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Development and validation of a green spectrophotometric method for simultaneous determination of combined pharmaceutical dosage form (paracetamol and caffeine) using chemometrics technique in comparison with HPLC

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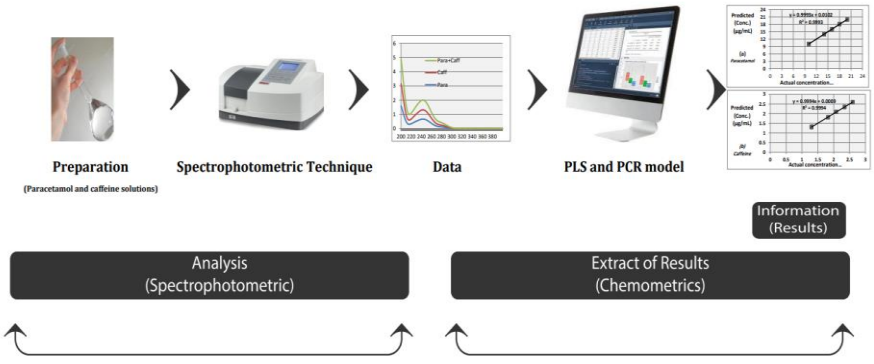
Abstract

A green analytical method, a simple, fast, and cost-effective simultaneous spectrophotometric method using two chemometric techniques, the partial least square regression (PLS) and principal component regression (PCR), for determining a combination of paracetamol and caffeine in pharmaceutical formulations was developed. Pretreatment and separation steps are not required in the proposed method. For model construction and validation, various drug concentrations and instrumental spectra of 25 mixed solutions of paracetamol and caffeine were analyzed. The UV analysis of the prepared mixtures was recorded for a selected solvent blank in the wavelength range of 210-300 nm. The digitized absorbance was sampled at 0.2-nm intervals. R^2 values of 0.9993 and 0.9994 assigned for the PLS of paracetamol and caffeine and 0.9995 and 0.9991 for the PCR of paracetamol and caffeine, respectively, exhibited greater prediction efficiencies. The obtained results were statistically compared with the results of the HPLC reference method. Concerning accuracy and precision, the statistical comparison revealed no significant differences between the suggested and reference HPLC approaches.



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Highlights

- Development and validation of a new eco-friendly chemometric spectrophotometric.
- The proposed methods are statistically compared with reported HPLC method.
- Can be used for the routine quality control of paracetamol and caffeine analysis.

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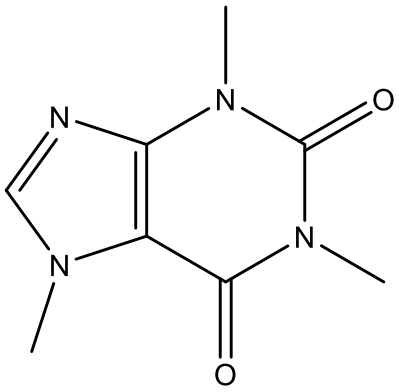
1. Introduction

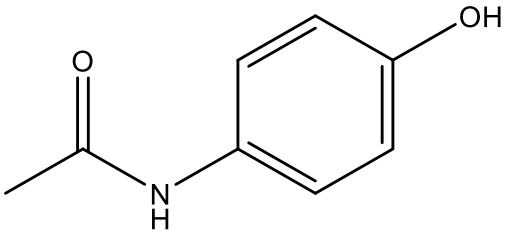
The combination of paracetamol and caffeine is commonly used as a pain reliever and antipyretic agent in pharmaceutical formulations (Uddin *et al.*, 2019). Chemically, paracetamol is (N-(4-hydroxyphenyl) acetamide (**Scheme 1a**). Paracetamol, also known as paracetamol, is one of the most popular medications commonly used to treat fever (antipyretic) and mild to moderate pain (analgesic agent) (Drugbank, 2005a; Glavanović *et al.*, 2016; Yehia and Mohamed, 2016). Caffeine is 1,3,7-Trimethyl-3,7-dihydro-1H-purine-2,6-dione and its chemical structure (**Scheme 1b**). It is one of the drugs mostly used worldwide as a Central Nervous System (CNS) stimulant of the methylxanthine class (Drugbank, 2005b; Uddin *et al.*, 2019).

1,3,7-Trimethylpurine-2,6-dione
Caffeine



a)

n-(4-hydroxyphenyl)acetamide
paracetamol



b)

Scheme 1. Chemical structure of paracetamol **(a)** and caffeine **(b)**.
Source: Adapted from Drugbank (2005a; b).

The field of chemometrics has had a significant impact on analytical chemistry, particularly in the area of spectral analysis, which is important in the quality control of mixed drugs and pharmaceutical formulations involving two or more medications of overlapping spectra (Eticha *et al.*, 2018; Glavanović *et al.*, 2016; K. Patel *et al.*, 2013a).

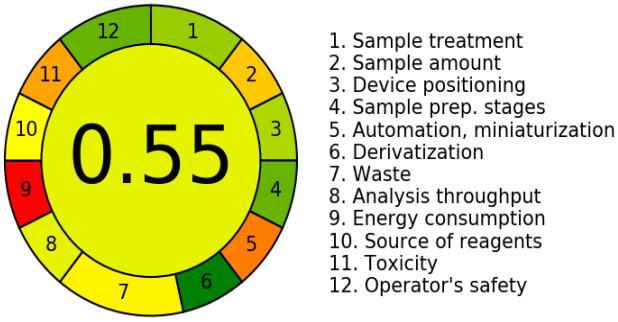
Chemometric methods depend on multivariate analysis, which means considering more than one variable at a time in UV Spectrophotometry techniques (Riddhi and Rajashree, 2019). Many wavelengths are taken as variables, and the absorbance at each wavelength is considered (Gandhi *et al.*, 2017; Riddhi and Rajashree, 2019). The most important chemometric methods used in multivariate analysis are Principal Component Regression (PCR) and Partial Least Squares (PLS). These methods use multivariate calibration using spectrophotometric data along with statistical tools, mathematical models, and software for the determination of combined drugs in pharmaceutical formulations (Riddhi and Rajashree, 2019). These methods also rely on the calibration of the mathematical model by using absorbance data of calibration standards with known concentrations and then predicting the concentration of unknown samples from their absorbance data (Gandhi *et al.*, 2017; Riddhi and Rajashree, 2019).

Chemometrics has multiple applications in spectroscopy, including UV-visible spectrophotometry (Ashour *et al.*, 2015; Attia *et al.*, 2018; Belal *et al.*, 2018; Darbandi *et al.*, 2020; Elfatatry *et al.*, 2016; Gholse *et al.*, 2022; Manouchehri *et al.*, 2016; Mattar and Sobhy, 2022; Moussa *et al.*, 2021; M. Patel *et al.*, 2013b; Phechkrajang *et al.*, 2015; Putri *et al.*, 2021; Sebaiy *et al.*, 2020; V. D. Singh and V. K. Singh, 2021; Vichare *et al.*, 2010), fluorescence spectroscopy (Manouchehri *et al.*, 2016; Salem *et al.*, 2019; Shinde and Divya, 2015; Walash *et al.*, 2011; Zhu *et al.*, 2016), NIR spectroscopy (Manouchehri *et al.*, 2016; Moroni *et al.*, 2022; Muntean *et al.*, 2021; Muntean *et al.*, 2017; Rahman *et al.*, 2020; Sun *et al.*, 2021), and FTIR spectroscopy (Rahman *et al.*, 2020). In addition, chromatography techniques such as Liquid Chromatography (Aminu *et al.*, 2019; Mohammed *et al.*, 2021; Tsvetkova *et al.*, 2012; Vu Dang *et al.*, 2020) as well as a variety of other analytical chemistry techniques, such as flow-injection analysis (Ortega-Barrales *et al.*, 2002; Silva *et al.*, 2011).

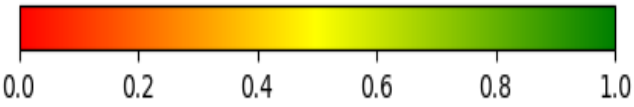
Uddin *et al.* (2019) reported that the classic UV spectral assay could not be used to determine most analytes of interest because they are accompanied in their dose forms by other substances that absorb in the same spectral area. Traditional procedures, such as extraction, are difficult to employ because they require a lot of solvent, which comes with hazards of analyte loss or contamination, as well as the likelihood of incomplete separation, which is costly and time-consuming. However, when paired with chemometric methods for determining a combined mixture in pharmaceutical quality control, spectrophotometry as a simple, precise, rapid, and low-cost method may be a great option. They provide benefits when the quality monitoring of pharmaceutical products demands reliable, accurate, and fast analytical procedures. This process avoids prior separation processes and is fast, accurate, and easy to use.

One of the tools used to assess the greenness of analytical procedures is the analytical Greenness Calculator, which is based on the 12 principles of Green Analytical Chemistry. It is a tool for assessing the environmental and occupational risks connected with a certain analytical technique applied in this study (Gałuszka *et al.*, 2013), as shown in **Scheme 2**. The criteria scores and the Analytical Greenness score are linked to a "traffic lights" red-yellow-green sequential color map, with red assigned to the lowest values and green to the highest values, and its value ranges from 0.0 (the lowest score) to 1.0 (perfect score) (Tobiszewski *et al.*, 2017), as shown in **Scheme 3**.

To the best of our knowledge, no published work has been conducted on developing and validating spectrophotometric methods for the examination of some combined pharmacological compounds using a chemometrics approach in the Yemeni market (The Republic of Yemen). Therefore, the present study aims to develop and validate an adequate and green simultaneous spectrophotometric assay method for the determination of paracetamol and caffeine in a combined pharmaceutical formulation-assisted chemometric technique.



Scheme 2. Annotated result of the generic assessment.



Scheme 3. The span of the colour map used in the graph and the corresponding values.

2. Materials and methods

2.1. Materials and reagents

The reference standard paracetamol and caffeine were obtained from Global Pharma Company, Sana'a, Yemen. All reagents and chemicals used for the spectrophotometric methods were of analytical grade, and HPLC grade was used for the HPLC method. Deionized water (with a specific conductance of 0.05 µS cm^{-1}) was in-house produced and used for the preparation of all sample solutions. Hydrochloric acid, sodium hydroxide, and benzoic acid were obtained from Shiba'a Pharma Company, Sana'a, Yemen.

