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Development and validation of a new spectrophotometric method for simultaneous determination of spiramycin and metronidazole in tablet pharmaceutical dosage forms using chemometrics technique in comparison with HPLC

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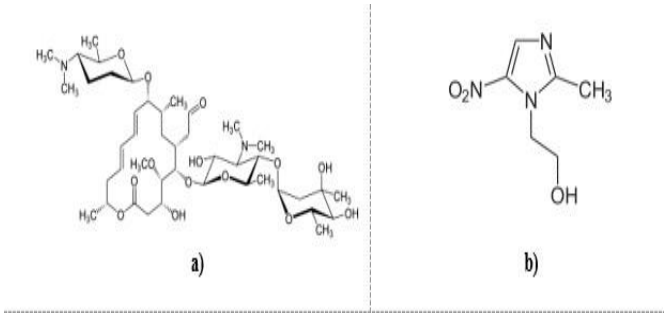
ABSTRACT: A new, easy, fast, and cost-effective simultaneous spectrophotometric method for determining the combined concentration of spiramycin and metronidazole in tablet pharmaceutical formulations was developed and proposed. The suggested method was validated using two chemometric techniques, namely partial least square regression (PLS) and principal component regression (PCR). The significance of the proposed method is that pretreatment or separation steps are not required. Various drug concentrations and instrumental spectra of 25 mixed solutions of spiramycin and metronidazole were used for model design and validation. The UV analysis of the prepared mixtures was recorded with respect to a selected solvent blank in the wavelength range of 200–375 nm. The digitized absorbance was sampled at 0.2 nm intervals. The R^2 values of 1 and 0.9998 assigned for the PLS of spiramycin and metronidazole, and those of 0.9991 and 0.9998 for the PCR of spiramycin and metronidazole, respectively, exhibited greater prediction efficiencies. The results were statistically compared to those of the HPLC reference method. The statistical comparison between the recommended (PLS and PCR) and reference HPLC method did not find any appreciable variations in accuracy or precision. The suggested approach is simple, effective, and trustworthy enough to be employed as an alternative analytical method for quality control of drug analysis in the pharmaceutical industry.



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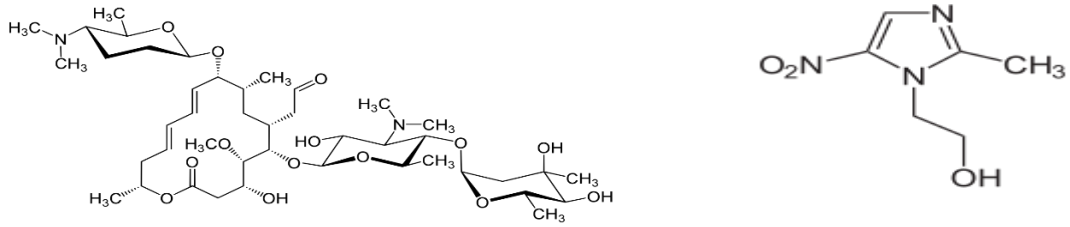
1.Introduction

Chemically, spiramycin is (4R,5S,6S,7R,9R,10R,11E,13E,16R)-6-[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β- D-glucopyranosyl]oxy]-4-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10- [[2,3,4,6 tetradeoxy-4-(dimethylamino) -D-erythro-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-on. It is used as antibacterial. (scheme 1a) [1, 2]. Metronidazole is 2-(2-Methyl-5-nitro-1H-imidazol-1-yl) ethanol. It is used as Imidazole antibacterial. Its chemical structure (scheme 1b) [2, 3].



a) **b)**

Scheme 1: Chemical structure of spiramycin (a) and metronidazole (b).

Analytical chemistry has been significantly influenced by the discipline of chemometrics, notably in the field of spectrum analysis, which is crucial for the quality control of pharmaceutical formulations including two or more medicines with overlapping spectra [4-6].

Chemometrics techniques have a variety of uses in analytical chemistry based on spectroscopy, including UV-visible spectrophotometry [7-19] , fluorescence spectroscopy [20-23], NIR spectroscopy [24-27], and the FTIR spectroscopic method [28]. In addition, chromatography techniques like liquid chromatography [29-31] and a variety of other analytical chemistry techniques, such as flow-injection analysis, are accessible for pharmaceutical formulations [32, 33].

Chemometrics methods rely on multivariate analysis, which requires UV Spectrophotometry methods to take into account more than one variable at a time [34]. There are several wavelengths considered variable, and each wavelength's absorbance is taken into account [34, 35]. The two most important chemometric methods used in multivariate analysis are principal component regression (PCR) and partial least squares (PLS). These methods are of multivariate calibration using spectrophotometric data along with statistical tools, mathematical models, and software for the determination of combined drugs in pharmaceutical formulations [34]. These methods likewise rely on the calibration of the mathematical model by utilizing the absorbance data of calibration standards with known concentrations to estimate the concentration of unknown samples from their absorbance data [34, 36].

Uddin, Mondol, et al. (2019) reported that high-performance liquid chromatography (HPLC) is a technique that gathers data from simultaneous separation and determination and is increasingly frequently employed in analytical techniques for the examination of pharmaceutical products. It has a number of disadvantages, including the possibility of harm to the environment and to human health. The HPLC assay also needed a lot of pricey chemicals and supplies. Additionally, they take a lot of time, which delays the marketing and production processes. The price of HPLC upkeep is likewise substantial. Spectrophotometry, which is simple, dependable, rapid, affordable, and most significantly, environmentally benign, may be a useful alternative for determining a complicated combination in pharmaceutical quality control laboratories. As shown by the research, spectrophotometry and

chemometrics coupling has a bright future and may be utilized as a substitute for HPLC for both quantitative and qualitative analysis.

Uddin, Mondol et al. 2019 have claimed [37] that conventional UV spectral measurements cannot be used to determine the majority of analytes of interest because they are accompanied in their dose forms by other substances that absorb in the same spectral area. Traditional techniques like extraction are challenging to use because they need a lot of solvent, which carries risks of analyte loss or contamination. They also run the risk of an incomplete separation, which is expensive and time-consuming. However, spectrophotometry, being a straightforward, accurate, quick, and inexpensive technology may be a wonderful choice when used with chemometric techniques for determining a mixed mixture in pharmaceutical quality control. Whereas trustworthy, accurate, and quick analytical processes are required for pharmaceutical product quality monitoring, they provide advantages. This procedure is quick, accurate, and simple to use while avoiding the usage of earlier separation processes.

Many methods for quantifying spiramycin and metronidazole have been published, including chromatographic [38-40] and spectrophotometric approaches [41, 42]. At the time of writing, we had the following information to our knowledge: there is no reference in the analytical literature reviews for the development and validation of simultaneous spectrophotometric method-assisted chemometrics methods for the determination of spiramycin and metronidazole in pharmaceutical dosage forms. Also, no published work has been conducted on developing and validating spectrophotometric methods for the examination of some combined pharmacological compounds utilizing a chemometrics approach in the Yemeni market (the Republic of Yemen). Therefore, the present study aims to develop and validate an adequate and reproducible simultaneous spectrophotometric assay method for the simultaneous determination of spiramycin and metronidazole in tablet pharmaceutical dosage forms using the chemometrics technique.

2. Materials and Methods

2.1. Materials and Reagents

The reference standards of spiramycin and metronidazole 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 materials were used for the HPLC method. De-ionized water (with a specific conductance of 0.05 S cm^{-1}) was produced in-house and used for the preparation of all sample solutions.

2.2. Instrumentation

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 with detector (UV-2070 Plus), pump (PU-2089), an auto sampler (AS-2055 Plus) and a column oven (CO-2067 Plus). Electronic balance (AA-160), Denver Instrument. Electronic balance (GH-252), AND. Electronic balance (GR-120), AND. pH meter (3520), Jenway. Centrifuge (Z326 K), Hermle were also used.

2.3. Development and validations procedures

In order to develop an accurate, precise, and reliable simultaneous spectrophotometric method assisted with the chemometrics technique, the analytical methods were established and developed to get the intended results for quantifying the targeted components.

2.3.1. Selection of Solvent

To choose the best solvents, the literature was reviewed to determine the appropriate solvents that aid in the dissolution of the targeted combined active pharmaceutical ingredients, and suitable solvents were selected through several trials and errors

attempts for the dissolution and extraction of the desired active pharmaceutical components without excipients. Other benefits for choosing the appropriate solvent, such as being available, easy to use, cheap, and for the spectrophotometric method's implementation, were given full consideration.

2.3.2. Selection of spectral zones analysis

After the phase of choosing the solvent and before the data is pre-processed, the individual pure and mixtures 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 interval. 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 [43].

2.3.3. Construction of the training set

A number of 25 different concentrations of spiramycin and metronidazole binary mixtures were prepared as the training set (calibration set) to construct the model. The absorbencies of these mixtures were measured between 200 nm and 400 nm at 0.2 nm intervals against a blank.

2.3.4. Construction of chemometric models

The two multivariate calibration models; the partial least square (PLS) and the principal component regression (PCR) analysis were constructed and 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.
- The PCR and PLS models were developed utilizing absorption data at selected spectral zones for analysis in the intervals of 0.2 nm using Minitab 17 programme.
- Leave-one-out (LOO) cross validation method was used to obtain 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 predicated concentrations.
- Finally, the predicted concentrations of the components were compared with the actual concentrations in each sample and the assay of binary mixture were calculated in 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 expression [43]:

$$\mathbf{RMSECV} = \sqrt{\frac{\sum(\boldsymbol{C}\mathbf{act}-\boldsymbol{C}\mathbf{pre})^2}{\boldsymbol{I}\mathbf{c}}}$$

Where:

RMSECV= Root mean square error of cross validation

C_{act} = Actual concentration of calibration set

C_{pre} = predicted concentration of calibration set

I_c = Total number of samples in calibration set

2.3.5. Validation and construction of the validation set

In order to validate and evaluate the performance of the proposed and developed spectrophotometric methods assisted chemometric models, these methods were applied to validation set. Additionally, according to the guidelines of the International Conference on Harmonization (ICH), the developed methods' linearity, accuracy, precision (repeatability), and specificity performance criteria were validated and subsequently established.

2.4. Developed analytical method procedures for spiramycin with metronidazole determination and comparing with reference method

The suitability of the proposed and developed method was determined in accordance with the method validation results. This method was studied and experimented for determination of spiramycin with metronidazole in marketed pharmaceutical formulations. And they were compared with analysis results of reference method.

2.4.1. Preparation of standard stock solution

Stock solutions of 1000 µg.mL^{-1} of spiramycin and 500 µg.mL^{-1} of metronidazole were individually prepared in a 250 ml volumetric flask by dissolving 250 mg spiramycin and 125 mg metronidazole separately in 0.1 mol L^{-1} HCl.

2.4.2. Preparation of working standard solution

2.4.2.1. Construction of the calibration (training) set

A number of 25 binary mixtures of spiramycin with metronidazole 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.

2.4.2.2. Construction of the validation set

A set of 11 binary mixtures of spiramycin and metronidazole was prepared by transferring different volumes into 50 ml volumetric flasks and the procedure under the construction of the training set was repeated (Table 4).

2.4.2.3. Preparation of test sample

A number of 20 tablets of a commercial pharmaceutical formulation tablet containing 250/125 mg of spiramycin / metronidazole, respectively was analyzed by the proposed chemometric methods. The sample 250/125 were weighed and finely powdered in a mortar. A quantity of powdered tablets equivalent to 183 mg of spiramycin and 125 mg of metronidazole was accurately weighed and transferred into a 250 ml volumetric flask containing 200 ml 0.1 HCl mol L^{-1}. The mixture was shaken for 10 minutes and with frequent shaking, the volume was completed to 250 ml with the selected solvent. The solution was then filtered through 0.45 µm filter paper. 1 ml of the filtrate was transferred into 50 ml volumetric flask 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.

