as:

$$SSr = \sum_{i=1}^{n}(y_i - \hat{y}_i)^2 \quad \text{Eq. 4}$$

And the Total Sum of Squares (**SST**) was calculated as:

$$SST = \sum_{i=1}^{n}(y_i - \bar{y}_i)^2 \quad \text{Eq. 5}$$

From these values, the coefficient of determination (R^2) was obtained using the equation:

$$R^2 = 1 - \frac{SQr}{SQT} \quad \text{Eq. 6}$$

The limit of detection (LOD) and a limit of quantification (LOQ) were calculated according to Souza *et al.*, [11,17]: the LOD was calculated as three times the standard deviation of the intercept (*s*) of the calibration curve divided by the slope of the calibration curve (*a*) (Eq. 7), while the LOQ was calculated to be 10 times the standard deviation of the intercept (*s*) of the calibration curve divided by the slope of the calibration curve (*a*), (Eq. 8).

$$\text{LOD} = 3 \times \frac{s}{a} \quad \text{Eq. 7}$$

$$\text{LOQ} = 10 \times \frac{s}{a} \quad \text{Eq. 8}$$

2.3 Sample preparation

Approximately 1.000 g of roasted ground coffee was weighed on an analytical balance. The sample was placed in a simple funnel lined with filter paper, and extraction was performed with 50 mL of distilled water heated to 90–98 °C (Figure 4). The extract was then diluted with distilled water at room temperature (25 °C) at a 1:25 ratio in a 25.0 mL volumetric flask.

Subsequently, the diluted extract was transferred into a beaker, and 25 mL of dichloromethane was added. The mixture was stirred at 300 rpm for 5 minutes using a magnetic stirrer. After resting, the aqueous and organic phases were observed, with the organic phase containing caffeine. The liquid–liquid extraction was performed in a separatory funnel, with the organic phase collected [7].

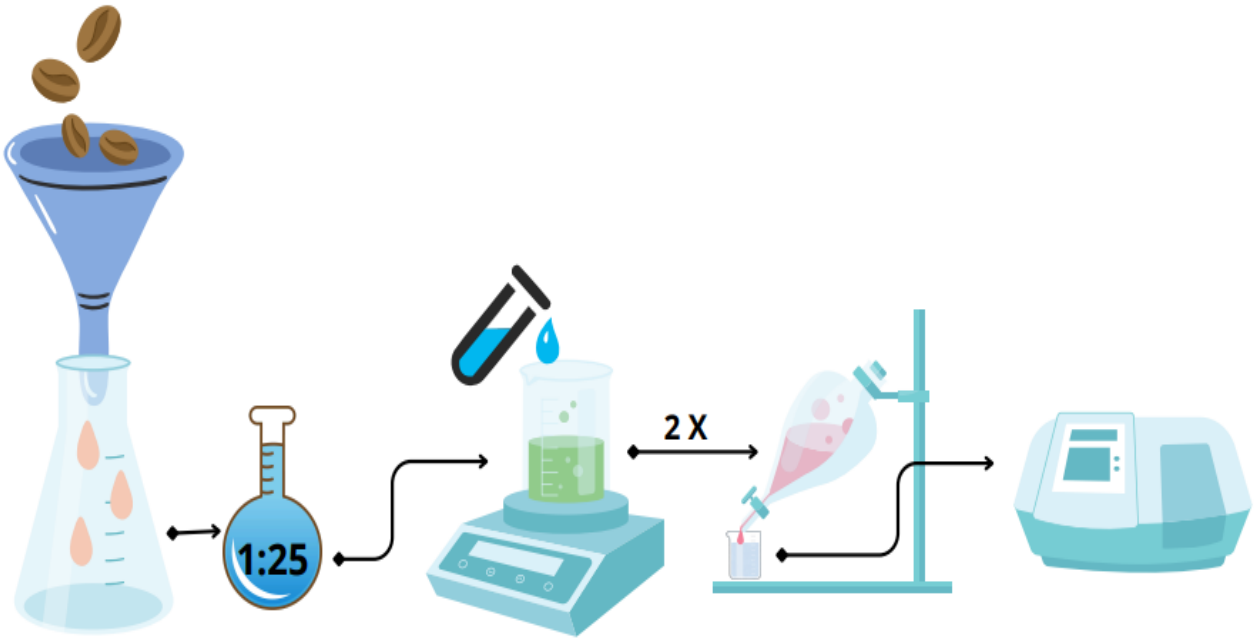

Fig. 4. Preparation of coffee samples for caffeine analysis. Source: Authors (2025).