- ***Preparation of standard stock solution:*** Stock solutions of 1000 µg mL^{-1} of paracetamol and 130 µg mL^{-1} of caffeine were individually prepared in a 100 mL volumetric flask by dissolving 100 mg paracetamol and 13 mg caffeine separately in water.
- ***Preparation of hydrochloric acid solution:*** it was prepared by diluting appropriate amounts of reagent in deionized water to make 0.1 mol L^{-1}.
- ***Preparation of sodium hydroxide solution:*** This solution was prepared by dissolving 4.00 g of NaOH pellet into a 1000 mL volumetric flask in deionized water to obtain a final concentration of 0.1 mol L^{-1}.
- ***Preparation of the benzoic acid solution:*** it was prepared by dissolving appropriate amounts of benzoic acid in methanol.

2.2. Instrumentation

A double beam UV-Vis spectrophotometer (analytik jena), Model (SPECORD 200) at Sana'a University-Faculty of Science was used for the absorbance measurements. The HPLC system was from JASCO and included a UV detector (UV-2070 Plus), pump (PU-2089), autosampler (AS-2055 Plus), column oven (CO-2067 Plus), and a C18 column (10 cm × 4.6 mm, 5 µm). Electronic balance (AA-160), Denver Instrument. Electronic balance (GH-252), AND. Electronic balance (GR-120), AND. pH meter (3520), Jenway. A centrifuge (Z326 K) and Hermle were also used.

2.3. Development procedures

To develop accurate, precise, and reliable simultaneous spectrophotometric methods assisted with the chemometrics technique, analytical methods were established and developed to obtain the intended results for quantifying the targeted components. The suitability of the proposed and developed method was decided based on the results of the validation method. This method was studied and experimented for the paracetamol determination with caffeine in marketed pharmaceutical formulations. They were compared to the results of the reference method.

2.3.1. Selection of the solvent

The effect of the solvent on solubility was studied to choose a suitable solvent. Solubility was checked in water, methanol, 0.1 mol L^{-1} NaOH, and 0.1 mol L^{-1} HCl. The targeted combined active pharmaceutical ingredients in this study were dissolved in volumetric flasks by adding appropriate amounts of selected solvents for the dissolution of the desired active pharmaceutical components without excipients.

2.3.2. Selection of the spectral zone analysis

After the solvent selection step and before pre-processing the data, the individual pure and mixture absorbance spectra of the targeted pharmaceutical components in an appropriately selected solvent were recorded in the range of 200–400 nm with 0.2 nm intervals. UV spectra of the mixtures analysis were selected among a suitable wavelength range against a solvent blank, providing the greatest amount of information about the two components (Shah and Jasani, 2017).

2.3.3. Construction of the training set

Twenty-five different concentrations of paracetamol and caffeine binary mixtures were prepared as the training set (calibration set) to construct the model. The absorbencies of these mixtures were measured between 200 and 400 nm at 0.2-nm intervals against a blank.

2.4. Validation of the chemometric analysis

2.4.1. Construction of the chemometric models

The two multivariate calibration models; the partial least square (PLS) and principal component regression (PCR), were developed as follows:

- The absorbencies of binary mixtures were measured against a blank, and the spectra were saved and extracted into MS Excel for model generation and merit figures to evaluate the obtained results;
- The PCR and PLS models were developed using absorption data at selected spectral zones for analysis at intervals of 0.2 nm using the Minitab 17 program;
- The leave-one-out (LOO) cross-validation method was used to obtain the necessary number of latent variables (optimum number of the principal factors);
- The calibration samples, constant, and coefficients at each wavelength were calculated to obtain the predicted concentrations;

- Finally, the predicted concentrations of the components were compared with the actual concentrations in each sample and the binary mixture was calculated for each sample;
- To determine the precision and accuracy of predictions for the models, the root mean square error of cross-validation (RMSECV), which must be as low as possible for a particular model, was calculated for each method using the following **Eq. 1** (Shah and Jasani, 2017):

$$RMSECV = \sqrt{\frac{\sum(C_{act} - C_{pre})^2}{I_c}} \quad (1)$$

where:
RMSECV = Root means square error of cross-validation
C_{act} = Actual concentration of the calibration set
C_{pre} = predicted concentration of the calibration set
I_c = Total number of samples in the calibration set

2.4.2. Validation method and construction of the validation set

To validate and evaluate the performance of the proposed and developed spectrophotometric methods assisted by chemometric models, these methods were applied to the validation set. In addition, the performance criteria of the developed methods, including linearity, accuracy, precision (repeatability), and specificity, were validated as per the recommendations of International Conference Harmonization (ICH) and hence determined.

2.5. Analytical method procedures

2.5.1. Construction of the calibration (training) set

Several 25 binary mixtures of paracetamol and caffeine were prepared by transferring different aliquots of their standard stock solutions into a series of 50 mL volumetric flasks (**Table 1**). The absorbencies of these mixtures were measured between 200 and 400 nm at 0.2 nm intervals against water as a blank.

2.5.2. Construction of the validation set

A set of 12 binary mixtures of paracetamol and caffeine was prepared by transferring different volumes into 50 mL volumetric flasks, and the procedure for the construction of the training set was repeated (**Table 2**).

2.5.3. Preparation of the test sample

Approximately 20 tablets of a commercial pharmaceutical formulation tablet containing 500/65 mg of paracetamol/caffeine, respectively, were analysed using the proposed chemometric methods. The sample 500/65 were weighed and finely powdered in a mortar. A quantity of powdered tablets equivalent to 100 mg of paracetamol and 13 mg of caffeine was accurately weighed and transferred into a 100 mL volumetric flask containing 50 ml of water. The mixture was shaken for 5 min, and with frequent shaking, the volume was completed to 100 mL with the selected solvent. The solution was then filtered through 0.45 µm filter paper. 0.8 mL of the filtrate was transferred into a 50 mL volumetric flask and then diluted by completion to 50 mL with water. The absorbance was measured between 200 and 400 nm at 0.2-nm intervals against water as a blank.

2.5.4. Preparation of spiked samples

Powdered tablets of 100 mg paracetamol and 13 mg caffeine in triplicates were accurately weighed and transferred to a 100 mL volumetric flask. Then, 50 mL of water was added, and the calculated amount of paracetamol and caffeine from standard solutions was spiked into the sample solution. The mixture was shaken for 5 min, and with frequent shaking the volume completion to 100 mL with the selected solvent was carried out. The solution was then filtered. A total of 0.8 mL of the filtrate was transferred into a 50 mL volumetric flask and then diluted with water up to 50 mL. The absorbance was then measured.

2.5.5. Analysis of the marketed formulations

The developed method was applied to the measurement of three commercially available samples. It was performed using the marketed formulation with a concentration of 500 mg paracetamol and 65 mg caffeine. The tablet solution prepared in the sample preparation section was diluted with water to prepare solutions with a concentration of 16 µg mL^{-1} paracetamol and 2.08 µg mL^{-1} caffeine. The spectra of the prepared solutions were recorded, and then the developed multivariate models PCR and PLS were applied to determine the concentrations of paracetamol and caffeine.

2.6. Comparing the suggested method with the reference method

Comparison was carried out with the recovery results of the newly developed methods and that of reference method for each of paracetamol with caffeine according to the United States Pharmacopeia (USP, 43). 100 µg mL^{-1} paracetamol with 13 µg mL^{-1} caffeine and 360 µg mL^{-1} of benzoic acid as internal standard solution were prepared by dissolving 100 mg paracetamol with 13 mg caffeine in methanol: glacial acetic acid (95:5) in a 100 mL volumetric flask as standard stock solution. The internal standard solution was prepared in a 100 mL volumetric flask by dissolving 600 mg of benzoic acid in methanol. 5 mL of paracetamol with the caffeine of the standard stock solution and 3 mL of internal standard solution were transferred in methanol: glacial acetic acid (95:5) in a 50 mL volumetric flask. A test sample was prepared by transferring a portion of the powder equivalent to 250 mg paracetamol with 32.5 mg caffeine from NLT 20 finely powdered tablets to a 100 mL volumetric flask. 75 mL of methanol: glacial acetic acid (95:5) as solvent was added as solvent and the solution was shaken for 30 min and then diluted with solvent. Two milliliters of this solution and 3 mL of internal standard solution were transferred into 50 mL volumetric flask and diluted with solvent. The standard and test samples of paracetamol with caffeine were injected through an HPLC system with a mixture of methanol: glacial acetic acid: and water (28: 3: 69) as the mobile phase at a flow rate of 2 mL/min. UV detection of paracetamol and caffeine was then carried out at 275 nm (United States Pharmacopeia and the National Formulary (USP 43 - NF 38). The United States Pharmacopeial Convention; 2020).

Table 1. Composition of the calibration set.

Mixture No.	Paracetamol (µg mL^{-1})	Caffeine (µg mL^{-1})	Mixture No.	Paracetamol (µg mL^{-1})	Caffeine (µg mL^{-1})
1	10	1.3	14	16	2.34
2	10	1.82	15	16	2.6
3	10	2.08	16	18	1.3
4	10	2.34	17	18	1.82
5	10	2.6	18	18	2.08
6	14	1.3	19	18	2.34
7	14	1.82	20	18	2.6
8	14	2.08	21	20	1.3
9	14	2.34	22	20	1.82
10	14	2.6	23	20	2.08
11	16	1.3	24	20	2.34
12	16	1.82	25	20	2.6
13	16	2.08	--	--	--

Table 2. Results of the predicted concent vrations with the recovery of paracetamol and caffeine in the binary mixture in each sample for the PLS model.

Name		Paracetamol			Caffeine	
Constant		**-0.20039**			**-0.02079**	
Mixture NO.	**Actual Conc. (µg mL^{-1})**	**Predicted Conc. (µg mL^{-1})**	**%Recovery**	**Actual Conc. (µg mL^{-1})**	**Predicted Conc. (µg mL^{-1})**	**%Recovery**
1	10	10.07	100.70	1.3	1.30	100.00
2	10	10.01	100.10	1.82	1.82	100.00
3	10	9.84	98.40	2.08	2.08	100.00
4	10	10.09	100.90	2.34	2.35	100.43
5	10	9.92	99.20	2.6	2.60	100.00
6	14	14.02	100.14	1.3	1.30	100.00
7	14	13.96	99.71	1.82	1.80	98.90
8	14	14.02	100.14	2.08	2.08	100.00
9	14	13.98	99.86	2.34	2.35	100.43
10	14	14.04	100.29	2.6	2.59	99.62
11	16	15.98	99.88	1.3	1.29	99.23
12	16	15.90	99.38	1.82	1.81	99.45
13	16	16.05	100.31	2.08	2.09	100.48
14	16	15.91	99.44	2.34	2.33	99.57
15	16	16.15	100.94	2.6	2.61	100.38
16	18	18.01	100.06	1.3	1.30	100.00
17	18	18.11	100.61	1.82	1.80	98.90
18	18	18.00	100.00	2.08	2.08	100.00
19	18	18.06	100.33	2.34	2.34	100.00
20	18	18.19	101.06	2.6	2.59	99.62
21	20	20.09	100.45	1.3	1.33	102.31
22	20	19.82	99.10	1.82	1.81	99.45
23	20	20.00	100.00	2.08	2.10	100.96
24	20	19.87	99.35	2.34	2.34	100.00
25	20	19.89	99.45	2.6	2.60	100.00
		Mean%	99.99		Mean%	99.99
		RSD%	0.64		RSD%	0.68
		RMSECV	0.093		RMSECV	0.011

3. Results and Discussion

3.1. Development procedures for paracetamol and caffeine determination

3.1.1. Selection of the solvent

To choose a suitable solvent, solubility was checked in water, methanol, 0.1 mol L^{-1} NaOH, and 0.1 mol L^{-1} HCl. The drug was found to be soluble in methanol, water, 0.1 mol L^{-1} NaOH, and 0.1 mol L^{-1} HCl. Therefore, water was selected as a diluent that has striking advantages such as being easily available, easy to handle, cheap, and environmentally friendly for implementing the spectrophotometric method, and **Fig. 1** shows the spectra of paracetamol and caffeine in water.