2.4.2.4. Preparation of spiked and recovery samples

Powdered tablets of 183 mg of spiramycin and 125 mg of metronidazole were accurately weighed, transferred to a 250 ml volumetric flask and then 200 ml of 0.1 HCl mol L^{-1} was added, calculated amount of spiramycin and metronidazole from standard solutions were spiked into sample solution. The mixture was shaken for 10 minutes and with frequent shaking the volume completion to 250 ml with the selected solvent was carried out. The solution was then filtered. A 0.5 ml of the filtrate was transferred into 50 ml volumetric flask and then diluted with water up to 50 ml. The absorbance was then measured.

2.4.2.5. Analysis of marketed formulations

The developed method was applied to the measurement of a commercially available samples. It was carried out using the marketed formulation with concentration of 250 mg spiramycin and 125 mg metronidazole. The tablets solution prepared in the sample preparation section was diluted with 0.1 HCl mol L^{-1} then water to prepare solutions with concentration of 14.62 µg.mL^{-1} spiramycin and of 10 µg.mL^{-1} metronidazole. The spectra of the prepared solutions were recorded and then the developed multivariate models PCR and PLS were applied to determine the concentrations of the spiramycin and metronidazole.

2.4.2.6. Comparing the suggested method with reference method

Comparison was carried out with the recovery results of the newly developed methods and that of reference method for each of spiramycin with metronidazole according to the in-house developed method. 173.5 µg.ml^{-1} spiramycin was prepared by dissolving 173.5 mg spiramycin in water: metthanol (70:30) in a 200 ml volumetric flask as standard stock solution. 10 ml of spiramycin of the standard stock solution was transferred in A potassium dihydrogen phosphate and potassium monohydrogen phosphate buffer solution pH=6.8 : acetonitrile: methanol: tetrahydrofuran (300:200:450:50) in 50 ml volumetric flask. A test sample was prepared by placing 4 tablets containing 3,000,000 IU of spiramycin to 200 ml volumetric flask. 200 ml of water: metthanol (70:30) as solvent was added and the solution was shaken for 30 min then a portion of the solution was centrifuged

for 10 min. 5 ml of of the supernatant solution was transferred into 100 ml volumetric flask and diluted with mobile phase. The standard and the test sample of spiramycin were injected through an HPLC system with a mixture of potassium dihydrogen phosphate and potassium monohydrogen phosphate buffer solution pH=6.8 : acetonitrile: methanol: tetrahydrofuran 300:200:450:50 as the mobile phase at flow rate of 1 ml/min through a C18 column (25 cm × 4.6 mm, 5 µm) and column temperature was 65°C. The UV detection of the spiramycin was then carried out at 232 nm.

Metronidazole was also determined, 500 µg.ml^{-1} metronidazole was prepared by dissolving 50 mg metronidazole in mobile phase potassium dihydrogen phosphate and potassium monohydrogen phosphate buffer solution pH=6.8 : methanol 800:200 in a 100 ml volumetric flask as standard stock solution. A test sample was prepared by placing 4 tablets containing 500 mg of metronidazole to 200 ml volumetric flask. 200 ml of methanol as solvent was added and the solution was shaken for 30 min then a portion of the solution was centrifuged for 10 min. 5 ml of of the supernatant solution was transferred into 25 ml volumetric flask and diluted with mobile phase. The standard and the test sample of metronidazole were injected through an HPLC system with a mixture of potassium dihydrogen phosphate and potassium monohydrogen phosphate buffer solution pH=6.8 : methanol 800:200 at flow rate of 1 ml/min through a C18 column (25 cm × 4.6 mm, 5 µm) and column temperature was 65°C. The UV detection of the metronidazole was then carried out at 254 nm.

3.Results and Discussion

3.1. Method development for spiramycin and metronidazole determination

3.1.1. Selection of solvent

To select 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 0.1 mol L^{-1} HCl. Therefore, 0.1 mol L^{-1} HCl was selected as diluent that has striking advantages such as easily available, easy to handle and a cheap for implementing the spectrophotometric method and figure 1 showed the spectra of the spiramycin and metronidazole in 0.1 mol L^{-1} HCl.

3.1.2. Selection of spectral zones for analysis

In order to determine the overlap spectral zones, the absorbance spectra of the pure spiramycin and metronidazole samples, and that sample of the mixed spiramycin with metronidazole in 0.1 mol L^{-1} HCl then water were recorded in the range of 200-400 nm with 0.2 nm interval. For the analysis, the UV spectra of the mixtures were selected for a suitable wavelength range (200-375 nm) against water blank. This range provided a great amount of information about the two components as shown in the spiramycin with metronidazole spectra (Figure 1).

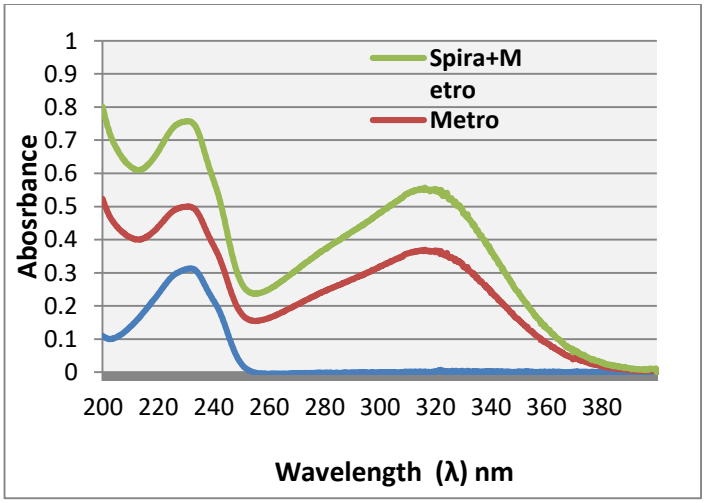


Figure 1:
UV Absorbance spectra of the pure and mixed samples of spiramycin and metronidazole in water solvent.

3.1.3. Construction of the training set

To determine the linear range from measuring the absorbance at different concentrations for spiramycin with metronidazole, the response was found to be linear in the range of 6-30 µg.mL^{-1} for spiramycin and 3-15 µg.mL^{-1} for metronidazole using twenty five different concentrations of spiramycin and metronidazole mixtures were prepared to construct the models as shown in Table 1.

Table 1: Composition of calibration set.

Mixture No.	Spiramycin (µg.mL^{-1})	Metronidazole (µg.mL^{-1})	Mixture No.	Spiramycin (µg.mL^{-1})	Metronidazole (µg.mL^{-1})
1	6	3	14	10	10
2	6	4	15	10	15
3	6	5	16	20	3
4	6	10	17	20	4
5	6	15	18	20	5
6	8	3	19	20	10
7	8	4	20	20	15
8	8	5	21	30	3
9	8	10	22	30	4
10	8	15	23	30	5
11	10	3	24	30	10
12	10	4	25	30	15
13	10	5			

3.1.4. Construction of chemometrics models

The spectra were saved and extracted into MS-Excel for model generation. The PCR and PLS models were developed utilizing the absorption data for the selected spectral zones using Minitab 17 software programme. After the PCR and PLS models have been constructed, the optimum number of principal components of spiramycin and metronidazole were obtained and given in Appendix 1 and 2 and 4.

3.1.5. Determination of the optimum number of the principal components of spiramycin and metronidazole for PLS

Choosing the proper number of principal components for the development of model was necessary to obtain good prediction. Leave-one-out (LOO) cross validation method was used to obtain the necessary optimum number of the principal factors for the PLS model. It was found that the optimum number of the principal components were eight for spiramycin and four for metronidazole as mentioned above and given in Appendix 1 and 2.

3.1.6. Determination of the constant and coefficients obtained at each wavelength of spiramycin and metronidazole for PLS models

The constant and coefficients at each wavelength were calculated using Minitab 17 programme as illustrated in Appendix 3.

3.1.7. Determination of the predicted concentrations and the recovery of spiramycin and metronidazole for PLS models

The predicted or calculated concentrations in µg.mL^{-1} of the spiramycin and metronidazole were worked out from the multiple regression equation:

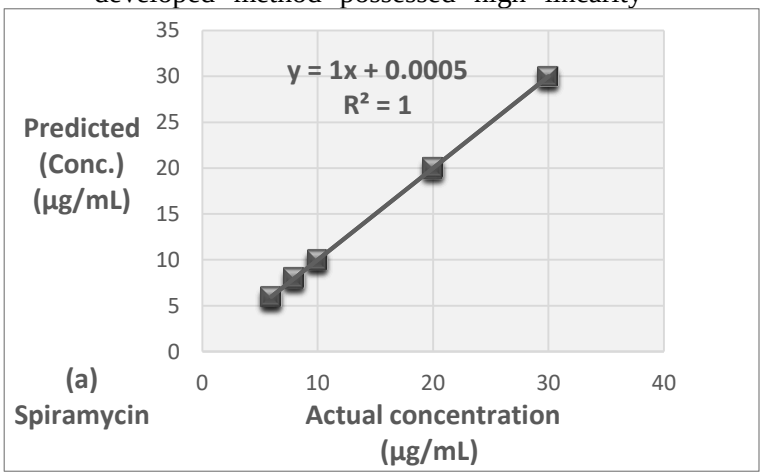
predicted (Calculated) = Constant + $\sum$ (Coefficient × Absorbance)

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

Table 2: Results of the predicted concentrations with the recovery of spiramycin and metronidazole in the binary mixture in each sample for PLS model

Name	Spiramycin			Metronidazole		
Constant	1.9872			-0.10806		
Mixture NO.	Actual Conc.	Predicted Conc.	%Recovery	Actual Conc.	Predicted Conc.	%Recovery
1	6.00	5.96	99.39	3.00	2.99	99.83
2	6.00	5.95	99.18	4.00	4.07	101.84
3	6.00	5.95	99.20	5.00	4.91	98.25
4	6.00	6.02	100.39	10.00	10.05	100.50
5	6.00	5.98	99.70	15.00	15.02	100.17
6	8.00	8.11	101.35	3.00	3.05	101.54
7	8.00	8.06	100.73	4.00	4.01	100.15
8	8.00	8.10	101.25	5.00	5.02	100.36
9	8.00	7.89	98.56	10.00	9.93	99.33
10	8.00	8.00	99.94	15.00	14.89	99.30
11	10.00	9.99	99.88	3.00	3.01	100.17
12	10.00	9.99	99.86	4.00	3.97	99.17
13	10.00	9.95	99.46	5.00	4.97	99.44
14	10.00	10.04	100.43	10.00	10.10	100.96
15	10.00	9.98	99.83	15.00	15.00	100.01
16	20.00	19.85	99.23	3.00	3.06	101.97
17	20.00	20.10	100.52	4.00	3.89	97.27
18	20.00	20.06	100.32	5.00	5.05	100.96
19	20.00	20.09	100.43	10.00	10.03	100.27
20	20.00	20.02	100.10	15.00	15.00	99.99
21	30.00	29.97	99.91	3.00	3.00	99.94
22	30.00	29.98	99.93	4.00	3.92	97.91
23	30.00	29.98	99.92	5.00	5.01	100.19
24	30.00	30.03	100.09	10.00	10.06	100.61
25	30.00	29.98	99.92	15.00	15.00	99.97
		Mean%	99.98		Mean%	100.00
		RSD%	0.63		RSD%	1.11
		RMSECV	0.063		RMSECV	0.055

The linearity of the developed method of PLS model was tested by constructing a cross-validation of the data in Table 2. The results obtained in Figure 2 indicated that the developed method possessed high linearity with $R^2 = 1$ within the method linear range (6 – 30 µg.mL^{-1}) for spiramycin and $R^2 = 0.9998$ within the method linear range (3–15 µg.mL^{-1}) for metronidazole.