The resulting sample extracts were then analyzed by UV/Vis molecular absorption spectrophotometry at 275 nm.

3. Results and Discussion

3.1 Caffeine Analytical Curve Result

After performing twelve spectrophotometric measurements, three columns were created in Microsoft Excel (Figure 5): the first containing the identification of the standard solutions, the second their concentrations (corresponding to the x-axis), and the third the absorbance values recorded (corresponding to the y-axis).

Concentration (mg/L) (Xi)	Absorbance (Yi)
0	0.000
0	0.000
1	0.064
1	0.071
2	0.138
2	0.144
4	0.303
4	0.312
6	0.450
6	0.446
8	0.586
8	0.584

Fig 5. Microsoft Excel table with concentration and absorbance values for caffeine. Source: Authors (2025).

Using these values, a calibration curve was constructed in Microsoft Excel by plotting absorbance *versus* concentration and performing linear regression. Following the methodology described by Souza [17], the concentration and absorbance values were plotted using the cell range B4:C15 in the Excel spreadsheet (see the Excel file in the Supplementary Material). With the two columns highlighted, the following sequence of commands was applied: *Insert* > *Chart* > *Scatter Plot*. A trendline was then added to the plot, displaying the regression equation and the coefficient of determination (R^2), as shown in Figure 6.

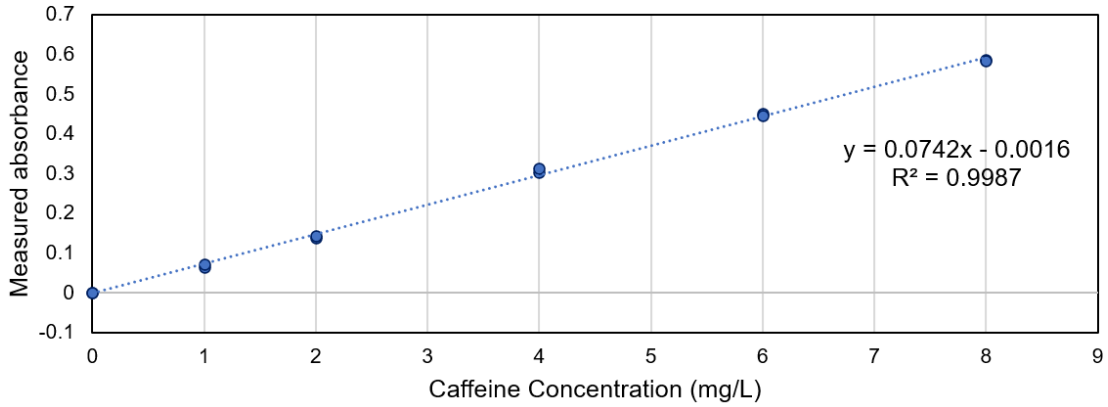

Fig. 6. Scatter plot of the caffeine calibration curve with the added trendline, showing the regression equation and the coefficient of determination (R^2). The curve was constructed in Microsoft Excel to illustrate the relationship between absorbance and caffeine concentration. Source: Authors (2025).

The linearity of the caffeine analytical curve was expressed using the coefficient of determination (R^2 = 0.9987), indicating a strong linear correlation (R^2 close to 1). However, for the validation of analytical procedures, it is recommended that linearity be further evaluated using statistical methods such as analysis of variance (ANOVA), as discussed by Souza *et al*., [11] and Silva *et al*. [7]. The coefficient of determination (R^2) represents the proportion of variance in the dependent variable (absorbance) that is explained by the independent variable (concentration) in a regression model [18]. The limits of detection (LOD) and quantification (LOQ), which were 0.15 mg L^{-1} and 0.50 mg L^{-1}, respectively, were calculated as described in Souza *et al*., [11].

3.1.1 Calculated Residuals for Caffeine Calibration Curve

Using the regression equation, the estimated absorbance values corresponding to the caffeine concentrations were calculated, allowing determination of the residuals for the constructed curve (Table 1).