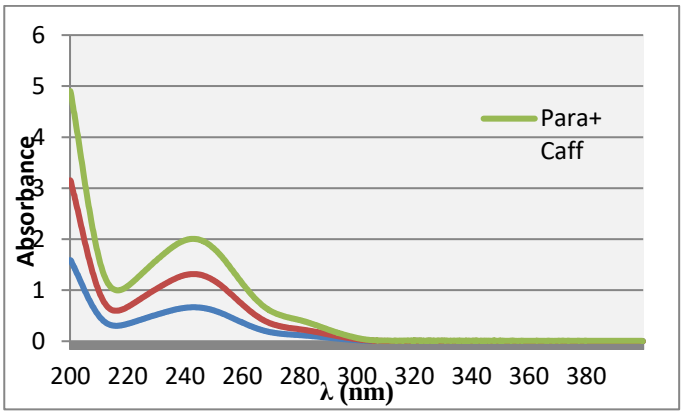


Figure 1. UV Absorbance spectra of pure and mixed samples of paracetamol and caffeine in water solvent.

3.1.2. Selection of the spectral zones for analysis

To determine the overlap spectral zones, the absorbance spectra of the pure paracetamol and caffeine samples and that of the sample of the mixed paracetamol with caffeine in water were recorded in the range of 200–400 nm with 0.2 nm intervals. For the analysis, the UV spectra of the mixtures were selected for a suitable wavelength range (210-300 nm) against the water blank. This range provided a great amount of information about the two components, as shown in the paracetamol and caffeine spectra (**Fig. 1**).

3.1.3. Construction of the training set

To determine the linear range from measuring the absorbance at different concentrations for paracetamol with caffeine, the response was found to be linear in the range of 10–20 μg mL^{-1} for paracetamol and 1.3–2.6 μg mL^{-1} for caffeine using 25 different concentrations of paracetamol and caffeine mixtures, as shown in **Table 1**.

3.2. Validation of the chemometric analysis for paracetamol and caffeine determination

3.2.1. Construction of chemometric models

The spectra were saved and extracted into MS Excel for model generation. The PCR and PLS models were developed using the absorption data for the selected spectral zones using the Minitab 17 software. After the PCR and PLS models were constructed, the optimum number of principal components of paracetamol and caffeine were obtained and given in **Table S1–S4 (Supplementary Material)**.

3.2.1.1. Determination of the optimum number of principal components of paracetamol and caffeine for PLS

Choosing the proper number of principal components for the development of the model was necessary to obtain good predictions. The leave-one-out (LOO) cross-validation method was used to obtain the necessary optimum number of principal factors for the PLS model. It was found that the optimum number of principal components was three for paracetamol and four for caffeine, as mentioned above and given in **Tables S1 and S2**.

3.2.1.2. Determination of constants and coefficients obtained at each wavelength of paracetamol and caffeine for PLS models

The constant and coefficients at each wavelength were calculated using the Minitab 17 program, as illustrated in **Table S3**.

3.2.1.3. Determination of predicted concentrations and recovery of paracetamol and caffeine in PLS models

The predicted or calculated concentrations in μg mL^{-1} of the paracetamol and caffeine were calculated from the multiple regression **Eq. 2**.

The predicted or calculated concentrations of the components were compared with the actual concentrations, and the assay of the binary mixture was performed. The root mean square error of cross-validation (RMSECV) was calculated and found to be low. The low values of RMSECV in **Table 2**indicate that both the precision and accuracy of the PLS model for paracetamol and caffeine were very high, and the R^2 values in **Fig. 2** were also of high linearity.

The linearity of the developed method of the PLS model was tested by constructing a cross-validation of the data in **Table 2**. The results obtained in **Fig. 2** indicate that the developed method possessed high linearity with R^2 = 0.9993 within the method linear range (10–20 μg mL^{-1}) for paracetamol and R^2 = 0.9994 within the method linear range (1.3–2.6 μg mL^{-1}) for caffeine. In comparison, Uddin *et al.* (2019) revealed less linearity with R^2 values of 0.9928 and 0.9933 assigned for the PLSR of paracetamol and caffeine in methanol solvent, respectively. In contrast, the other study (Aktaş and Kitiş, 2014) that was carried out in 0.1 mol L^{-1} HCl revealed linearity almost similar to our eco-friendly developed method.

$$\text{Predicted (Calculated)} = \text{Constant} + \sum(\text{Coefficient} \times \text{Absorbance}) \quad (2)$$

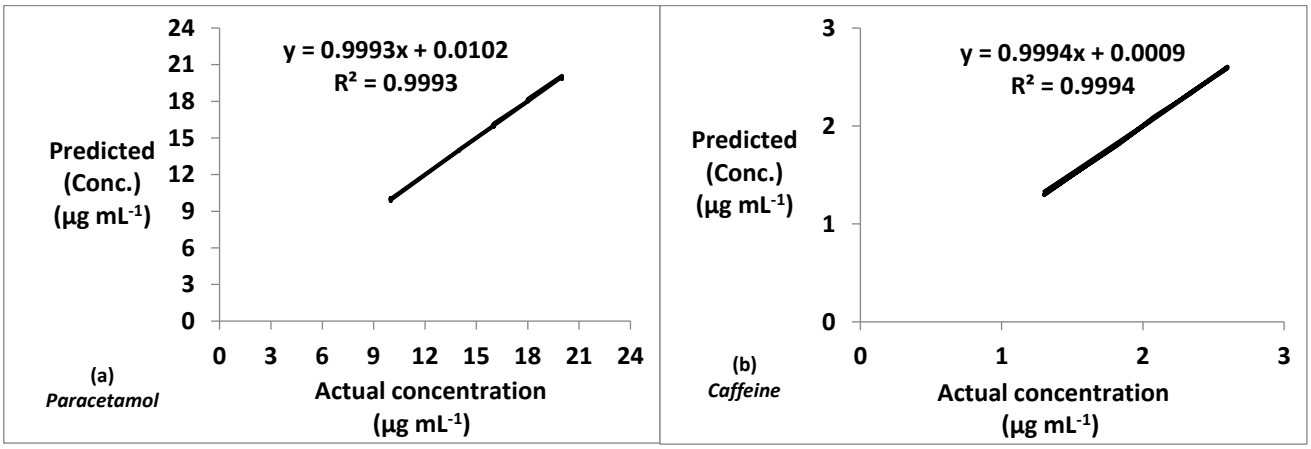


Figure 2. PLS cross-validation for the calibration set of the actual vs. predicted concentration.

3.2.1.4. Determination of the optimum number of principals components and their coefficients of paracetamol and caffeine for PCR

The PCR was computed using six principal components (PCs) and a regression analysis of these PCs with a concentration was performed to determine the PC coefficients of paracetamol and caffeine for the PCR model, as shown in **Table S4**. From the treatment of the principal component's coefficients in (**Table S4**) using the Minitab 17 program. Regression equations for paracetamol and caffeine were obtained and used to calculate the predicted concentrations, as shown below.

Response variable (Predicted concentration) of paracetamol

(3) -0.177 + 1.23301 Z1 + 1.1417 Z2 + 3.102 Z3 + 0.81 Z4 + 2.94 Z5 + 16.91 Z6

Response variable (Predicted concentration) of caffeine

(4) 0.0284 + 0.01374 Z1 + 1.5390 Z2 + 3.531 Z3 + 1.016 Z4 + 1.491 Z5 + 1.30 Z6

where: Z is the principal component coefficients.

3.2.1.5. Determination of the predicted concentrations and recovery of paracetamol and caffeine in the PCR models

The predicted or calculated concentrations in μg mL^{-1} of the paracetamol and caffeine were calculated from the above regression equations.

The predicted or calculated concentrations of paracetamol and caffeine were compared with the actual concentrations, and the assay for binary mixture was performed for each sample. The root mean square error of cross-validation (RMSECV) was calculated and found to be minimal. The small RMSECV values in **Table 3** indicate that both the precision and accuracy of the PCR model for paracetamol and caffeine were very great, with the R^2 values in **Fig. 3** showing very strong linearity.

3.2.2 Validation procedures and construction of the validation set for paracetamol and caffeine determination

3.2.2.1 Linearity method

The linearity of the developed methods for both the PLS and PCR models was tested by constructing a cross-validation of the data, as shown in **Table 4**. The results obtained (**Figs. 4** and **5**) indicated that the developed method possessed high linearity: R^2 = 0.9989 and 0.9988 for the PLS and PCR models, respectively, within the method linear range (10 – 20 μg mL^{-1}) of paracetamol. Whereas R^2 = 0.9989 and 0.9987 for the PLS and PCR models, respectively, within the method linear range (1.3–2.6 μg mL^{-1}) of caffeine. The linearity of the developed method was better than that of the method in Uddin *et al.* (2019). In addition, another study by Alam *et al.* (2022) showed less linearity with R^2 values of 0.9970 and 0.9928 assigned for the linear regression analysis of paracetamol and caffeine using the greener normal-phase HPTLC technique, respectively, and with R^2 values of 0.9966 and 0.9976 assigned for the linear regression analysis of paracetamol and caffeine using the greener reversed-phase HPTLC technique, respectively.

3.2.2.2. Construction of validation set

The results of the prediction and the percentage recoveries are presented in **Table 4**. The predictive abilities of the models were evaluated by plotting the actual known concentrations against the predicted concentrations shown in **Figs. 4** and **5**. A tremendous agreement between the predicted (calculated) and actual paracetamol and caffeine concentrations for the PLS and PCR models can be observed in **Figs. 4** and **5**.