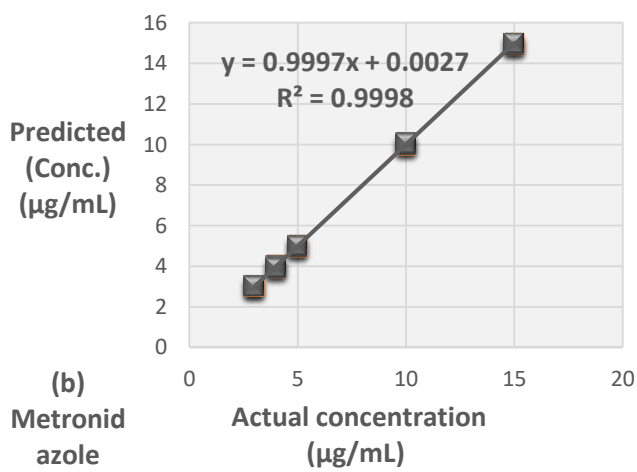


Figure 2: The PLS cross validation for the calibration set of the actual vs. predicted concentration

3.1.8. Determination of the optimum number of the principal components and their coefficients of spiramycin and metronidazole for PCR

The PCR was computed by using a few principal components (PCs) and performed regression analysis of these PCs with concentration in order to determine the principal components coefficients of spiramycin and metronidazole for PCR model as shown in Appendix 4.

From the treatment of the principal components coefficients in (Appendix 4) using Minitab 17 programme. Regression equations of spiramycin and metronidazole were obtained and used to calculate the predicted concentration as shown below.

Regression equations of spiramycin
2.141 + 1.7645 Z1 - 2.6024 Z2 - 2.231 Z3 - 4.757 Z4 + 27.43 Z5 + 2.12 Z6
Regression Equation of metronidazole
-0.051 + 0.75186 Z1 + 1.51667 Z2 + 0.3532 Z3 - 1.292 Z4 + 4.084 Z5 + 2.28 Z6

where Z: The principal components coefficients.

3.1.9. Determination of the predicted concentrations and recovery of spiramycin and metronidazole for PCR models

The predicted or calculated concentrations in $\mu g.mL^{-1}$ of the spiramycin and metronidazole were calculated from above regression equations.

The predicted or calculated concentrations of the spiramycin and metronidazole were compared with the actual concentrations and the assay for binary mixture were calculated in each sample. Root mean square error of cross-validation (RMSECV) was calculated and found to be low. The RMSECV low values in Table 3 indicate that both the precision and accuracy of PCR model for spiramycin and metronidazole were very high, with the R^2 values in Figure 3 of very high linearity.

Table 3: Results of the predicted concentrations with recovery of spiramycin and metronidazole in binary mixture in each sample for PCR models

Name	Spiramycin			Metronidazole		
Constant	2.141			-0.051		
Mixture NO.	Actual Conc.	Predicted Conc.	%Recovery	Actual Conc.	Predicted Conc.	%Recovery
1	6.00	5.95	99.21	3.00	2.99	99.69
2	6.00	6.26	104.39	4.00	3.99	99.76
3	6.00	5.77	96.08	5.00	4.93	98.62
4	6.00	5.97	99.54	10.00	10.04	100.40
5	6.00	6.13	102.10	15.00	15.03	100.19
6	8.00	8.30	103.80	3.00	3.03	100.99
7	8.00	7.87	98.40	4.00	4.01	100.28
8	8.00	7.53	94.12	5.00	5.05	100.90
9	8.00	7.76	97.01	10.00	9.98	99.80
10	8.00	7.79	97.37	15.00	14.82	98.83
11	10.00	9.89	98.88	3.00	3.02	100.83
12	10.00	10.41	104.11	4.00	4.00	99.89
13	10.00	10.01	100.06	5.00	4.98	99.65
14	10.00	10.12	101.19	10.00	10.09	100.85
15	10.00	10.05	100.49	15.00	15.02	100.12
16	20.00	19.59	97.95	3.00	3.04	101.38
17	20.00	20.27	101.36	4.00	3.92	97.99

Name	Spiramycin			Metronidazole		
Constant	2.141			-0.051		
Mixture NO.	Actual Conc.	Predicted Conc.	%Recovery	Actual Conc.	Predicted Conc.	%Recovery
18	20.00	19.91	99.54	5.00	5.05	100.95
19	20.00	20.75	103.75	10.00	10.06	100.57
20	20.00	20.15	100.75	15.00	15.01	100.09
21	30.00	30.04	100.12	3.00	2.98	99.19
22	30.00	29.92	99.73	4.00	3.91	97.70
23	30.00	29.97	99.89	5.00	5.04	100.80
24	30.00	29.98	99.94	10.00	10.00	99.98
25	30.00	29.61	98.71	15.00	15.02	100.15
		Mean%	99.94		Mean%	99.98
		RSD%	2.50		RSD %	0.93
		RSEMV	0.266		RSEMV	0.055

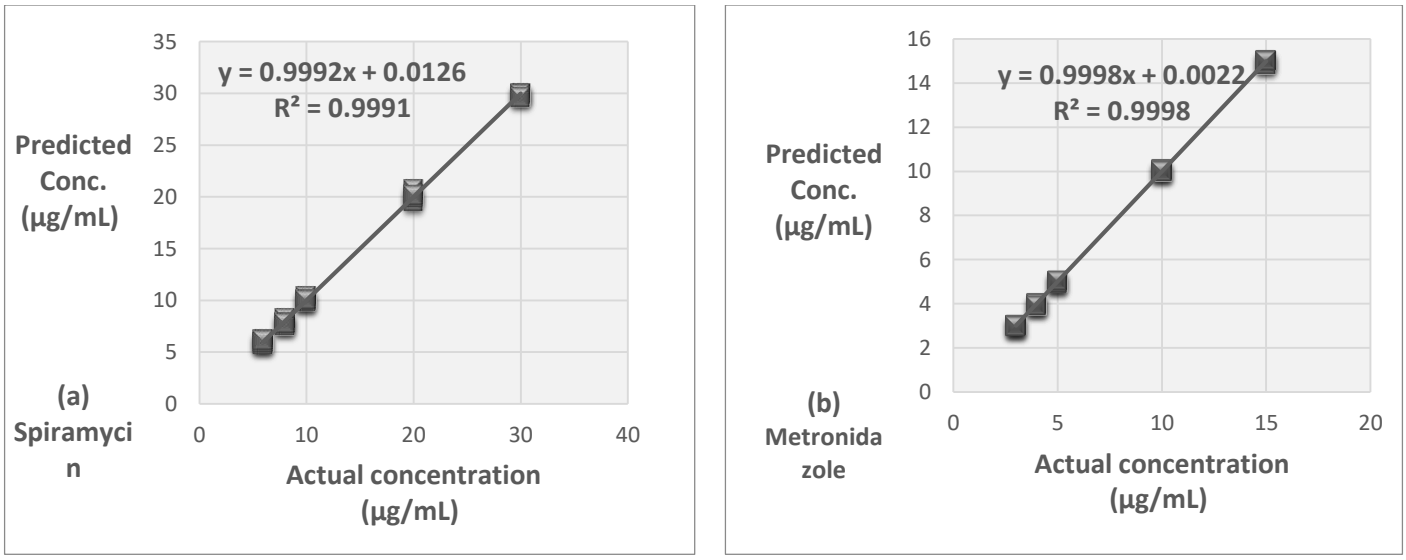


Figure 3: The PCR cross validation for calibration set of the actual vs. predicted concentration

3.2 Validation method for of spiramycin and metronidazole

3.2.1 Linearity method

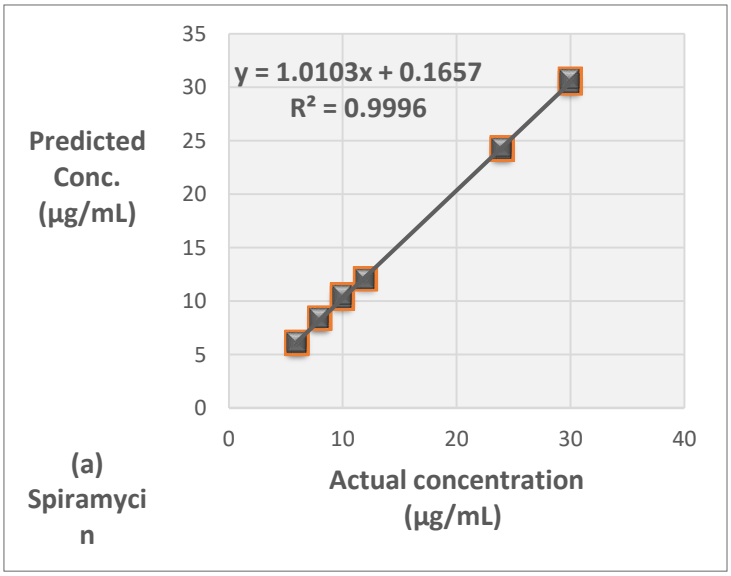
Building a cross-validation of the data as shown in Table 4 allowed researchers to assess the linearity of the created approaches for both PLS and PCR models. The results obtained (Figures 4 and 5) indicated that the developed method possessed high linearity: R^2 = 0.9996 and 0.9998 for PLS and PCR models respectively, within the method linear range (6 – 30 µg.mL^{-1}) of spiramycin. Whereas R^2 = 0.9974 and 0.9974 for PLS and PCR models respectively, within the method linear range (3 – 15 µg.mL^{-1}) of metronidazole.

3.2.2 Construction of validation set

Table 4 displays the predictions' results and recovery percentages. By comparing the actual known concentrations to the predicted concentrations depicted in Figures 4 and 5, the models' prediction abilities were evaluated. Figures 4 and 5 show excellent agreement between calculated and actual spiramycin and metronidazole concentrations for PLS and PCR models.