Table 1. Caffeine concentration and absorbance were measured at 275 nm using a spectrophotometer. Calculation details are provided in the Supplementary Material.

Sample	Observed Concentration (mg/L)	Absorbance (Yi)	Estimated absorbance (y')	Residues
Blank	0	0.000	-0.0016	0.0016
Blank	0	0.000	-0.0016	0.0016
1	1	0.064	0.0726	-0.0086
1	1	0.071	0.0726	-0.0016
2	2	0.138	0.1468	-0.0088
2	2	0.144	0.1468	-0.0028
3	4	0.303	0.2953	0.0077
3	4	0.312	0.2953	0.0167
4	6	0.450	0.4437	0.0063
4	6	0.446	0.4437	0.0023
5	8	0.586	0.5922	-0.0062
5	8	0.584	0.5922	-0.0082
			SQr:	0.000657

Source: Authors (2025)

In linear regression analysis, the vertical deviations between observed and estimated values from the fitted line are called residuals. The least-squares method determines the line that best represents the experimental data by minimizing the sum of squared residuals, ensuring the fitted line closely follows the central trend of the data. In addition to providing the regression coefficients (slope "***b***" and intercept "***a***"), it also allows estimation of the standard deviations of these coefficients, offering a measure of modeling uncertainty and statistical reliability [12].

The constructed model exhibited strong linearity, as evidenced by the very low sum of squared residuals, indicating that the experimental points closely align with the regression line. This measure represents the portion of variability in the dependent variable (*y*) not explained by its linear relationship with the independent variable (*x*) [19]. Squaring residuals assign greater weight to larger deviations, so smaller sums of squared residuals indicate a better fit and more accurate predictions. This is particularly important in chemical analyses, where inadequate fitting may compromise the quantification of unknown samples [20].

The correlation coefficient (R), which is the square root of the coefficient of determination (R^2), was 0.9993 for the model, confirming a strong linear relationship. This indicates that the instrumental response increases proportionally with analyte concentration, resulting in a linear distribution of points. The correlation coefficient ranges from -1 to +1, with values close to +1 indicating a strong positive association between variables. A high R^2 thus reflects both the linearity and the reliability of the analytical curve for quantitative purposes [21].

3.2 Analysis of Caffeine Concentration in Samples

Seven coffee samples from different origins were analyzed. The samples were labeled with numbers from 1 to 7 to ensure impartial evaluation (Table 2). Caffeine was extracted and quantified using a UV/Vis molecular absorption spectrophotometer at 275 nm. The calibration curve equation was applied to estimate caffeine concentrations prior to dilution.

All measured caffeine concentrations fall within regulatory standards. According to International Numbering System (SNI) 01-3542-2004, coffee powder should contain 0.45–2.00% w/w caffeine. This ensures product quality and prevents health risks. Current guidelines (SNI 01-7152-2006) recommend a daily caffeine intake of 50–150 mg [22].

Table 2. Caffeine concentration in coffee samples. Calculation details are provided in the Supplementary Material.

Sample	Absorbance	Sample weight (mg)	Caffeine Concentration (mg/L)	Caffeine Concentration % (w/w)
1	0.169	990.6	114.4	0.58
2	0.186	1006.9	126.8	0.63
3	0.183	995.9	123.8	0.62
4	0.167	1007.1	113.1	0.56
5	0.223	1014.8	150.8	0.74
6	0.183	1007.4	123.8	0.62
7	0.197	1030.1	133.3	0.65

Source: Authors (2025)

The calibration curve showed high linearity (R^2 = 0.9987) and a very low sum of squared residuals (SQr = 0.00065), confirming its reliability for caffeine quantification. Measured concentrations ranged from 114.9 to 151.3 mg L^{-1} (0.56–0.74% w/w), consistent with regulatory requirements and literature values obtained via liquid–liquid extraction. Macheiner et al. [23] reported caffeine levels of 114–188 mg L^{-1} in coffee beans from nine countries, similar to our findings of 114–151 mg L^{-1} for national samples. Merecz et al. [24] observed 114 mg L^{-1} in filtered *Robusta coffee*, highlighting the influence of extraction method on caffeine concentration. Ihsan et al. [25] reported up to 8% w/w in lightly roasted *Robusta* beans, decreasing with higher roasting degrees.