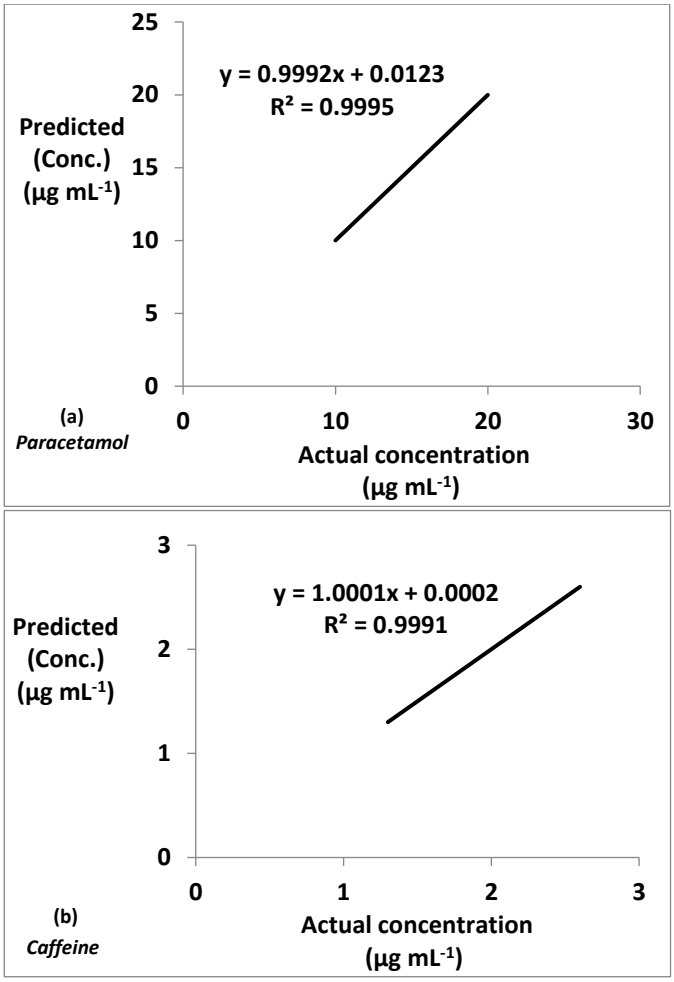


Figure 3. PCR cross-validation for the calibration set of the actual vs. predicted concentrations.

Table 3. Results of the predicted concentrations with the recovery of paracetamol and caffeine in the binary mixture in each sample for the PCR models.

Name		Paracetamol			Caffeine	
Constant		-0.177			0.0284	
Mixture NO.	Actual Conc. (μg mL^{-1})	Predicted Conc. (μg mL^{-1})	%Recovery	Actual Conc. (μg mL^{-1})	Predicted Conc. (μg mL^{-1})	%Recovery
1	10	10.09	100.90	1.3	1.30	100.00
2	10	10.04	100.40	1.82	1.82	100.00
3	10	9.85	98.50	2.08	2.08	100.00
4	10	10.10	101.00	2.34	2.35	100.43
5	10	9.93	99.30	2.6	2.60	100.00
6	14	13.97	99.79	1.3	1.31	100.77
7	14	13.94	99.57	1.82	1.80	98.90
8	14	14.02	100.14	2.08	2.09	100.48
9	14	13.99	99.93	2.34	2.35	100.43
10	14	14.07	100.50	2.6	2.59	99.62
11	16	15.99	99.94	1.3	1.30	100.00
12	16	15.88	99.25	1.82	1.81	99.45
13	16	16.05	100.31	2.08	2.10	100.96
14	16	15.90	99.38	2.34	2.32	99.15
15	16	16.15	100.94	2.6	2.61	100.38
16	18	18.01	100.06	1.3	1.30	100.00
17	18	18.05	100.28	1.82	1.78	97.80
18	18	17.91	99.50	2.08	2.08	100.00
19	18	18.04	100.22	2.34	2.34	100.00
20	18	18.11	100.61	2.6	2.59	99.62
21	20	20.08	100.40	1.3	1.32	101.54
22	20	20.00	100.00	1.82	1.81	99.45
23	20	19.98	99.90	2.08	2.10	100.96
24	20	19.87	99.35	2.34	2.35	100.43
25	20	19.96	99.80	2.6	2.61	100.38
		Mean%	100.00		Mean%	100.03
		RSD%	0.60		RSD %	0.75
		RMSECV	0.079		RMSECV	0.014

Table 4. Results of the validation set of paracetamol and caffeine for the PLS and PCR models.

NO.	METHOD			PLS				PCR		
	Para.		Caff.	Para.		Caff.		Para.		Caff.
	Actual (μg mL^{-1})	Predicted (μg mL^{-1})	%R	Predicted (μg mL^{-1})	%R	Predicted (μg mL^{-1})	%R	Predicted (μg mL^{-1})	%R	Predicted (μg mL^{-1})	%R
1	10	2.34	10.178	101.78	2.344	100.17	10.247	102.47	2.312	98.80
2	10	2.60	10.030	100.30	2.610	100.38	10.096	100.96	2.573	98.96
3	16	1.82	15.912	99.45	1.800	98.90	15.900	99.38	1.784	98.02
4	16	2.08	15.876	99.23	2.056	98.85	15.896	99.35	2.040	98.08
5	20	1.30	19.798	98.99	1.257	96.69	19.746	98.73	1.248	96.00
6	20	2.60	19.905	99.53	2.575	99.04	19.893	99.47	2.545	97.88
7	12	2.808	11.806	98.38	2.864	101.99	11.852	98.77	2.837	101.03
8	12	3.12	11.802	98.35	3.155	101.12	11.829	98.58	3.136	100.51
9	19.2	2.184	18.847	98.16	2.181	99.86	18.812	97.98	2.162	98.99
10	19.2	2.496	19.445	101.28	2.470	98.96	19.431	101.20	2.444	97.92
11	24	1.56	23.865	99.44	1.530	98.08	23.795	99.15	1.514	97.05
12	24	3.12	23.946	99.78	3.129	100.29	23.895	99.56	3.094	99.17
			Mean%	99.55	--	99.53	Mean%	99.63	--	98.54
			RSD%	1.12	--	1.42	RSD%	1.29	--	1.40

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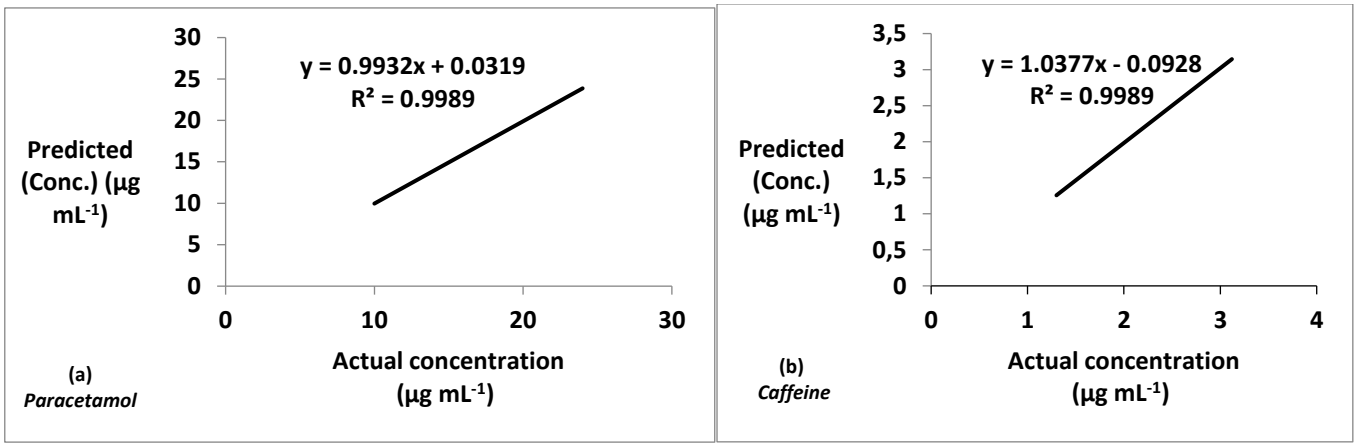

Figure 4. PLS cross-validation for the validation set of the actual vs. predicted concentrations.

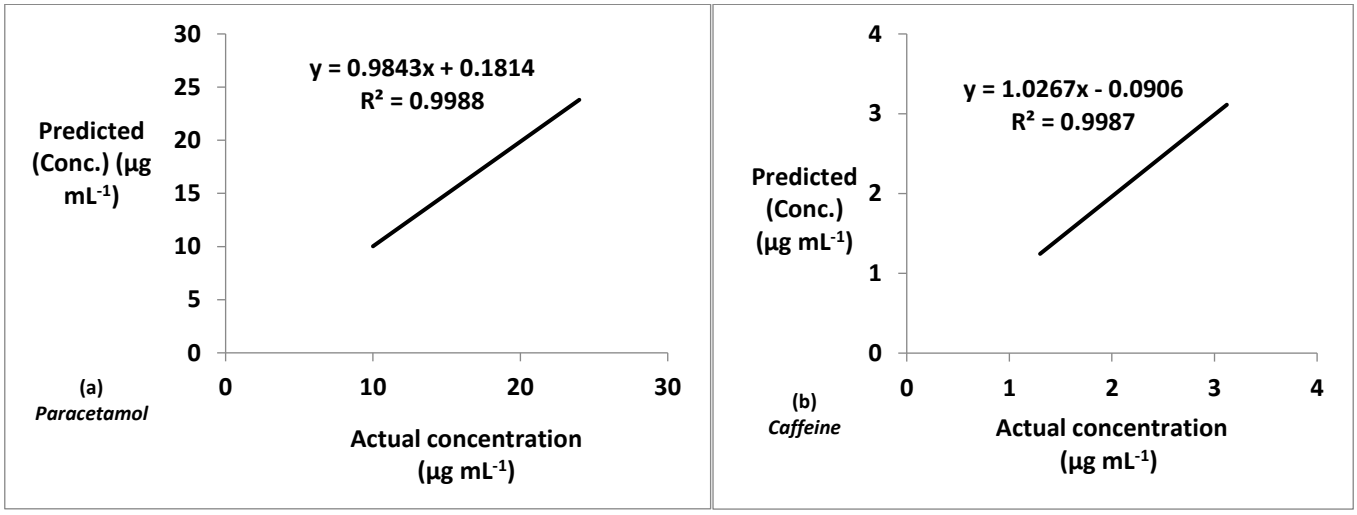

Figure 5. PCR cross-validation for the validation set of the actual vs. predicted concentrations.