Table 4: Results of validation set of spiramycin and metronidazole for PLS and PCR model

NO.	METHOD		PLS				PCR			
	Spira.	Metr.	Spira.		Metr.		Spira.		Metr.	
	Actual ($\mu g.mL^{-1}$)		Predicted ($\mu g.mL^{-1}$)	R%	Predicted ($\mu g.mL^{-1}$)	R%	Predicted ($\mu g.mL^{-1}$)	R %	Predicted ($\mu g.mL^{-1}$)	R%
1	8	8	8.4	105.00	7.85	98.13	7.80	97.50	7.76	97.00
2	8	12	8.4	105.00	11.76	98.00	7.78	97.25	11.67	97.25
3	24	8	24.2	100.83	7.98	99.75	23.00	95.83	7.74	96.75
4	24	12	24.34	101.42	12.13	101.08	22.90	95.42	11.93	99.42
5	6	10	6.03	100.50	9.84	98.40	6.00	100.00	9.84	98.40
6	6	15	6.13	102.17	14.81	98.73	5.80	96.67	14.80	98.67
7	10	10	10.26	102.60	9.96	99.60	9.70	97.00	9.84	98.40
8	10	15	10.57	105.70	15.16	101.07	9.80	98.00	15.12	100.80
9	30	10	30.39	101.30	10.08	100.80	29.00	96.67	9.79	97.90
10	30	15	30.75	102.50	14.92	99.47	28.50	95.00	14.64	97.60
11	12	12	12.08	100.67	12.06	100.50	11.55	96.25	11.99	99.92
			Mean%	102.52		99.59	Mean%	96.87		98.37
			RSD%	1.84		1.17	RSD%	1.41		1.29



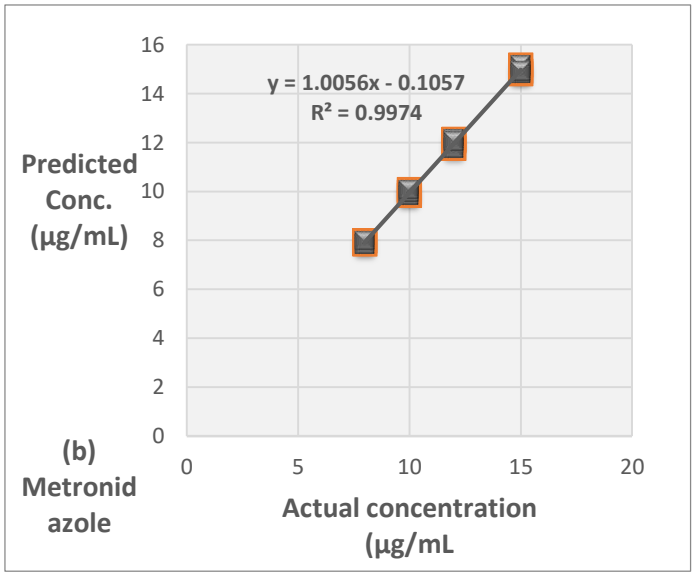


Figure 4: The PLS cross-validation for validation set of the actual vs. predicted concentration

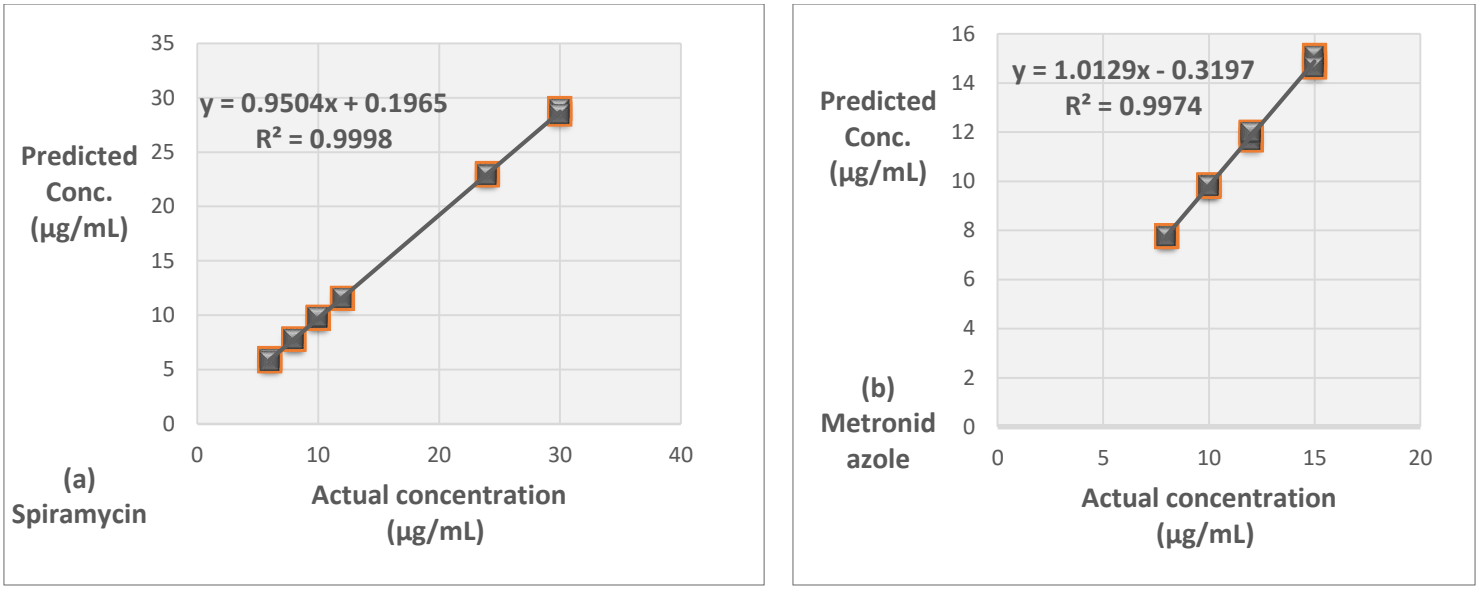


Figure 5: The PCR cross-validation for validation set of the actual vs. predicted concentration

3.2.3 Precision (Repeatability)

The repeatability (intraday precision) of the developed method was carried out by determining the binary mixture at three different concentrations for spiramycin and metronidazole in bulk using three different concentrations (i.e. 6/3, 10/10 and 30/15 µg.mL^{-1} of spiramycin / metronidazole, respectively) in triplicates sequentially. The results were reported as % RSD. The low values of % RSD were indicative of 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.

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

Amount taken (Actual Conc.) µg.mL^{-1}		Predicted Conc. µg.mL^{-1}				% Recovery				Acceptable % RSD NMT 2%			
Spira.	Metro.	PLS		PCR		PLS		PCR		PLS		PCR	
		Spira.	Metro.	Spira.	Metro.	Spira.	Metro.	Spira.	Metro.	Spira.	Metro.	Spira.	Metro.
6	3	5.96	2.99	5.95	2.99	99.33	99.67	99.17	99.67	0.63	1.07	1.87	0.88
6	3	5.95	3.05	5.77	3.03	99.17	101.67	96.17	101.00				
6	3	6.02	3.00	5.97	2.98	100.33	100.00	99.50	99.33				
10	10	9.95	10.05	10.01	10.04	99.50	100.50	100.10	100.40	0.46	0.87	0.55	0.55
10	10	10.04	10.10	10.12	9.98	100.40	101.00	101.20	99.80				
10	10	9.98	9.93	10.05	10.09	99.80	99.30	100.50	100.90				
30	15	29.98	15.02	29.92	15.03	99.93	100.13	99.73	100.20	0.10	0.10	0.11	0.06
30	15	29.98	15.00	29.97	15.02	99.93	100.00	99.90	100.13				
30	15	30.03	14.99	29.98	15.01	100.10	99.93	99.93	100.07				

% Recovery = Predicted Conc. (µg.mL-1) / Actual Conc. (µg.mL-1) ×100

3.2.4 Accuracy

Recovery tests were used to examine the approach's accuracy using the standard addition method for three different percentage levels (i.e., 80, 100, and 120%). Known amounts of standard solutions containing spiramycin and metronidazole were added to sample solutions under investigation to make

up solutions of 80%, 100% and 120% levels in triplicates and scanned at the range 200-400 nm. The amount of the drugs recovered at each percentage level were estimated by using the developed PCR and PLS models. The mean percentage recovery for each percentage level was showed low values of % RSD and the percentage recovery was within the acceptable limit (90-110%) as suggested by USP pharmacopeia. This indicates a high accuracy method at all three levels, and the accuracy results are shown in Tables 6 and 7.

Table 6: Accuracy data of spiramycin by PCR and PLS models

%Level	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
80%	5.86	10	15.86	16.40	15.67	103.40	98.80	0.94	0.76
				16.50	15.89	104.04	100.19		
				16.20	15.86	102.14	100.00		
100%	7.32	10	17.32	18.40	18.12	106.24	104.62	0.38	0.75
				18.54	18.30	107.04	105.66		
				18.45	18.39	106.52	106.18		
120%	9	10	19	20.90	21.07	110.00	110.89	0.39	0.21
				20.75	20.99	109.21	110.47		
				20.77	21.06	109.32	110.84		

Table 7: Accuracy data of metronidazole by PCR and PLS models

%Level	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
80%	4	5	9	9.11	8.94	101.22	99.33	0.38	0.29
				9.11	8.95	101.22	99.44		
				9.05	8.90	100.56	98.89		
100%	5	5	10	10.11	10.00	101.10	100.00	0.21	0.15
				10.07	10.01	100.70	100.10		
				10.10	10.03	101.00	100.30		
120%	6	5	11	11.03	10.98	100.27	99.82	0.19	0.27
				11.00	10.93	100.00	99.36		
				10.99	10.93	99.91	99.36		

3.2.5 Specificity (Spiking Method)

The specificity of the method was tested by adding a known amount of spiramycin and metronidazole standard into known amount of marketed sample solution as described earlier (i.e. Methodology). Specificity data are displayed in Table 8 and 9.

Table 8: Results of specificity for spiramycin using the developed PCR and PLS models

Name of 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
Spirazine	7.32	10	17.32	18.29	18.06	105.60	104.27	0.43	0.24
				18.40	18.12	106.24	104.62		
Spiradent	7.32	10	17.32	18.54	18.30	107.04	105.66	0.34	0.35
				18.45	18.39	106.52	106.18		
Dentazole	7.32	10	17.32	18.47	18.42	106.64	106.35	0.15	1.07
				18.51	18.70	106.87	107.97		

Table 9: Results of specificity for metronidazole using the developed PCR and PLS models

Name of 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
Spirazine	5	5	10	10.10	9.99	101.00	99.90	0.07	0.07
				10.11	10.00	101.10	100.00		
Spiradent	5	5	10	10.07	10.01	100.70	100.10	0.21	0.14
				10.10	10.03	101.00	100.30		
Dentazole	5	5	10	10.07	9.99	100.70	99.90	0.14	0.49
				10.09	10.06	100.90	100.60		

As can be seen from these data, recovery for spiramycin and metronidazole using the developed PCR and PLS models is 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 results indicate that the method is simple, rapid, economical, precise, and accurate, in addition to being eco-friendly. Therefore, it can be used for routine analysis in quality control of mixtures and commercial products containing spiramycin and metronidazole.

3.2.6. Analysis of marketed formulations

The applicability of the developed methods for quantifying spiramycin and metronidazole in marketed formulations was tested using a marketed formulation of 250 mg spiramycin with a metronidazole concentration of 125 mg collected from local pharmacies in the capital Sana'a. Tables 10 and 11 summarized the data obtained for spiramycin and metronidazole in the marketed formulations under consideration.