Observed differences among samples likely reflect variations in bean type and processing methods, rather than origin alone. The extraction step is crucial, as transferring caffeine to an organic phase minimizes spectral interference and demonstrates the impact of solvent choice on selectivity and yield. Dichloromethane, for example, efficiently separates caffeine from sugars, acids, and polyphenols, yielding a purer extract [26]. Moreover, the choice of solvent influences key operational parameters, including pH, temperature, and extraction time, allowing optimization for laboratory, industrial, or pharmaceutical applications [27].

3.3 Comparison with alternative methods and interdisciplinary context.

Although UV–Vis spectrophotometry provides an accessible, low-cost route for teaching quantitative analysis, caffeine in coffee can also be determined by more selective instrumental techniques, such as HPLC [28]. In this work, the UV–Vis approach yielded caffeine contents of 0.56-0.74% (w/w) (Table 2), demonstrating that the proposed didactic protocol is suitable for routine quantification exercises and for discussing real sample variability. HPLC typically offers higher selectivity and, in many validated applications, improved precision and lower detection limits. From an educational perspective, this experiment strengthens the interdisciplinary connection between Analytical Chemistry (sample preparation, extraction, instrumental measurement), data treatment/statistics (calibration by linear regression, residual analysis, and validation figures of merit such as LOD/LOQ and repeatability), and contextualized learning.

3.4 Educational Application

It is evident that expository–memorization-based approaches are neither the only nor the most effective strategy for teaching Chemistry. This methodological limitation contributes to many students failing to establish

connections between the content taught and phenomena in their daily lives. Such disconnection tends to be exacerbated when the learning process is restricted to memorizing symbols, formulas, equations, and laws, without adequate contextualization and practical application [29].

The Brazilian National Common Curricular Base (BNCC) emphasizes that contextualizing school content is a fundamental principle to ensure meaningful learning. An essential strategy for the effectiveness of the educational process is relating curricular content to the students' sociocultural, historical, environmental, and temporal reality, adopting methodologies that present, exemplify, connect, and reframe knowledge, thereby making it relevant and applicable to everyday life [30,31].

Moreover, employing complex approaches in a contextualized manner highlights the presence of Chemistry in students' daily lives and enables the contextualization of theoretical content, making the teaching process more dynamic, visual, and experimental. This approach fosters interest, stimulates the pursuit of knowledge, and encourages a critical understanding of the reality in which students are embedded [29].

One way to link everyday life with relevant Chemistry topics is to analyze culturally and socially meaningful issues in students' surroundings. Considering that Brazil is the world's largest coffee producer and exporter, accounting for about 33–35% of global production, and that the coffee sector employs millions of people, especially in small family farms [32], the theme of coffee analysis provides an accessible alternative for addressing Analytical Chemistry techniques. According to the Brazilian Coffee Industry Association (ABIC), Brazilians consume approximately 5 times the global average. Using coffee analysis as a thematic approach in the classroom thus becomes a valuable resource.

4. Conclusions

This study (Part II) achieved its objectives by constructing a linear calibration model and applying it to quantify caffeine in coffee samples using UV–Vis spectrophotometry. In addition, the study effectively reinforced fundamental regression concepts within a practical and accessible teaching context, complementing the didactic proposal presented. The analytical curve for caffeine exhibited strong linearity, as evidenced by the coefficient of determination (R^2 = 0.9987), confirming a strong linear relationship ($R \approx 1$), and the model showed a low sum of squared residuals. All samples analyzed complied with legislative standards, ranging from 0.45% to 2.00% (w/w). Beyond the analytical results, this work highlights the value of integrating real-world examples, such as coffee analysis, into the teaching of Analytical Chemistry, promoting student engagement and enhancing understanding of key quantitative concepts. The proposed approach demonstrates clear potential as an effective educational tool, capable of fostering meaningful improvements in the teaching–learning process of Analytical Chemistry.

Author Contributions

Gilmara da Silva Rangel, Tayná da Silva Picanço and Rafael Pinheiro Caetano Damasceno contributed to conceptualization, methodology, investigation, formal analysis, data curation and writing – original draft. Sávio da Silva Berilli, Ana Carla Rangel Rosa and Mario Ferreira Conceicão Santos contributed to data curation and writing – original draft. Murilo de Oliveira Souza contributed to data curation and writing – original draft, writing – review & editing, supervision, project administration, formal analysis and funding acquisition.

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