3.2.2.3. Precision (Repeatability)

The repeatability (intraday precision) of the developed method was determined by determining the binary mixture at three different concentrations for paracetamol and caffeine in bulk using three different concentrations (i.e., 10/1.3, 16/1.82 and 20/2.6 µg mL^{-1} of paracetamol/caffeine, respectively) sequentially in triplicates. The results are reported as percentage RSD. The low values of percentage RSD indicated the high precision of the method. The %RSD values of the developed method were within the acceptable limit as suggested by the USP pharmacopeia, and the results are presented in **Table 5**.

3.2.2.4. Accuracy

The accuracy of the method was investigated using the standard addition method for three different percentage levels (i.e., 80, 100, and 120%) by recovery experiments. Known amounts of standard solutions containing paracetamol and caffeine were added to sample solutions under investigation to make up solutions of 80%, 100%, and 120% levels in triplicate and scanned in the range 200–400 nm. The quantity of drugs recovered at each percentage level was determined using the developed PCR and PLS models. The mean percentage recovery for each percentage level showed low values of percentage RSD, and the percentage recovery was within the acceptable limit (90–110%) as suggested by the USP pharmacopeia. This indicates a high accuracy method at all three levels, and the accuracy data are given in **Tables 6** and **7**.

3.2.2.5. Specificity (spiking method)

The specificity of the method was checked by adding a certain amount of paracetamol and caffeine standard into a known amount of the marketed sample solution, as described earlier (i.e., Methodology). Specificity data are shown in **Tables 8** and **9**.

As can be seen from these data, recovery for paracetamol and caffeine using the developed PCR and PLS models are within the acceptable limit (90-110%). This suggests that the methods are free from interference due to the excipients used in the commercial formulation.

The above validation indicates the method is simple, rapid, economical, precise, and accurate in addition to being eco-friendly. Therefore, it can be used for routine analysis in the quality control of mixtures and commercial products containing paracetamol and caffeine.

Table 5. Results of repeatability and Intraday precision using the developed PLS and PCR models.

Amount taken (Actual Conc.) µg/ml		Predicted Conc. µg mL^{-1}				% Recovery				Acceptable % RSD NMT 2%			
Para.	Caff.	PLS		PCR		PLS		PCR		PLS		PCR	
		Para.	Caff.	Para.	Caff.	Para.	Caff.	Para.	Caff.	Para.	Caff.	Para.	Caff.
10	1.3	9.973	1.291	10.059	1.281	99.73	99.31	100.59	98.54				
10	1.3	9.984	1.299	10.048	1.286	99.84	99.92	100.48	98.92	0.12	0.32	0.08	0.28
10	1.3	9.996	1.297	10.063	1.288	99.96	99.77	100.63	99.08				
16	1.82	16.382	1.912	16.358	1.91	102.39	105.05	102.24	104.95				
16	1.82	16.396	1.914	16.357	1.91	102.48	105.16	102.23	104.95	0.08	0.28	0.04	0.30
16	1.82	16.370	1.904	16.347	1.90	102.31	104.62	102.17	104.40				
20	2.6	20.365	2.721	20.282	2.715	101.83	104.65	101.41	104.42				
20	2.6	20.387	2.707	20.275	2.714	101.94	104.12	101.38	104.38	0.16	0.41	0.11	0.50
20	2.6	20.324	2.699	20.239	2.691	101.62	103.81	101.20	103.50				

Note: % Recovery = Predicted Conc. (µg/ml) / Actual Conc. (µg/ml) ×100.

Table 6. Accuracy data for paracetamol by PCR and PLS models.

%Level	Sample Conc. µg mL^{-1}	Amount of standard paracetamol µg mL^{-1}	Total Conc. µg mL^{-1}	Predicted Conc. µg mL^{-1}		% Recovery		% RSD	
				PLS	PCR	PLS	PCR	PLS	PCR
80%	10	8	18	18.427	18.549	102.37	103.05	0.16	0.12
				18.464	18.547	102.58	103.04		
				18.487	18.586	102.71	103.26		
100%	10	10	20	20.291	20.302	101.46	101.51	0.21	0.17
				20.367	20.361	101.84	101.81		
				20.362	20.366	101.81	101.83		
120%	10	12	22	22.465	22.416	102.11	101.89	0.08	0.14
				22.429	22.403	101.95	101.83		
				22.445	22.358	102.02	101.63		

Table 7. Accuracy data for caffeine by PCR and PLS models.

%Level	Sample Conc. µg mL^{-1}	Amount of standard caffeine µg mL^{-1}	Total Conc. µg mL^{-1}	Predicted Conc. µg mL^{-1}		% Recovery		% RSD	
				PLS	PCR	PLS	PCR	PLS	PCR
80%	1.3	1.04	2.34	2.377	2.333	101.58	99.70	0.49	0.68
				2.395	2.355	102.35	100.64		
100%	1.3	1.3	2.6	2.399	2.364	102.52	101.03	0.37	0.52
				2.690	2.670	103.46	102.69		
				2.692	2.683	103.54	103.19		
120%	1.3	1.56	2.86	2.708	2.698	104.15	103.77	0.26	0.28
				2.981	2.970	104.23	103.85		
				2.966	2.965	103.71	103.67		
				2.971	2.981	103.88	104.23		

Table 8. Results of specificity for paracetamol using the developed PCR and PLS models.

Name of the marketed sample	Sample Conc. µg mL^{-1}	Amount added µg mL^{-1}	Total Conc. µg mL^{-1}	Predicted Conc. µg mL^{-1}		% Recovery		% RSD	
				PLS	PCR	PLS	PCR	PLS	PCR
Panadol	16	16	32	31.590	31.524	98.72	98.51	1.64	1.5
				32.329	32.206	101.03	100.64		
Ramol	16	16	32	32.478	32.451	101.49	101.41	1.85	2.0
				31.639	31.532	98.87	98.54		
Amol	16	16	32	31.625	31.514	98.83	98.48	1.58	1.8
				32.339	32.332	101.06	101.04		

Table 9. Results of specificity for caffeine using the developed PCR and PLS models.

Name of the marketed sample	Sample Conc. µg mL^{-1}	Amount added µg mL^{-1}	Total Conc. µg mL^{-1}	Predicted Conc. µg mL^{-1}		% Recovery		% RSD	
				PLS	PCR	PLS	PCR	PLS	PCR
Panadol	2.08	2.08	4.16	4.125	4.044	99.16	97.21	1.17	0.21
				4.194	4.056	100.82	97.50		
Ramol	2.08	2.08	4.16	4.130	4.039	99.28	97.09	0.20	0.47
				4.142	4.066	99.57	97.74		
Amol	2.08	2.08	4.16	4.171	4.091	100.26	98.34	0.73	0.36
				4.214	4.112	101.30	98.85		

3.3. Analysis of the marketed formulations

The applicability of the developed methods for the quantification of paracetamol and caffeine in marketed formulations was evaluated using the marketed formulation of 500 mg paracetamol with 65 mg caffeine concentration collected from the local pharmacies in the capital Sana'a. **Tables 10** and **11** summarize the data obtained for paracetamol and caffeine in the analyzed marketed formulations.

As can be seen from these data, the paracetamol and caffeine concentrations were within the acceptable limit (90-110%) according to the United States Pharmacopeia (USP).

3.4. Comparison with the reference method

A comparison was carried out with the aid of the SPSS program using F-Test to ensure a non-significant difference between the recovery results of the newly developed methods and that of the reference method for both paracetamol and caffeine. The significance level indicated that the null hypothesis was acceptable because the P-value was greater than the significance level (**Table 12**). As for reference methods, paracetamol and caffeine were determined according to the United States Pharmacopeia (USP), as described earlier in the methodology.

In addition, the chromatograms in **Fig. 6** show the results of the analysis for the reference method for the determination of paracetamol and caffeine.

Table 10. Assay results for paracetamol and caffeine in tablets (marketed sample) using the proposed PLS method.

Name of the marketed sample	METHOD		PLS					
	Para.	Caff.	Para.			Caff.		
	Actual (µg mL^{-1})		Predicted (µg mL^{-1})	% Recovery	% RSD	Predicted (µg mL^{-1})	% Recovery	% RSD
Panadol	16	2.08	16.135	100.84	1.30	2.088	100.38	2
	16	2.08	16.434	102.71		2.023	97.26	
Amol	16	2.08	15.654	97.84	0.03	2.053	98.70	0
	16	2.08	15.660	97.88		2.053	98.70	
Ramol	16	2.08	15.597	97.48	2	2.039	98.03	2
	16	2.08	16.132	100.83		2.098	100.87	

Table 11. Assay results for paracetamol and caffeine in tablets (Marketed Sample) by the PCR proposed method.

Name of the marketed sample	METHOD		PCR					
	Para.	Caff.	Para.			Caff.		
	Actual (µg mL^{-1})		Predicted (µg mL^{-1})	% Recovery	% RSD	Predicted (µg mL^{-1})	% Recovery	% RSD
Panadol	16	2.08	16.253	101.58	0.93	2.007	96.49	1.21
	16	2.08	16.469	102.93		1.973	94.86	
Amol	16	2.08	15.653	97.83	0.01	2.024	97.31	0.03
	16	2.08	15.656	97.85		2.025	97.36	
Ramol	16	2.08	15.620	97.63	2.6	1.996	95.96	1.40
	16	2.08	16.215	101.34		2.036	97.88	

Table 12. Results of statistical comparison between the newly developed and reference methods.