Table 10: Assay result for spiramycin and metronidazole in tablet (Marketed Sample) by PLS proposed method

Name of marketed sample	METHOD		PLS					
	Spira.	Metr.	Spira.			Metr.		
	Actual ($\mu g.mL^{-1}$)		Predicted ($\mu g.mL^{-1}$)	% Recovery	% RSD	Predicted ($\mu g.mL^{-1}$)	% Recovery	% RSD
Spirazine	14.62	10	15.11	103.35	0.10	10.51	105.10	0.27
	14.62	10	15.09	103.21		10.55	105.50	
Spiradent	14.62	10	15.48	105.88	0.55	10.48	104.80	0.54
	14.62	10	15.60	106.70		10.40	104.00	
Dentazole	14.62	10	15.50	106.02	1.80	10.45	104.50	0.40
	14.62	10	15.11	103.35		10.51	105.10	

Table 11: Assay result for spiramycin and metronidazole in tablet (Marketed Sample) by PCR proposed method

Name of marketed sample	METHOD		PCR					
	Spira.	Metr.	Spira.			Metr.		
	Actual ($\mu g.mL^{-1}$)		Predicted ($\mu g.mL^{-1}$)	% Recovery	% RSD	Predicted ($\mu g.mL^{-1}$)	% Recovery	% RSD
Spirazine	14.62	10	13.80	94.39	0.52	10.37	103.70	0.14
	14.62	10	13.90	95.08		10.39	103.90	
Spiradent	14.62	10	13.70	93.71	0.82	10.26	102.60	0.41
	14.62	10	13.86	94.80		10.32	103.20	
Dentazole	14.62	10	13.90	95.08	0.50	10.24	102.40	0.89
	14.62	10	14.00	95.76		10.37	103.70	

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

3.2.7 Comparing with reference method

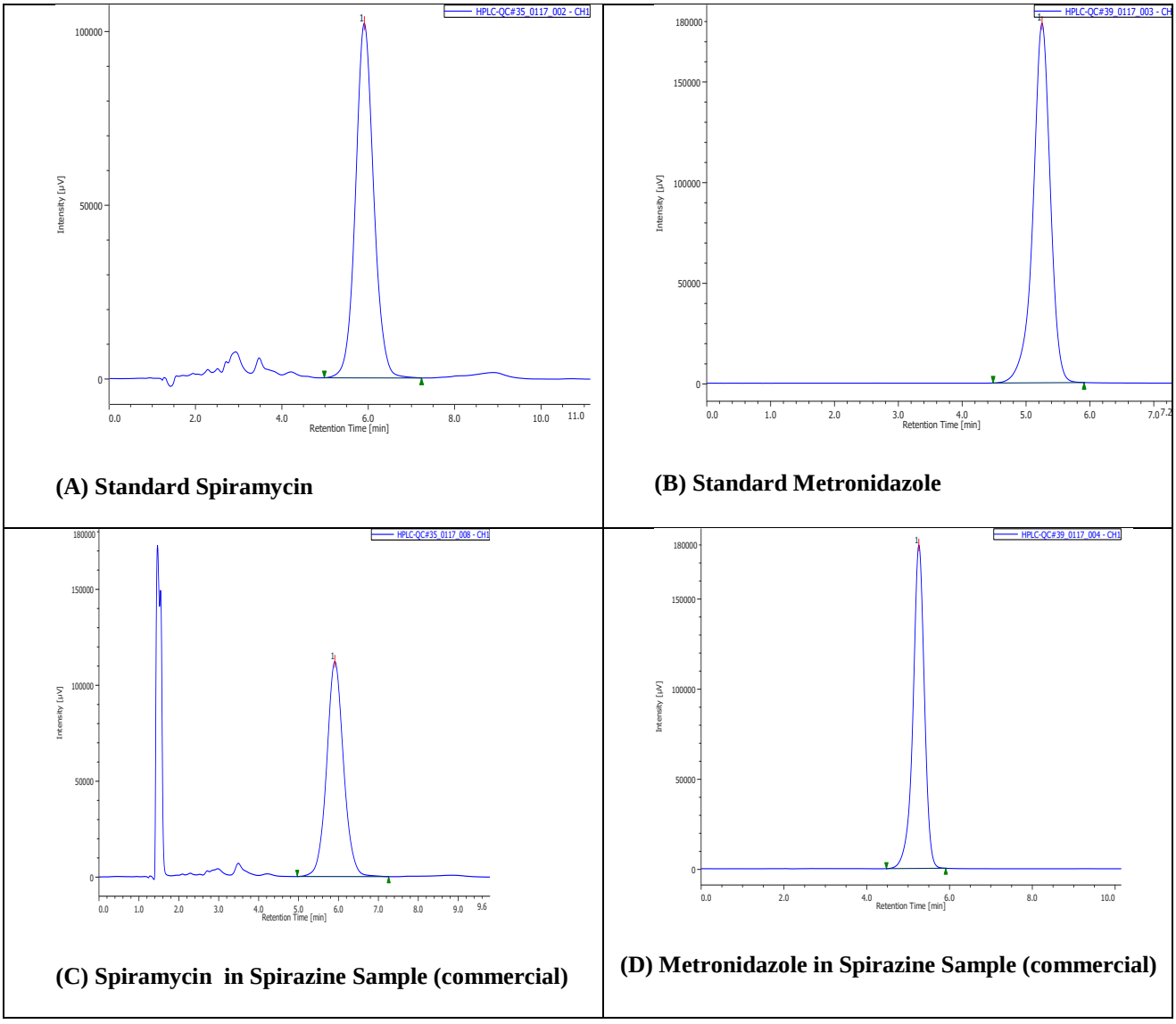
A comparison was carried out with the aid of the SPSS program using F-Test to assure a non-significant difference between the recovery results of the newly developed methods and those of the reference method for both spiramycin and metronidazole. The significance level indicated that the null hypothesis was acceptable since the P-value was greater than the significance level (Table 12). As for reference methods, spiramycin and metronidazole were determined according to an in-house developed method, as described earlier in the methodology.

Table (12): Results of statistical comparison between newly developed method and reference method

Name of marketed sample	Component	Spiramycin			Metronidazole		
	Methods	Reference method (HPLC)	PLS	PCR	Reference method HPLC	PLS	PCR
Spirazine		103.10	103.35	94.39	103.44	105.10	103.70
		97.20	103.21	95.08	104.69	105.50	103.90
	F-value		0.40	0.21		0.21	0.716
Dentazole		100.11	106.02	95.08	103.40	104.50	102.40
		103.01	103.35	95.76	103.21	105.10	103.70
	F-value		0.254	0.054		0.042	0.735

p -value =0.01

Also, the chromatograms in Figure 6 show the results of the analysis for the reference method for the determination of spiramycin and metronidazole.

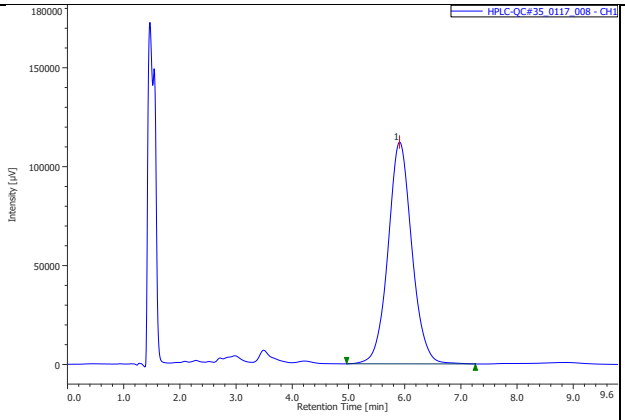


(A) Standard Spiramycin

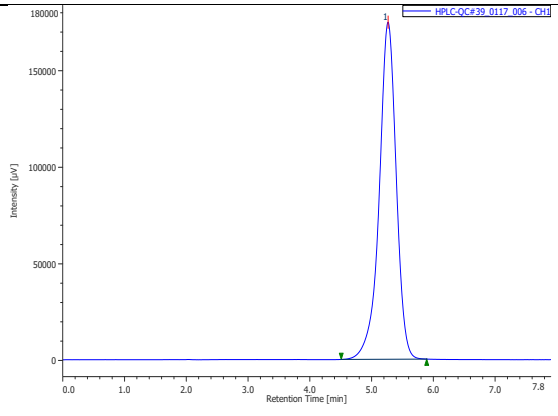
(B) Standard Metronidazole

(C) Spiramycin in Spirazine Sample (commercial)

(D) Metronidazole in Spirazine Sample (commercial)



(E) Spiramycin in Dentazole Sample (commercial)



(F) Metronidazole in Dentazole Sample (commercial)

Figure 6: Chromatogram of spiramycin and metronidazole standard and commercial samples

Conclusions

The suggested chemometrics models (PLS and PCR) allow for the simultaneous determination of spiramycin and metronidazole in binary combinations in pharmaceutical dosage forms without the necessity for physical separation of the two medications beforehand or excipient interference. Spectral and concentration data matrices were used to create multivariate calibration models. Excellent results were obtained from the validation of the two models and their application to a commercial pharmaceutical dosage form. Thus, the proposed methods may be used to conduct routine quality checks on the prescribed drugs in their combination dose form in pharmaceutical labs.

Data Availability

The data used to support the findings of this study are included within the article and the supplementary information file.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Appendix 1: Results of optimum number of principal factors of spiramycin for PLS models

Method	Components to evaluate	Number of components evaluated	Number of components selected		
Cross-validation (Leave-one-out)	Set	10	8		
Model selection and validation for spiramycin					
Components	X Variance	Error	R-sq	Press	R-sq (Pred)
1	0.331105	267.161	0.86800	509.540	0.748251
2	0.995048	31.721	0.98433	40.428	0.980026
3	0.997917	10.274	0.99492	16.241	0.991976
4	0.999602	3.806	0.99812	6.254	0.996910
5	0.999823	2.442	0.99879	4.724	0.997666
6	0.999863	1.135	0.99944	5.146	0.997457
7	0.999885	0.246	0.99988	3.252	0.998393
8	0.999903	0.101	0.99995	2.801	0.998616
9	--	0.027	0.99999	2.891	0.998572
10	--	0.002	1.00000	2.971	0.998532

Appendix 2: Results of optimum number of principal factors of metronidazole 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 metronidazole					
Components	X Variance	Error	R-sq	Press	R-sq (Pred)
1	0.791328	7.09674	0.98597	8.98458	0.982244
2	0.995031	2.08673	0.99588	2.79341	0.994479
3	0.998326	0.26120	0.99948	0.37149	0.999266
4	0.999608	0.07479	0.99985	0.12087	0.999761
5	--	0.06441	0.99987	0.13277	0.999738
6	--	0.01700	0.99997	0.19409	0.999616
7	--	0.00793	0.99998	0.15422	0.999695
8	--	0.00432	0.99999	0.17472	0.999655
9	--	0.00100	1.00000	0.17725	0.999650
10	--	0.00012	1.00000	0.18688	0.999631

Appendix 3: The constant and coefficients at each wavelength of spiramycin and metronidazole for PLS models

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
375	40.4608	287.4	-0.2201	375	-2.45317	287.4	0.10084
374.8	35.6593	287.2	-0.2075	374.8	-2.29171	287.2	0.10112
374.6	41.9783	287	-0.1743	374.6	-2.37157	287	0.10042
374.4	-58.8424	286.8	-0.2509	374.4	-2.49219	286.8	0.10243
374.2	36.0237	286.6	-0.1163	374.2	-2.21381	286.6	0.10073
374	65.2703	286.4	-0.6674	374	-2.14306	286.4	0.10055
373.8	-53.5219	286.2	-0.218	373.8	-2.00174	286.2	0.10152
373.6	-3.9296	286	-0.3851	373.6	-2.42354	286	0.10094
373.4	27.7787	285.8	-0.0136	373.4	-2.08307	285.8	0.10414
373.2	-7.6274	285.6	-0.2559	373.2	-2.03824	285.6	0.10078
373	9.1554	285.4	-0.0458	373	-1.88407	285.4	0.10236
372.8	40.8262	285.2	-0.105	372.8	-1.78675	285.2	0.10345
372.6	-32.7623	285	-0.0466	372.6	-1.48681	285	0.10373
372.4	-11.8975	284.8	-0.0738	372.4	-1.6229	284.8	0.10435
372.2	-5.449	284.6	-0.2115	372.2	-1.56595	284.6	0.10291
372	-11.2261	284.4	-0.3164	372	-1.25058	284.4	0.10221
371.8	-9.7872	284.2	-0.4815	371.8	-1.39214	284.2	0.10236
371.6	-14.2632	284	-0.3119	371.6	-1.43179	284	0.10447
371.4	-12.1379	283.8	-0.14	371.4	-1.38212	283.8	0.10426
371.2	7.3642	283.6	-0.0087	371.2	-1.10849	283.6	0.10536
371	31.4386	283.4	-0.2357	371	-1.23693	283.4	0.10284