Name of the marketed sample	Components	paracetamol			Caffeine		
	Methods	Reference method (HPLC)	PLS	PCR	Reference method (HPLC)	PLS	PCR
Panadol	Mean%	102.12	100.84	101.58	99.27	100.38	96.49
		101.67	102.71	102.93	99.32	97.26	94.86
		101.90	101.78	102.26	99.30	98.82	95.68
	Significance level		0.912	0.663	--	0.790	0.047
Ramol	Mean%	100.08	97.48	97.63	97.75	98.03	95.96
		100.02	100.83	101.34	97.35	100.87	97.88
		100.05	99.16	99.49	97.55	99.45	96.92
	Significance level (α)		0.647	0.789	--	0.316	0.586

Note: p-value = 0.01.

https://doi.org/10.26850/1678-4618.eq.v49.2024.e1515

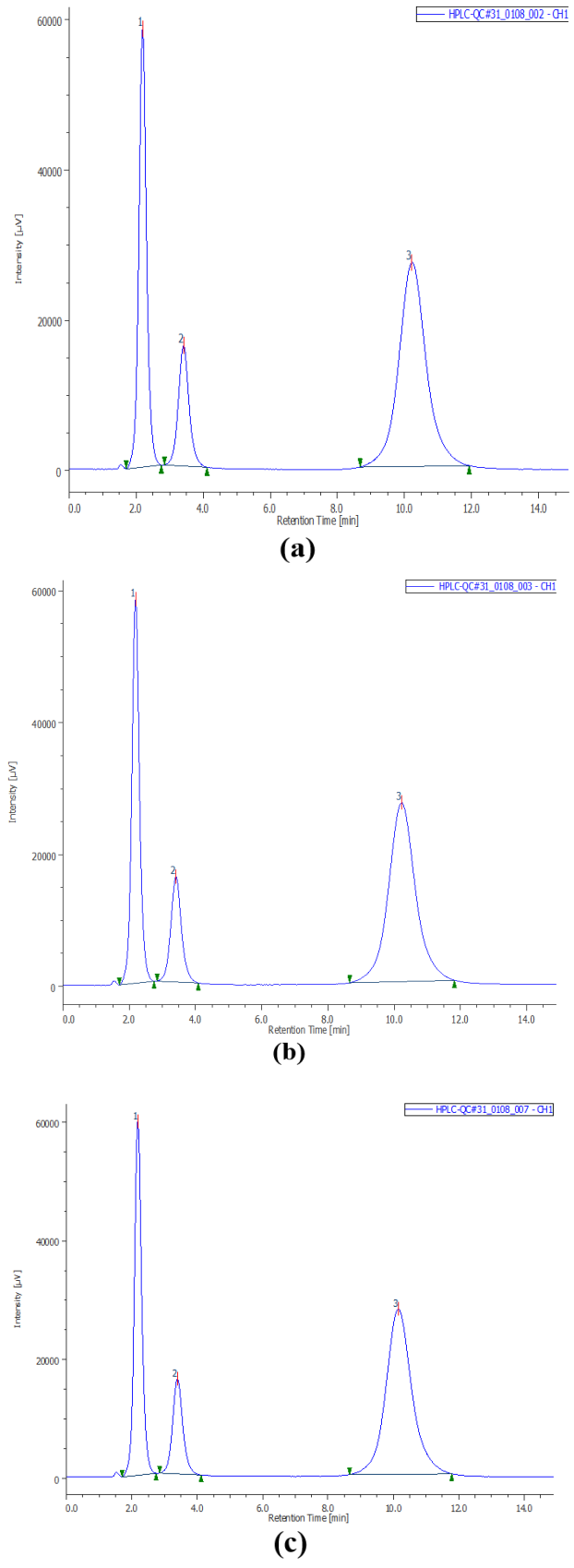

Figure 6. Chromatogram of paracetamol and caffeine standard with Benzoic acid as the internal standard and commercial samples. **(a)** Standard paracetamol and caffeine with benzoic acid as the internal standard; **(b)** Panadol Extra Sample (commercial); **(c)** Ramol Extra Sample (commercial).

3.5. Greenness evaluation of the developed methods

Modern analytical chemistry provides various methods and tools for identifying a specific analyte in various samples. The main objectives of greening analytical methods are to minimize energy consumption, eliminate or reduce the use of chemical substances (solvents, reagents, preservatives, additives for pH adjustment, and others), and properly manage analytical waste while increasing operator safety. Most of these problems demand reductions, e.g., sample number, reagents, energy, waste, risk, and hazard (Gałuszka *et al.*, 2013). This study introduces green analytical methods in the field of pharmaceutical analysis. In this study, water was used as a solvent to prepare the stock solution of one of the analytes and further dilutions to determine paracetamol with caffeine. Water is a safe solvent for health, safety, and environmental hazards. The instrument used was a spectrophotometer; hence, the energy used by these methods is safe. The proposed method in this study generates only a small volume of waste compared with the reference HPLC method. Another important issue is that the toxicity of waste was negligible. In general, AGREE considers UV-chemometrics methods to be the greenest methods compared to HPLC methods. According to the AGREE scale, the UV-chemometrics method shows a very intense greenness of 0.87. However, the HPLC method is less green and shows a very weak intense greenness, 0.45. This comparison is based on the 12 green analytical chemistry principles as follows:

1. Sample treatment;
2. Sample amount;
3. Device Positioning;
4. Sample pre. Stages;
5. Automation, miniaturization;
6. Derivization;
7. Waste;
8. Analysis throughput;
9. Energy consumption;
10. Source of the reagents;
11. Toxicity;
12. Operator's safety;

A comparison of the results obtained by UV chemometrics and those obtained by HPLC methods for the AGREE program scale is shown in **Fig. 7**.

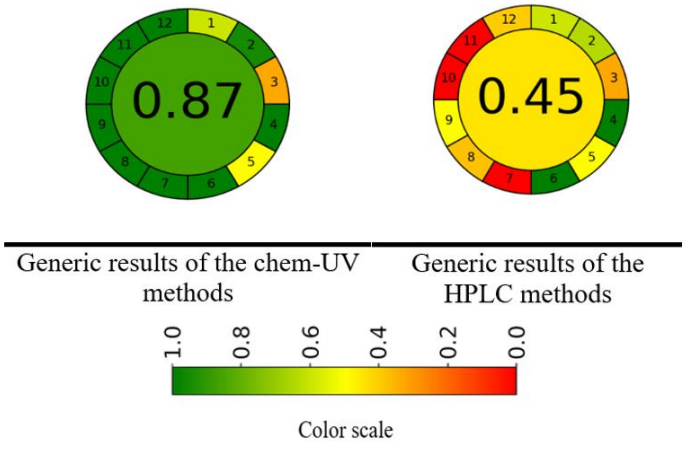

Figure 7. Generic result of assessment (left) and the corresponding color scale for reference for the comparison of the developed UV-chemometrics and reference HPLC methods of paracetamol with caffeine according to the 12 principles of green analytical chemistry, performed using the AGREE program.

4. Conclusions

The use of dangerous chemicals has been discouraged using green analytical chemistry. To determine the combined amounts of caffeine and paracetamol in pharmaceutical formulations, a green spectrophotometric method for simultaneous determination-assisted chemometrics that is simple, quick, and cost-effective has been developed. The proposed chemometric models (PLS and PCR) can be used to simultaneously determine paracetamol and caffeine in binary mixtures in pharmaceutical dosage forms without excipient interference or from each other, and there is no need for prior physical separation of the two drugs. Multivariate calibration models were generated using spectral and concentration matrices. Validation of the two models and their application to a commercial pharmaceutical dosage form gave excellent results. As a result, the suggested techniques can be applied to regular quality control of the specified medications in their combination dosage form in standard laboratories.

Authors' contributions

Conceptualization: Bushra Alattab; Fares Abdullah Alarbagi; **Data curation:** Maher Ali Almaqtari; Entesar Alhuraishi; **Formal Analysis:** Bushra Alattab; Fares Abdullah Alarbagi; **Funding acquisition**: Not applicable; **Investigation**: Bushra Alattab; Fares Abdullah Alarbagi; **Methodology**: Fares Abdullah Alarbagi; **Project administration**: Bushra Alattab; Fares Abdullah Alarbagi; **Resources**: Not applicable; **Software**: Entesar Alhuraishi; Hussein Al-Maydama; **Supervision**: Bushra Alattab; Fares Abdullah Alarbagi; **Validation**: Bushra Alattab; Fares Abdullah Alarbagi; Maher Ali Almaqtari; **Visualization**: Fares Abdullah Alarbagi; **Writing – original draft**: Fares Abdullah Alarbagi; **Writing – review & editing**: Hussein Al-Maydama.

Data availability statement

All data sets were generated or analyzed in the current study.

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Supplementary Material

Table S1. Results of the optimum number of principal factors of paracetamol for PLS models.

Method	Components to evaluate	Number of components evaluated		Number of components selected	
Cross-validation (Leave-one-out)	Set	10		3	
Model selection and validation for paracetamol					
Components	X Variance	Error	R-sq	Press	R-sq (Pred)
1	0.966084	14.5473	0.95085	17.0743	0.942316
2	0.990946	0.4642	0.99843	0.6627	0.997761
3	0.999871	0.2161	0.99927	0.3068	0.998964
4	--	0.0555	0.99981	0.3730	0.998740
5	--	0.0308	0.99990	0.3141	0.998939
6	--	0.0126	0.99996	0.3219	0.998913
7	--	0.0032	0.99999	0.3245	0.998904
8	--	0.0007	1.00000	0.3327	0.998876
9	--	0.0002	1.00000	0.3332	0.998874
10	--	0.0000	1.00000	0.3298	0.998886

Table S2. Results of the optimum number of principal factors of caffeine for PLS models.

Method	Components to evaluate	Number of components evaluated		Number of components selected	
Cross-validation (Leave-one-out)	Set	10		4	
Model selection and validation for caffeine					
Components	X Variance	Error	R-sq	Press	R-sq (Pred)
1	0.952542	4.50078	0.10028	4.98888	0.002703
2	0.990880	0.14035	0.97194	0.18389	0.963240
3	0.999871	0.00689	0.99862	0.00937	0.998128
4	0.999899	0.00334	0.99933	0.00779	0.998442
5	--	0.00097	0.99981	0.00858	0.998285
6	--	0.00029	0.99994	0.00821	0.998359
7	--	0.00012	0.99998	0.00840	0.998320
8	--	0.00002	1.00000	0.00834	0.998332
9	--	0.00001	1.00000	0.00848	0.998304
10	--	0.00000	1.00000	0.00846	0.998310

Table S3. The constant and coefficients at each wavelength of paracetamol and caffeine for PLS models.

Paracetamol				Caffeine			
Constant			-0.20039	Constant			-0.02079
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
300	-3.17198	254.8	0.11817	300	-2.54573	254.8	-0.06339
299.8	-2.62108	254.6	0.11705	299.8	-0.88698	254.6	-0.06383
299.6	-2.39074	254.4	0.11657	299.6	-2.97845	254.4	-0.05558
299.4	-1.71683	254.2	0.11665	299.4	5.04438	254.2	-0.05372
299.2	-1.9243	254	0.11566	299.2	-0.38125	254	-0.06811
299	-1.97862	253.8	0.11516	299	-4.09387	253.8	-0.05123
298.8	-1.58562	253.6	0.11491	298.8	-3.29	253.6	-0.05448
298.6	-1.20244	253.4	0.11407	298.6	0.94618	253.4	-0.07226
298.4	-1.25856	253.2	0.11396	298.4	1.81124	253.2	-0.06115
298.2	-1.0679	253	0.11314	298.2	-0.54598	253	-0.05667
298	-0.98847	252.8	0.11242	298	-2.58962	252.8	-0.057
297.8	-0.77672	252.6	0.1115	297.8	-1.24952	252.6	-0.05138
297.6	-0.77833	252.4	0.1115	297.6	-2.48049	252.4	-0.05338
297.4	-0.55081	252.2	0.11087	297.4	-0.1358	252.2	-0.05592
297.2	-0.51919	252	0.11071	297.2	-0.67162	252	-0.05017
297	-0.51479	251.8	0.10941	297	-1.30059	251.8	-0.06055
296.8	-0.47054	251.6	0.10994	296.8	-0.95739	251.6	-0.06112

296.6	-0.30712	251.4	0.10891	296.6	-1.44424	251.4	-0.04166
296.4	-0.27819	251.2	0.10796	296.4	-0.45932	251.2	-0.06577
296.2	-0.29218	251	0.1086	296.2	-0.49553	251	-0.04374
296	-0.06107	250.8	0.10768	296	0.76776	250.8	-0.04445
295.8	-0.29063	250.6	0.10673	295.8	-0.9825	250.6	-0.05228
295.6	-0.2163	250.4	0.1067	295.6	-0.64288	250.4	-0.04955
295.4	-0.18514	250.2	0.10583	295.4	-0.70114	250.2	-0.05665
295.2	-0.19596	250	0.10582	295.2	-0.68644	250	-0.05365
295	-0.09683	249.8	0.10498	295	-0.74669	249.8	-0.04801
294.8	-0.12287	249.6	0.10461	294.8	-1.46849	249.6	-0.05607
294.6	-0.04307	249.4	0.10457	294.6	-1.12768	249.4	-0.04357
294.4	-0.13292	249.2	0.10466	294.4	-1.62163	249.2	-0.04086
294.2	-0.11608	249	0.10343	294.2	0.36611	249	-0.04269
294	-0.02925	248.8	0.10342	294	0.5644	248.8	-0.04174
293.8	-0.05058	248.6	0.10329	293.8	-0.12767	248.6	-0.0353
293.6	-0.09462	248.4	0.10269	293.6	-0.13326	248.4	-0.05478
293.4	-0.01126	248.2	0.1022	293.4	-0.80982	248.2	-0.04107
293.2	-0.03762	248	0.1018	293.2	0.19936	248	-0.03153
293	-0.0456	247.8	0.10148	293	-0.32957	247.8	-0.03954
292.8	-0.06734	247.6	0.10127	292.8	-0.09365	247.6	-0.04458
292.6	-0.08343	247.4	0.10153	292.6	-0.31811	247.4	-0.05439
292.4	-0.07732	247.2	0.10077	292.4	0.18434	247.2	-0.06205
292.2	-0.07333	247	0.09953	292.2	0.08145	247	-0.04007
292	-0.0856	246.8	0.10028	292	-0.59123	246.8	-0.04153
291.8	-0.06925	246.6	0.09982	291.8	-0.50905	246.6	-0.03187
291.6	-0.08663	246.4	0.09978	291.6	0.40509	246.4	-0.04485
291.4	-0.0139	246.2	0.09973	291.4	0.41079	246.2	-0.03161
291.2	-0.05657	246	0.09897	291.2	-0.09501	246	-0.04129
291	-0.09089	245.8	0.09884	291	-0.18023	245.8	-0.0361
290.8	-0.11682	245.6	0.09866	290.8	0.12179	245.6	-0.02679
290.6	-0.11116	245.4	0.09886	290.6	-0.19209	245.4	-0.03814
290.4	-0.12019	245.2	0.09797	290.4	0.1637	245.2	-0.04417
290.2	-0.10109	245	0.09785	290.2	0.13566	245	-0.04607
290	-0.13797	244.8	0.09808	290	-0.13265	244.8	-0.04249
289.8	-0.13573	244.6	0.09791	289.8	-0.12967	244.6	-0.03722
289.6	-0.13253	244.4	0.0977	289.6	0.02555	244.4	-0.02913
289.4	-0.15148	244.2	0.0972	289.4	-0.21064	244.2	-0.03327
289.2	-0.16285	244	0.09755	289.2	-0.41242	244	-0.03707
289	-0.14923	243.8	0.0974	289	-0.25736	243.8	-0.03062
288.8	-0.14669	243.6	0.09711	288.8	0.24968	243.6	-0.02654
288.6	-0.158	243.4	0.09708	288.6	0.12284	243.4	-0.03557
288.4	-0.17086	243.2	0.09676	288.4	0.1785	243.2	-0.02865
288.2	-0.1695	243	0.09642	288.2	0.19158	243	-0.03372
288	-0.17493	242.8	0.09652	288	0.10924	242.8	-0.03226
287.8	-0.15086	242.6	0.09603	287.8	0.37266	242.6	-0.0202
287.6	-0.18811	242.4	0.09645	287.6	-0.04277	242.4	-0.03252
287.4	-0.1742	242.2	0.09654	287.4	0.21131	242.2	-0.03506
287.2	-0.17609	242	0.09626	287.2	0.24657	242	-0.02784
287	-0.17017	241.8	0.09619	287	0.12559	241.8	-0.02424
286.8	-0.20714	241.6	0.09668	286.8	0.0962	241.6	-0.02498
286.6	-0.18553	241.4	0.0959	286.6	0.3877	241.4	-0.03212
286.4	-0.18549	241.2	0.0963	286.4	0.42973	241.2	-0.02749
286.2	-0.19396	241	0.09613	286.2	0.42634	241	-0.0204
286	-0.18352	240.8	0.09632	286	0.2337	240.8	-0.02946
285.8	-0.20782	240.6	0.09636	285.8	0.40371	240.6	-0.01848
285.6	-0.19629	240.4	0.09577	285.6	0.27051	240.4	-0.02891
285.4	-0.19592	240.2	0.09586	285.4	0.40861	240.2	-0.02316
285.2	-0.19647	240	0.09622	285.2	0.2303	240	-0.02554
285	-0.20083	239.8	0.0961	285	0.45941	239.8	-0.02212
284.8	-0.1987	239.6	0.09582	284.8	0.49869	239.6	-0.02385
284.6	-0.18508	239.4	0.09597	284.6	0.43238	239.4	-0.02365
284.4	-0.19714	239.2	0.09633	284.4	0.3339	239.2	-0.02832
284.2	-0.20147	239	0.09659	284.2	0.36469	239	-0.0268
284	-0.20639	238.8	0.09663	284	0.38612	238.8	-0.01485
283.8	-0.20381	238.6	0.09651	283.8	0.34181	238.6	-0.01829
283.6	-0.19359	238.4	0.09665	283.6	0.38542	238.4	-0.01225
283.4	-0.21951	238.2	0.09657	283.4	0.16936	238.2	-0.01526
283.2	-0.19123	238	0.09689	283.2	0.40077	238	-0.02151
283	-0.2012	237.8	0.09708	283	0.56637	237.8	-0.01965
282.8	-0.19456	237.6	0.097	282.8	0.32709	237.6	-0.02355
282.6	-0.21331	237.4	0.09702	282.6	0.18173	237.4	-0.01789

282.4	-0.20475	237.2	0.09722	282.4	0.20358	237.2	-0.01263
282.2	-0.20026	237	0.09784	282.2	0.36444	237	-0.00706
282	-0.20709	236.8	0.0974	282	0.37242	236.8	-0.02062
281.8	-0.20313	236.6	0.09773	281.8	0.19249	236.6	-0.02412
281.6	-0.20848	236.4	0.098	281.6	0.22699	236.4	-0.00733
281.4	-0.2103	236.2	0.09772	281.4	0.2773	236.2	-0.01646
281.2	-0.19703	236	0.09784	281.2	0.32308	236	-0.01026
281	-0.20881	235.8	0.09841	281	0.22468	235.8	-0.0138
280.8	-0.19285	235.6	0.0985	280.8	0.16909	235.6	-0.01194
280.6	-0.19189	235.4	0.09848	280.6	0.30375	235.4	-0.0138
280.4	-0.20417	235.2	0.09878	280.4	0.15414	235.2	-0.01678
280.2	-0.19544	235	0.09894	280.2	0.26435	235	-0.02103
280	-0.1989	234.8	0.09934	280	0.23919	234.8	-0.01126
279.8	-0.19093	234.6	0.099	279.8	0.29626	234.6	-0.00774
279.6	-0.17525	234.4	0.09917	279.6	0.51439	234.4	-0.00784
279.4	-0.18638	234.2	0.09925	279.4	0.29315	234.2	-0.00805
279.2	-0.18813	234	0.10003	279.2	0.21955	234	-0.01253
279	-0.18442	233.8	0.09998	279	0.30854	233.8	-0.01755
278.8	-0.18587	233.6	0.10022	278.8	0.30478	233.6	-0.01027
278.6	-0.19019	233.4	0.10092	278.6	0.23387	233.4	-0.0081
278.4	-0.18618	233.2	0.10075	278.4	0.35055	233.2	-0.00561
278.2	-0.18654	233	0.10136	278.2	0.29053	233	0.00352
278	-0.18201	232.8	0.10152	278	0.4192	232.8	-0.00622
277.8	-0.18923	232.6	0.10141	277.8	0.26233	232.6	-0.00823
277.6	-0.17961	232.4	0.1018	277.6	0.31118	232.4	-0.00879
277.4	-0.17806	232.2	0.10175	277.4	0.36514	232.2	-0.00428
277.2	-0.17741	232	0.10261	277.2	0.31819	232	-0.00043
277	-0.16926	231.8	0.10291	277	0.3386	231.8	-0.00164
276.8	-0.17351	231.6	0.10292	276.8	0.30991	231.6	-0.00177
276.6	-0.17669	231.4	0.10296	276.6	0.33439	231.4	0.0056
276.4	-0.17355	231.2	0.10333	276.4	0.33753	231.2	-0.00369
276.2	-0.17091	231	0.10374	276.2	0.34312	231	0.00428
276	-0.16386	230.8	0.10418	276	0.37346	230.8	0.0035
275.8	-0.1659	230.6	0.10479	275.8	0.21794	230.6	-0.00563
275.6	-0.16337	230.4	0.10472	275.6	0.35442	230.4	0.00489
275.4	-0.16673	230.2	0.10542	275.4	0.24724	230.2	-0.00581
275.2	-0.15572	230	0.10552	275.2	0.27426	230	0.00595
275	-0.15385	229.8	0.10596	275	0.33961	229.8	0.0065
274.8	-0.15432	229.6	0.10618	274.8	0.27547	229.6	0.00228
274.6	-0.15271	229.4	0.10638	274.6	0.28833	229.4	-0.00461
274.4	-0.14574	229.2	0.10656	274.4	0.33196	229.2	-0.00303
274.2	-0.14712	229	0.10721	274.2	0.18674	229	-0.00132
274	-0.1453	228.8	0.10755	274	0.26149	228.8	0.00793
273.8	-0.13354	228.6	0.10785	273.8	0.27242	228.6	0.01306
273.6	-0.12801	228.4	0.10842	273.6	0.32901	228.4	0.00594
273.4	-0.12673	228.2	0.10859	273.4	0.27983	228.2	0.00814
273.2	-0.11965	228	0.10872	273.2	0.29935	228	0.0032
273	-0.11852	227.8	0.10924	273	0.25227	227.8	0.0105
272.8	-0.1106	227.6	0.10905	272.8	0.31885	227.6	0.00853
272.6	-0.10752	227.4	0.1093	272.6	0.21985	227.4	0.00929
272.4	-0.10465	227.2	0.10985	272.4	0.24164	227.2	0.00172
272.2	-0.09522	227	0.11046	272.2	0.25001	227	0.01777
272	-0.09216	226.8	0.1106	272	0.26687	226.8	0.01083
271.8	-0.08578	226.6	0.11071	271.8	0.24861	226.6	0.00384
271.6	-0.08373	226.4	0.1115	271.6	0.21936	226.4	0.00374
271.4	-0.0755	226.2	0.11167	271.4	0.26732	226.2	-0.00048
271.2	-0.07275	226	0.11182	271.2	0.19673	226	0.01165
271	-0.0614	225.8	0.11199	271	0.16119	225.8	0.00911
270.8	-0.06158	225.6	0.11254	270.8	0.18653	225.6	0.00852
270.6	-0.05443	225.4	0.11265	270.6	0.14512	225.4	0.01667
270.4	-0.05089	225.2	0.11323	270.4	0.14119	225.2	0.01592
270.2	-0.04055	225	0.11303	270.2	0.17367	225	0.0127
270	-0.03647	224.8	0.11357	270	0.20174	224.8	0.02353
269.8	-0.03366	224.6	0.11363	269.8	0.09564	224.6	0.00963
269.6	-0.03094	224.4	0.11357	269.6	0.17085	224.4	0.01553
269.4	-0.02401	224.2	0.11429	269.4	0.15675	224.2	0.00863
269.2	-0.01523	224	0.11355	269.2	0.1152	224	0.01504
269	-0.00982	223.8	0.11403	269	0.12943	223.8	0.0105
268.8	-0.00526	223.6	0.11424	268.8	0.102	223.6	0.02661
268.6	-0.00312	223.4	0.11402	268.6	0.06177	223.4	0.01975
268.4	0.00565	223.2	0.11427	268.4	0.06742	223.2	0.0261