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
370.8	1.1946	283.2	-0.277	370.8	-1.27106	283.2	0.10559
370.6	-21.5484	283	-0.2999	370.6	-1.38032	283	0.10432
370.4	-5.6364	282.8	0.0954	370.4	-1.1413	282.8	0.10521
370.2	18.5791	282.6	0.1623	370.2	-0.9344	282.6	0.10541
370	-27.5308	282.4	0.0891	370	-0.91755	282.4	0.10419
369.8	57.9875	282.2	-0.1599	369.8	-0.95877	282.2	0.10495
369.6	29.57	282	-0.3474	369.6	-1.17305	282	0.10449
369.4	-16.1046	281.8	-0.1426	369.4	-0.90038	281.8	0.10602
369.2	-34.2927	281.6	0.1546	369.2	-1.13768	281.6	0.10637
369	-45.0789	281.4	-0.1536	369	-1.07058	281.4	0.10621
368.8	-41.1764	281.2	-0.0249	368.8	-0.87568	281.2	0.10658
368.6	-28.3345	281	-0.2997	368.6	-0.97256	281	0.10566
368.4	-29.7417	280.8	-0.1037	368.4	-0.86542	280.8	0.10672
368.2	10.9382	280.6	-0.0345	368.2	-0.56378	280.6	0.10779
368	-11.0083	280.4	-0.2424	368	-0.5647	280.4	0.10574
367.8	-34.1882	280.2	0.0544	367.8	-0.66638	280.2	0.10696
367.6	-38.0884	280	-0.1337	367.6	-0.63057	280	0.10655
367.4	-25.6079	279.8	0.214	367.4	-0.60836	279.8	0.1078
367.2	8.7277	279.6	0.0088	367.2	-0.53728	279.6	0.10741
367	-15.5487	279.4	-0.0345	367	-0.50083	279.4	0.10741
366.8	-13.2211	279.2	-0.0648	366.8	-0.5191	279.2	0.1074
366.6	-20.6931	279	0.0006	366.6	-0.42533	279	0.1088
366.4	17.8575	278.8	-0.1161	366.4	-0.3934	278.8	0.10903
366.2	-9.7144	278.6	-0.0095	366.2	-0.28494	278.6	0.1093
366	22.2344	278.4	-0.098	366	-0.372	278.4	0.10857
365.8	20.4369	278.2	-0.1007	365.8	-0.20177	278.2	0.10975
365.6	-21.1011	278	-0.0318	365.6	-0.31631	278	0.10881
365.4	-11.3704	277.8	-0.1838	365.4	-0.1245	277.8	0.10911
365.2	-3.1948	277.6	0.1822	365.2	-0.23585	277.6	0.11002
365	-15.0869	277.4	-0.152	365	-0.32437	277.4	0.10978
364.8	22.3033	277.2	-0.1611	364.8	-0.1664	277.2	0.10903
364.6	-4.5155	277	-0.0058	364.6	-0.28555	277	0.10929
364.4	0.2981	276.8	0.0511	364.4	-0.14756	276.8	0.1103
364.2	-12.3712	276.6	-0.202	364.2	-0.19013	276.6	0.11097
364	-21.954	276.4	-0.2108	364	-0.19976	276.4	0.11002
363.8	0.1042	276.2	-0.137	363.8	-0.30188	276.2	0.11129
363.6	0.8079	276	-0.0651	363.6	-0.19207	276	0.10958
363.4	20.4023	275.8	-0.0889	363.4	-0.07763	275.8	0.11005
363.2	-6.7801	275.6	-0.2546	363.2	-0.27068	275.6	0.11
363	11.6493	275.4	-0.0014	363	-0.1344	275.4	0.11232
362.8	-4.4714	275.2	0.0596	362.8	-0.15968	275.2	0.11124

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
362.6	3.9379	275	0.0655	362.6	-0.07146	275	0.11216
362.4	-10.9351	274.8	-0.0296	362.4	-0.15219	274.8	0.11267
362.2	5.4798	274.6	0.1913	362.2	-0.03405	274.6	0.1123
362	-12.8642	274.4	0.2135	362	-0.12595	274.4	0.11389
361.8	-15.9853	274.2	-0.0519	361.8	-0.09501	274.2	0.11246
361.6	7.6022	274	0.2115	361.6	-0.10551	274	0.1127
361.4	-6.7874	273.8	0.2898	361.4	-0.04103	273.8	0.11438
361.2	-5.5953	273.6	0.3696	361.2	-0.11679	273.6	0.11447
361	-0.6972	273.4	0.2529	361	-0.1033	273.4	0.11308
360.8	-7.0139	273.2	0.1342	360.8	-0.08415	273.2	0.11524
360.6	-0.2077	273	-0.0712	360.6	-0.05682	273	0.11504
360.4	-1.0854	272.8	0.0457	360.4	0.01362	272.8	0.11389
360.2	0.5487	272.6	-0.0555	360.2	-0.10538	272.6	0.11299
360	-14.9672	272.4	0.0147	360	-0.03421	272.4	0.11613
359.8	-10.9381	272.2	0.1797	359.8	-0.07408	272.2	0.11533
359.6	5.7257	272	-0.0337	359.6	-0.03021	272	0.11578
359.4	7.4489	271.8	0.3796	359.4	0.02204	271.8	0.1182
359.2	0.1308	271.6	0.0324	359.2	-0.03992	271.6	0.11792
359	-7.7681	271.4	0.1415	359	-0.08887	271.4	0.11397
358.8	4.4946	271.2	-0.1192	358.8	0.04493	271.2	0.11678
358.6	-13.2485	271	0.0531	358.6	-0.06475	271	0.11748
358.4	-0.0825	270.8	0.1176	358.4	-0.01343	270.8	0.1169
358.2	-2.5763	270.6	0.1312	358.2	0.00388	270.6	0.11726
358	2.9557	270.4	0.1047	358	0.01871	270.4	0.11775
357.8	-10.1328	270.2	0.2815	357.8	-0.03606	270.2	0.11956
357.6	1.6786	270	0.1765	357.6	0.02424	270	0.11882
357.4	-4.9856	269.8	0.1713	357.4	0.03366	269.8	0.11851
357.2	8.5973	269.6	0.068	357.2	0.03447	269.6	0.12041
357	-5.8164	269.4	0.3178	357	0.0081	269.4	0.12006
356.8	-3.9699	269.2	0.4481	356.8	-0.02894	269.2	0.12217
356.6	-3.8207	269	-0.0818	356.6	0.07213	269	0.12015
356.4	-1.1833	268.8	0.4078	356.4	0.00586	268.8	0.12178
356.2	2.5949	268.6	0.1709	356.2	0.06469	268.6	0.12093
356	-4.5674	268.4	0.235	356	0.02953	268.4	0.12279
355.8	-3.1282	268.2	0.2959	355.8	0.0576	268.2	0.12199
355.6	-9.3841	268	0.2284	355.6	-0.03448	268	0.12162
355.4	5.8831	267.8	0.4321	355.4	0.05287	267.8	0.12371
355.2	-5.0662	267.6	0.0705	355.2	0.05823	267.6	0.12143
355	-2.5031	267.4	0.6326	355	0.05749	267.4	0.12275
354.8	-3.162	267.2	0.4619	354.8	0.04498	267.2	0.12613
354.6	0.4881	267	0.4821	354.6	0.08418	267	0.1245

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
354.4	5.5762	266.8	0.278	354.4	0.06511	266.8	0.1246
354.2	1.6508	266.6	0.1821	354.2	0.11271	266.6	0.12371
354	2.5895	266.4	0.1424	354	0.06895	266.4	0.1249
353.8	-10.875	266.2	0.1699	353.8	0.02127	266.2	0.12568
353.6	3.309	266	0.4125	353.6	0.09268	266	0.12586
353.4	4.8961	265.8	0.3242	353.4	0.07103	265.8	0.126
353.2	-1.6608	265.6	0.2828	353.2	0.04623	265.6	0.12597
353	3.2458	265.4	0.3314	353	0.0917	265.4	0.12724
352.8	0.6885	265.2	-0.0066	352.8	0.08046	265.2	0.12549
352.6	-2.8829	265	0.5442	352.6	0.07666	265	0.12904
352.4	2.2046	264.8	0.4186	352.4	0.08978	264.8	0.12908
352.2	-4.2988	264.6	0.5	352.2	0.0549	264.6	0.12923
352	6.3149	264.4	0.3761	352	0.0826	264.4	0.12899
351.8	3.2131	264.2	0.4761	351.8	0.06637	264.2	0.12987
351.6	0.1626	264	0.2465	351.6	0.06092	264	0.12968
351.4	-9.6075	263.8	0.3948	351.4	0.0479	263.8	0.13056
351.2	-1.3219	263.6	0.4988	351.2	0.10402	263.6	0.13309
351	-1.4291	263.4	0.5157	351	0.08133	263.4	0.13105
350.8	-0.5552	263.2	0.3911	350.8	0.07357	263.2	0.13149
350.6	-0.1555	263	0.582	350.6	0.07271	263	0.13107
350.4	4.5958	262.8	0.4876	350.4	0.08599	262.8	0.13029
350.2	4.5621	262.6	0.4228	350.2	0.08753	262.6	0.13105
350	8.2667	262.4	0.3665	350	0.09712	262.4	0.13097
349.8	-0.4962	262.2	0.5409	349.8	0.0861	262.2	0.13317
349.6	-1.5243	262	0.4778	349.6	0.0507	262	0.13227
349.4	-0.7547	261.8	0.6513	349.4	0.08665	261.8	0.1351
349.2	2.4917	261.6	0.5572	349.2	0.08961	261.6	0.13159
349	1.7085	261.4	0.5262	349	0.10296	261.4	0.13325
348.8	-0.8067	261.2	0.5256	348.8	0.08412	261.2	0.13451
348.6	-0.6275	261	0.4816	348.6	0.07349	261	0.13504
348.4	1.0159	260.8	0.3313	348.4	0.10673	260.8	0.13365
348.2	2.5501	260.6	0.4084	348.2	0.10409	260.6	0.1367
348	2.4993	260.4	0.3668	348	0.0879	260.4	0.13615
347.8	-2.5684	260.2	0.6158	347.8	0.08622	260.2	0.13646
347.6	-0.5678	260	0.0301	347.6	0.10269	260	0.13549
347.4	0.7468	259.8	0.328	347.4	0.0948	259.8	0.13607
347.2	3.4047	259.6	0.3528	347.2	0.10479	259.6	0.13697
347	-1.3395	259.4	0.3366	347	0.08739	259.4	0.13529
346.8	2.2899	259.2	0.3876	346.8	0.11255	259.2	0.13715
346.6	-3.1364	259	0.3248	346.6	0.07608	259	0.13798
346.4	-0.0792	258.8	0.2481	346.4	0.10092	258.8	0.13615