268.2	0.01076	223	0.11387	268.2	0.04745	223	0.03155
268	0.01655	222.8	0.11357	268	0.03964	222.8	0.02794
267.8	0.02367	222.6	0.1138	267.8	0.02999	222.6	0.02827
267.6	0.02674	222.4	0.11355	267.6	0.04151	222.4	0.0255
267.4	0.03211	222.2	0.11266	267.4	0.06309	222.2	0.03409
267.2	0.03769	222	0.11243	267.2	0.00752	222	0.03115
267	0.04393	221.8	0.11142	267	0.02221	221.8	0.03609
266.8	0.04813	221.6	0.11134	266.8	0.02363	221.6	0.02762
266.6	0.0536	221.4	0.11087	266.6	0.03962	221.4	0.03691
266.4	0.05645	221.2	0.1096	266.4	0.01931	221.2	0.04239
266.2	0.06129	221	0.10846	266.2	-0.01085	221	0.04582
266	0.06514	220.8	0.10765	266	0.02409	220.8	0.04515
265.8	0.06951	220.6	0.10606	265.8	-0.00922	220.6	0.03088
265.6	0.07342	220.4	0.10516	265.6	0.00656	220.4	0.04215
265.4	0.07518	220.2	0.1038	265.4	-0.05403	220.2	0.05323
265.2	0.07984	220	0.10136	265.2	0.00241	220	0.05013
265	0.0856	219.8	0.10028	265	-0.03753	219.8	0.05219
264.8	0.08882	219.6	0.09751	264.8	0.02459	219.6	0.04387
264.6	0.09165	219.4	0.09576	264.6	-0.02507	219.4	0.04725
264.4	0.09424	219.2	0.09329	264.4	-0.03678	219.2	0.04766
264.2	0.09598	219	0.09091	264.2	-0.0252	219	0.04726
264	0.09936	218.8	0.08817	264	-0.04011	218.8	0.0572
263.8	0.10157	218.6	0.08536	263.8	-0.02593	218.6	0.0517
263.6	0.10486	218.4	0.08192	263.6	-0.03444	218.4	0.06626
263.4	0.10778	218.2	0.08004	263.4	-0.02858	218.2	0.05415
263.2	0.10915	218	0.07485	263.2	-0.03984	218	0.05407
263	0.11181	217.8	0.07177	263	-0.05378	217.8	0.07554
262.8	0.1132	217.6	0.06893	262.8	-0.05115	217.6	0.06741
262.6	0.11405	217.4	0.06297	262.6	-0.06987	217.4	0.0635
262.4	0.11492	217.2	0.05933	262.4	-0.06584	217.2	0.06013
262.2	0.11651	217	0.05469	262.2	-0.05916	217	0.05869
262	0.11885	216.8	0.05017	262	-0.05737	216.8	0.06929
261.8	0.11934	216.6	0.04544	261.8	-0.04062	216.6	0.06265
261.6	0.12013	216.4	0.04113	261.6	-0.06852	216.4	0.06484
261.4	0.12107	216.2	0.03564	261.4	-0.07011	216.2	0.06679
261.2	0.12092	216	0.03101	261.2	-0.07297	216	0.07003
261	0.1224	215.8	0.02575	261	-0.07327	215.8	0.06925
260.8	0.12341	215.6	0.02124	260.8	-0.04862	215.6	0.06546
260.6	0.12328	215.4	0.01721	260.6	-0.06958	215.4	0.07782
260.4	0.12459	215.2	0.01193	260.4	-0.07804	215.2	0.06201
260.2	0.12375	215	0.00741	260.2	-0.07093	215	0.05706
260	0.12403	214.8	0.00206	260	-0.07518	214.8	0.05957
259.8	0.12459	214.6	-0.00082	259.8	-0.07188	214.6	0.05726
259.6	0.12496	214.4	-0.00438	259.6	-0.09861	214.4	0.05672
259.4	0.12614	214.2	-0.00753	259.4	-0.04769	214.2	0.05022
259.2	0.12568	214	-0.01116	259.2	-0.08026	214	0.04857
259	0.12558	213.8	-0.01397	259	-0.07046	213.8	0.03835
258.8	0.12502	213.6	-0.01572	258.8	-0.05786	213.6	0.05278
258.6	0.12489	213.4	-0.01956	258.6	-0.06397	213.4	0.0418
258.4	0.12508	213.2	-0.02124	258.4	-0.08428	213.2	0.03143
258.2	0.1246	213	-0.02194	258.2	-0.07583	213	0.02191
258	0.12487	212.8	-0.02278	258	-0.06488	212.8	0.03108
257.8	0.12414	212.6	-0.02329	257.8	-0.07595	212.6	0.0144
257.6	0.12361	212.4	-0.02442	257.6	-0.07015	212.4	0.00553
257.4	0.12398	212.2	-0.02323	257.4	-0.06395	212.2	0.01013
257.2	0.12308	212	-0.02229	257.2	-0.06097	212	-0.0053
257	0.12292	211.8	-0.02257	257	-0.06829	211.8	0.00233
256.8	0.12224	211.6	-0.02102	256.8	-0.07187	211.6	-0.01371
256.6	0.12199	211.4	-0.01973	256.6	-0.07402	211.4	-0.01745
256.4	0.12101	211.2	-0.01759	256.4	-0.07965	211.2	-0.02744
256.2	0.12101	211	-0.01646	256.2	-0.06801	211	-0.0351
256	0.12132	210.8	-0.01478	256	-0.07327	210.8	-0.03423
255.8	0.12035	210.6	-0.01303	255.8	-0.06154	210.6	-0.03908
255.6	0.11904	210.4	-0.00943	255.6	-0.06224	210.4	-0.06083
255.4	0.11973	210.2	-0.00852	255.4	-0.05225	210.2	-0.04575
255.2	0.1186	210	-0.00525	255.2	-0.0549	210	-0.02839
255	0.11763			255	-0.06462		

Table S4. Results of the principal components coefficients of paracetamol and caffeine for the PCR model.

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Mixture No.	Paracetamol (μg mL^{-1})	Caffeine (μg mL^{-1})	Z1	Z2	Z3	Z4	Z5	Z6
1	10	1.3	9.146716	0.922621	0.11385	0.218591	0.064549	-0.00164
2	10	1.82	9.338489	1.227304	0.100951	0.223531	0.066993	-0.00205
3	10	2.08	9.354737	1.388513	0.100658	0.224171	0.060107	-0.00185
4	10	2.34	9.754932	1.57956	0.107429	0.220398	0.058897	-0.0019
5	10	2.6	9.74823	1.72816	0.099278	0.214642	0.059679	-0.0013
6	14	1.3	12.40446	0.933764	0.141302	0.260088	0.057792	0.001265
7	14	1.82	12.64458	1.256145	0.142028	0.25596	0.062195	-0.00074
8	14	2.08	12.89527	1.456187	0.143263	0.240196	0.066623	-0.00077
9	14	2.34	13.23925	1.710256	0.17538	0.204584	0.056079	-0.00052
10	14	2.6	13.279	1.804253	0.149894	0.224501	0.067227	-0.0009
11	16	1.3	14.17486	0.998982	0.167127	0.211254	0.059428	8.95E-05
12	16	1.82	14.41187	1.337918	0.166771	0.208607	0.064014	0.00127
13	16	2.08	14.67569	1.505969	0.163074	0.221245	0.063982	0.000802
14	16	2.34	14.5841	1.607437	0.143083	0.228941	0.070436	0.001214
15	16	2.6	14.91319	1.774835	0.141138	0.240823	0.070132	7.25E-05
16	18	1.3	15.72434	0.95616	0.156724	0.231376	0.065447	0.000162
17	18	1.82	15.91465	1.176127	0.109593	0.212343	0.065423	0.004414
18	18	2.08	15.9445	1.334762	0.10192	0.225312	0.058374	0.005089
19	18	2.34	16.1327	1.493542	0.099248	0.231075	0.058385	0.001423
20	18	2.6	16.36708	1.642257	0.095751	0.233073	0.055706	0.004384
21	20	1.3	17.24382	0.860645	0.108718	0.209636	0.062819	0.002135
22	20	1.82	17.25686	1.14882	0.104635	0.221209	0.063474	-0.01031
23	20	2.08	17.59386	1.363286	0.105134	0.217987	0.079396	0.003633
24	20	2.34	17.55533	1.472502	0.08927	0.228363	0.06883	0.001512
25	20	2.6	17.82869	1.683043	0.114235	0.230266	0.062232	-0.00376