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
346.2	-3.3567	258.6	0.4316	346.2	0.07863	258.6	0.13442
346	-0.6095	258.4	0.5426	346	0.07775	258.4	0.13721
345.8	-0.277	258.2	0.3038	345.8	0.09371	258.2	0.13794
345.6	0.8825	258	0.5439	345.6	0.09052	258	0.13942
345.4	-0.1917	257.8	0.3216	345.4	0.07796	257.8	0.13651
345.2	-1.8692	257.6	0.2237	345.2	0.07087	257.6	0.13458
345	0.7034	257.4	0.4605	345	0.08427	257.4	0.13831
344.8	-0.0254	257.2	0.5027	344.8	0.08233	257.2	0.13797
344.6	0.9423	257	0.7171	344.6	0.08359	257	0.13838
344.4	-1.6405	256.8	0.7389	344.4	0.09216	256.8	0.13889
344.2	-1.0528	256.6	0.3591	344.2	0.07631	256.6	0.13844
344	3.766	256.4	0.3949	344	0.08391	256.4	0.13706
343.8	0.3832	256.2	0.5192	343.8	0.06704	256.2	0.13847
343.6	0.1366	256	0.2552	343.6	0.07846	256	0.13625
343.4	-3.0984	255.8	0.4697	343.4	0.07071	255.8	0.13639
343.2	0.9255	255.6	0.0885	343.2	0.07995	255.6	0.13426
343	-1.5266	255.4	0.5427	343	0.07683	255.4	0.13445
342.8	0.5879	255.2	0.4172	342.8	0.07806	255.2	0.13584
342.6	-1.2196	255	0.515	342.6	0.08622	255	0.13737
342.4	2.113	254.8	0.3839	342.4	0.08482	254.8	0.13679
342.2	-0.452	254.6	0.3335	342.2	0.09101	254.6	0.13444
342	2.0142	254.4	0.0552	342	0.09501	254.4	0.1348
341.8	2.063	254.2	0.2379	341.8	0.09225	254.2	0.13516
341.6	-0.4033	254	0.1398	341.6	0.08389	254	0.13312
341.4	-1.1297	253.8	0.1547	341.4	0.0799	253.8	0.13109
341.2	0.5788	253.6	0.0124	341.2	0.09058	253.6	0.13096
341	-0.3431	253.4	0.1075	341	0.06763	253.4	0.12883
340.8	2.1587	253.2	-0.0774	340.8	0.0831	253.2	0.12663
340.6	0.1925	253	-0.4055	340.6	0.09918	253	0.12498
340.4	-0.1709	252.8	0.0518	340.4	0.07913	252.8	0.12596
340.2	1.292	252.6	-0.2246	340.2	0.08184	252.6	0.12672
340	0.6179	252.4	-0.1527	340	0.08849	252.4	0.1242
339.8	-0.7556	252.2	-0.3463	339.8	0.07425	252.2	0.12304
339.6	-0.4991	252	-0.4644	339.6	0.07902	252	0.11949
339.4	-0.4489	251.8	-0.3766	339.4	0.06583	251.8	0.11937
339.2	-1.9792	251.6	-0.2582	339.2	0.07223	251.6	0.11774
339	2.597	251.4	-0.6242	339	0.07875	251.4	0.11435
338.8	0.1272	251.2	-0.6241	338.8	0.07208	251.2	0.11226
338.6	0.6587	251	-0.3734	338.6	0.08033	251	0.11031
338.4	-0.9787	250.8	-0.3192	338.4	0.07096	250.8	0.10944
338.2	0.3162	250.6	-1.2694	338.2	0.07472	250.6	0.10354

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
338	-2.2809	250.4	-0.5563	338	0.07894	250.4	0.10116
337.8	-0.0096	250.2	-1.0166	337.8	0.06987	250.2	0.09703
337.6	1.0028	250	-0.837	337.6	0.10552	250	0.09594
337.4	0.1381	249.8	-1.0136	337.4	0.07645	249.8	0.09204
337.2	0.3336	249.6	-0.9415	337.2	0.08123	249.6	0.08897
337	1.7578	249.4	-1.1425	337	0.08403	249.4	0.08414
336.8	-0.5493	249.2	-0.682	336.8	0.06524	249.2	0.08565
336.6	-0.3544	249	-1.5837	336.6	0.07878	249	0.07494
336.4	1.1249	248.8	-1.1157	336.4	0.08498	248.8	0.07325
336.2	0.2583	248.6	-1.212	336.2	0.07724	248.6	0.06959
336	1.6184	248.4	-1.338	336	0.08557	248.4	0.06529
335.8	2.3078	248.2	-1.3639	335.8	0.08235	248.2	0.06053
335.6	0.5604	248	-1.2698	335.6	0.08431	248	0.05796
335.4	-0.0659	247.8	-1.1942	335.4	0.07412	247.8	0.05035
335.2	-0.1971	247.6	-1.3745	335.2	0.07732	247.6	0.04709
335	0.7589	247.4	-1.2283	335	0.08287	247.4	0.04467
334.8	1.5555	247.2	-1.3708	334.8	0.07796	247.2	0.03904
334.6	0.5613	247	-1.3155	334.6	0.07926	247	0.03597
334.4	-0.8189	246.8	-1.2655	334.4	0.0604	246.8	0.03223
334.2	-2.0152	246.6	-1.2731	334.2	0.06883	246.6	0.02859
334	1.9941	246.4	-1.2037	334	0.0892	246.4	0.02569
333.8	3.7905	246.2	-1.0763	333.8	0.09029	246.2	0.02169
333.6	2.6867	246	-1.0118	333.6	0.07545	246	0.01974
333.4	0.0205	245.8	-0.894	333.4	0.07975	245.8	0.0167
333.2	0.2925	245.6	-0.7615	333.2	0.08414	245.6	0.01443
333	0.0147	245.4	-0.7095	333	0.07133	245.4	0.01199
332.8	0.8327	245.2	-0.6918	332.8	0.07416	245.2	0.01083
332.6	1.252	245	-0.7351	332.6	0.07722	245	0.00895
332.4	2.6102	244.8	-0.6816	332.4	0.09157	244.8	0.00666
332.2	-1.3555	244.6	-0.6573	332.2	0.07674	244.6	0.00543
332	0.7393	244.4	-0.5025	332	0.06636	244.4	0.00397
331.8	0.63	244.2	-0.482	331.8	0.06588	244.2	0.00279
331.6	-0.9878	244	-0.3954	331.6	0.07231	244	0.00229
331.4	-0.5016	243.8	-0.2671	331.4	0.07325	243.8	0.00132
331.2	0.9578	243.6	-0.2764	331.2	0.07002	243.6	0.00011
331	0.4027	243.4	-0.1566	331	0.07532	243.4	-0.00017
330.8	-1.289	243.2	-0.1266	330.8	0.06253	243.2	-0.00063
330.6	0.7138	243	-0.062	330.6	0.08375	243	-0.00095
330.4	1.1416	242.8	-0.1542	330.4	0.06121	242.8	-0.00186
330.2	-0.7412	242.6	-0.0541	330.2	0.08522	242.6	-0.00202
330	2.0207	242.4	0.0946	330	0.07258	242.4	-0.00223

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
329.8	0.821	242.2	0.0493	329.8	0.07882	242.2	-0.00294
329.6	-0.6269	242	0.1732	329.6	0.08257	242	-0.00273
329.4	-0.2374	241.8	0.1508	329.4	0.07873	241.8	-0.003
329.2	-0.8513	241.6	0.1269	329.2	0.07741	241.6	-0.00342
329	-0.2262	241.4	0.2266	329	0.07706	241.4	-0.00344
328.8	1.4978	241.2	0.1944	328.8	0.08294	241.2	-0.00385
328.6	-1.4872	241	0.2536	328.6	0.07758	241	-0.00423
328.4	1.2829	240.8	0.2611	328.4	0.07149	240.8	-0.00392
328.2	0.5158	240.6	0.2389	328.2	0.07746	240.6	-0.00409
328	1.5695	240.4	0.2573	328	0.06767	240.4	-0.00432
327.8	-0.0103	240.2	0.29	327.8	0.07151	240.2	-0.00439
327.6	0.5873	240	0.3071	327.6	0.08125	240	-0.00455
327.4	0.4005	239.8	0.3515	327.4	0.08433	239.8	-0.00467
327.2	-0.7992	239.6	0.2658	327.2	0.07081	239.6	-0.00498
327	2.9953	239.4	0.2784	327	0.08916	239.4	-0.00472
326.8	1.2791	239.2	0.2696	326.8	0.07302	239.2	-0.00517
326.6	-1.3479	239	0.2231	326.6	0.07779	239	-0.00546
326.4	-1.9172	238.8	0.2835	326.4	0.06824	238.8	-0.0052
326.2	0.6011	238.6	0.2693	326.2	0.06747	238.6	-0.00553
326	-0.8483	238.4	0.2722	326	0.04693	238.4	-0.00525
325.8	0.1334	238.2	0.1888	325.8	0.06943	238.2	-0.00587
325.6	0.1757	238	0.2399	325.6	0.0715	238	-0.0057
325.4	1.2789	237.8	0.2698	325.4	0.07681	237.8	-0.00573
325.2	2.8346	237.6	0.2019	325.2	0.07923	237.6	-0.00603
325	1.4471	237.4	0.2442	325	0.06787	237.4	-0.00614
324.8	-0.9128	237.2	0.2535	324.8	0.07334	237.2	-0.0061
324.6	-0.6076	237	0.2138	324.6	0.06101	237	-0.00635
324.4	-1.5579	236.8	0.2411	324.4	0.07927	236.8	-0.00628
324.2	0.6986	236.6	0.1973	324.2	0.08736	236.6	-0.0065
324	0.2073	236.4	0.2067	324	0.06775	236.4	-0.00629
323.8	2.6369	236.2	0.1952	323.8	0.06919	236.2	-0.00667
323.6	0.6936	236	0.2529	323.6	0.08401	236	-0.00641
323.4	0.5448	235.8	0.26	323.4	0.07037	235.8	-0.0065
323.2	0.0104	235.6	0.2453	323.2	0.07746	235.6	-0.00648
323	2.0739	235.4	0.2257	323	0.0695	235.4	-0.0065
322.8	1.7189	235.2	0.2623	322.8	0.08398	235.2	-0.00658
322.6	-3.4428	235	0.2623	322.6	0.07101	235	-0.00648
322.4	2.165	234.8	0.2561	322.4	0.08441	234.8	-0.00647
322.2	-1.2818	234.6	0.318	322.2	0.05945	234.6	-0.00619
322	1.9655	234.4	0.2703	322	0.08001	234.4	-0.00659
321.8	2.2955	234.2	0.3102	321.8	0.09321	234.2	-0.00649

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
321.6	-0.6117	234	0.3629	321.6	0.07817	234	-0.00619
321.4	-0.0423	233.8	0.3809	321.4	0.06944	233.8	-0.00617
321.2	1.1121	233.6	0.3562	321.2	0.07892	233.6	-0.00621
321	-0.0186	233.4	0.3585	321	0.0681	233.4	-0.00619
320.8	0.5186	233.2	0.3871	320.8	0.06618	233.2	-0.00596
320.6	-0.5378	233	0.3362	320.6	0.07556	233	-0.00607
320.4	1.6657	232.8	0.4529	320.4	0.05594	232.8	-0.00586
320.2	2.7984	232.6	0.3976	320.2	0.08124	232.6	-0.00585
320	0.696	232.4	0.3969	320	0.07709	232.4	-0.00573
319.8	0.0341	232.2	0.417	319.8	0.06148	232.2	-0.00586
319.6	0.3279	232	0.4351	319.6	0.06149	232	-0.00561
319.4	-0.3436	231.8	0.4134	319.4	0.05942	231.8	-0.00563
319.2	0.314	231.6	0.4088	319.2	0.06539	231.6	-0.00573
319	0.2849	231.4	0.4137	319	0.06377	231.4	-0.00574
318.8	0.0903	231.2	0.4632	318.8	0.06325	231.2	-0.00552
318.6	0.669	231	0.4381	318.6	0.06732	231	-0.00567
318.4	0.2769	230.8	0.402	318.4	0.06434	230.8	-0.00571
318.2	0.4723	230.6	0.471	318.2	0.06429	230.6	-0.00554
318	-0.0965	230.4	0.5086	318	0.06403	230.4	-0.00543
317.8	1.0527	230.2	0.384	317.8	0.06399	230.2	-0.00576
317.6	0.5982	230	0.4897	317.6	0.06732	230	-0.00549
317.4	-0.0052	229.8	0.5075	317.4	0.06628	229.8	-0.00538
317.2	0.6172	229.6	0.4538	317.2	0.06515	229.6	-0.00548
317	0.245	229.4	0.4439	317	0.06564	229.4	-0.00562
316.8	0.4169	229.2	0.4409	316.8	0.06454	229.2	-0.00564
316.6	0.6306	229	0.4734	316.6	0.06628	229	-0.00543
316.4	0.2383	228.8	0.3855	316.4	0.06006	228.8	-0.00552
316.2	0.6405	228.6	0.4734	316.2	0.06049	228.6	-0.00554
316	0.3801	228.4	0.5217	316	0.0641	228.4	-0.00534
315.8	-0.3757	228.2	0.3668	315.8	0.05875	228.2	-0.0058
315.6	0.2761	228	0.5018	315.6	0.06145	228	-0.00535
315.4	0.0394	227.8	0.4359	315.4	0.06165	227.8	-0.00558
315.2	0.1801	227.6	0.4733	315.2	0.05976	227.6	-0.00559
315	-0.0734	227.4	0.4447	315	0.06433	227.4	-0.00562
314.8	-0.0041	227.2	0.4961	314.8	0.06008	227.2	-0.00542
314.6	-0.4808	227	0.4989	314.6	0.06193	227	-0.00559
314.4	0.3764	226.8	0.4736	314.4	0.0625	226.8	-0.00566
314.2	0.0453	226.6	0.4946	314.2	0.06163	226.6	-0.00559
314	-0.2915	226.4	0.5074	314	0.06405	226.4	-0.00537
313.8	0.1185	226.2	0.4956	313.8	0.06565	226.2	-0.00547
313.6	0.2733	226	0.5367	313.6	0.06707	226	-0.00546

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
313.4	0.5073	225.8	0.4483	313.4	0.06204	225.8	-0.00575
313.2	-0.3951	225.6	0.5651	313.2	0.06358	225.6	-0.00537
313	0.2988	225.4	0.5024	313	0.0674	225.4	-0.00548
312.8	0.0677	225.2	0.5666	312.8	0.06485	225.2	-0.00524
312.6	-0.017	225	0.4934	312.6	0.06611	225	-0.00537
312.4	0.5876	224.8	0.5332	312.4	0.06605	224.8	-0.00545
312.2	-0.2292	224.6	0.5825	312.2	0.06701	224.6	-0.00522
312	-0.0765	224.4	0.5545	312	0.06374	224.4	-0.00522
311.8	0.2067	224.2	0.5398	311.8	0.0645	224.2	-0.00527
311.6	-0.3391	224	0.6077	311.6	0.0623	224	-0.00516
311.4	0.1055	223.8	0.5041	311.4	0.06523	223.8	-0.00541
311.2	0.2436	223.6	0.5629	311.2	0.06995	223.6	-0.00516
311	0.2032	223.4	0.608	311	0.06644	223.4	-0.00511
310.8	-0.1153	223.2	0.5831	310.8	0.06695	223.2	-0.00523
310.6	0.0925	223	0.5548	310.6	0.0678	223	-0.00534
310.4	0.1892	222.8	0.5575	310.4	0.0674	222.8	-0.00505
310.2	0.1225	222.6	0.5549	310.2	0.06764	222.6	-0.00504
310	0.7835	222.4	0.521	310	0.06648	222.4	-0.00524
309.8	0.3324	222.2	0.5834	309.8	0.07082	222.2	-0.00482
309.6	0.4824	222	0.5836	309.6	0.07181	222	-0.00481
309.4	-0.0117	221.8	0.5385	309.4	0.06772	221.8	-0.00518
309.2	0.0085	221.6	0.5155	309.2	0.06707	221.6	-0.00491
309	0.0581	221.4	0.5573	309	0.06873	221.4	-0.00469
308.8	0.3937	221.2	0.6144	308.8	0.07223	221.2	-0.00459
308.6	0.0193	221	0.5268	308.6	0.07077	221	-0.00503
308.4	0.1528	220.8	0.5566	308.4	0.06964	220.8	-0.00494
308.2	0.0269	220.6	0.4935	308.2	0.07035	220.6	-0.00484
308	0.1719	220.4	0.5538	308	0.07229	220.4	-0.00445
307.8	0.5604	220.2	0.5509	307.8	0.07053	220.2	-0.00463
307.6	0.1889	220	0.5463	307.6	0.07408	220	-0.00473
307.4	0.0501	219.8	0.4998	307.4	0.0736	219.8	-0.00503
307.2	-0.1834	219.6	0.5295	307.2	0.07013	219.6	-0.00456
307	0.2456	219.4	0.5939	307	0.07338	219.4	-0.0045
306.8	0.0622	219.2	0.4926	306.8	0.0716	219.2	-0.00455
306.6	-0.1038	219	0.4915	306.6	0.07396	219	-0.00466
306.4	-0.4088	218.8	0.5846	306.4	0.07327	218.8	-0.0042
306.2	0.1472	218.6	0.5019	306.2	0.07504	218.6	-0.00437
306	0.1084	218.4	0.5152	306	0.07311	218.4	-0.00433
305.8	0.1204	218.2	0.433	305.8	0.07213	218.2	-0.00431
305.6	-0.3359	218	0.4967	305.6	0.07396	218	-0.00464
305.4	0.0268	217.8	0.4439	305.4	0.07595	217.8	-0.00423

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
305.2	-0.0759	217.6	0.4169	305.2	0.0759	217.6	-0.00399
305	-0.0768	217.4	0.4188	305	0.07498	217.4	-0.00432
304.8	-0.1543	217.2	0.3703	304.8	0.07691	217.2	-0.00423
304.6	0.3259	217	0.4221	304.6	0.07767	217	-0.00413
304.4	0.0529	216.8	0.3833	304.4	0.0772	216.8	-0.00423
304.2	-0.4828	216.6	0.4689	304.2	0.07763	216.6	-0.00379
304	-0.0787	216.4	0.3541	304	0.07863	216.4	-0.0039
303.8	0.7395	216.2	0.3996	303.8	0.08139	216.2	-0.00354
303.6	-0.1151	216	0.4028	303.6	0.07851	216	-0.00293
303.4	0.4273	215.8	0.3319	303.4	0.07962	215.8	-0.00339
303.2	-0.3383	215.6	0.3147	303.2	0.07959	215.6	-0.00338
303	-0.0304	215.4	0.3215	303	0.08014	215.4	-0.00322
302.8	-0.2219	215.2	0.3054	302.8	0.07783	215.2	-0.00315
302.6	-0.1841	215	0.2503	302.6	0.08046	215	-0.00305
302.4	-0.4552	214.8	0.1994	302.4	0.08184	214.8	-0.00305
302.2	0.4984	214.6	0.2643	302.2	0.08207	214.6	-0.00247
302	-0.592	214.4	0.1863	302	0.07866	214.4	-0.00275
301.8	-0.2463	214.2	0.096	301.8	0.08168	214.2	-0.00246
301.6	-0.6249	214	0.1983	301.6	0.07943	214	-0.00238
301.4	0.3538	213.8	0.1799	301.4	0.08346	213.8	-0.00266
301.2	0.1309	213.6	0.0639	301.2	0.08303	213.6	-0.00233
301	-0.4886	213.4	0.0419	301	0.08094	213.4	-0.00211
300.8	-0.1951	213.2	0.0548	300.8	0.0832	213.2	-0.00164
300.6	-0.3184	213	-0.0211	300.6	0.08253	213	-0.00186
300.4	0.4446	212.8	0.0028	300.4	0.08405	212.8	-0.00127
300.2	-0.6186	212.6	0.0676	300.2	0.08394	212.6	-0.00085
300	-0.053	212.4	-0.0393	300	0.0838	212.4	-0.00087
299.8	0.5906	212.2	-0.0208	299.8	0.08881	212.2	-0.00042
299.6	0.0123	212	-0.1364	299.6	0.08765	212	-0.00047
299.4	-0.3212	211.8	-0.1935	299.4	0.08536	211.8	0.00027
299.2	0.0869	211.6	-0.2801	299.2	0.08594	211.6	-0.00036
299	-0.464	211.4	-0.221	299	0.08747	211.4	0.00022
298.8	0.0139	211.2	-0.2817	298.8	0.08752	211.2	0.00021
298.6	-0.3509	211	-0.2292	298.6	0.08679	211	0.00065
298.4	-0.0448	210.8	-0.2365	298.4	0.08979	210.8	0.00138
298.2	0.0422	210.6	-0.2999	298.2	0.08809	210.6	0.00168
298	0.1482	210.4	-0.2566	298	0.08954	210.4	0.00225
297.8	0.238	210.2	-0.2147	297.8	0.09184	210.2	0.00251
297.6	0.0853	210	-0.3723	297.6	0.08785	210	0.00207
297.4	0.0564	209.8	-0.2776	297.4	0.09	209.8	0.00258
297.2	-0.4802	209.6	-0.2834	297.2	0.08811	209.6	0.0036

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
297	-0.3643	209.4	-0.3478	297	0.08822	209.4	0.00401
296.8	-0.1162	209.2	-0.3691	296.8	0.09205	209.2	0.00392
296.6	-0.4165	209	-0.5246	296.6	0.08899	209	0.00417
296.4	-0.0577	208.8	-0.4677	296.4	0.09067	208.8	0.00472
296.2	0.1899	208.6	-0.4224	296.2	0.09012	208.6	0.00426
296	-0.062	208.4	-0.4408	296	0.09095	208.4	0.00519
295.8	-0.0698	208.2	-0.4178	295.8	0.09331	208.2	0.00564
295.6	-0.4823	208	-0.4223	295.6	0.09239	208	0.00673
295.4	0.1287	207.8	-0.3913	295.4	0.09348	207.8	0.00738
295.2	-0.3611	207.6	-0.4375	295.2	0.09426	207.6	0.00676
295	-0.213	207.4	-0.4501	295	0.09336	207.4	0.00785
294.8	0.0499	207.2	-0.3651	294.8	0.09355	207.2	0.00776
294.6	-0.0222	207	-0.4751	294.6	0.09304	207	0.00771
294.4	-0.3098	206.8	-0.4542	294.4	0.09195	206.8	0.008
294.2	0.3647	206.6	-0.5493	294.2	0.0958	206.6	0.00807
294	0.232	206.4	-0.5365	294	0.0966	206.4	0.0092
293.8	-0.3125	206.2	-0.627	293.8	0.09331	206.2	0.00871
293.6	-0.0362	206	-0.4748	293.6	0.0952	206	0.00966
293.4	-0.2056	205.8	-0.6234	293.4	0.09376	205.8	0.00986
293.2	-0.4714	205.6	-0.4739	293.2	0.09371	205.6	0.01099
293	-0.2515	205.4	-0.4908	293	0.09616	205.4	0.01036
292.8	-0.1997	205.2	-0.3874	292.8	0.09463	205.2	0.01119
292.6	-0.0112	205	-0.5226	292.6	0.09519	205	0.01126
292.4	0.0361	204.8	-0.5708	292.4	0.09735	204.8	0.01102
292.2	-0.1097	204.6	-0.4125	292.2	0.09725	204.6	0.0117
292	-0.3981	204.4	-0.4975	292	0.09517	204.4	0.01223
291.8	-0.5977	204.2	-0.4103	291.8	0.09584	204.2	0.01332
291.6	-0.6284	204	-0.4349	291.6	0.09569	204	0.01269
291.4	-0.1959	203.8	-0.4664	291.4	0.09692	203.8	0.01278
291.2	-0.4413	203.6	-0.5806	291.2	0.09617	203.6	0.01242
291	-0.4503	203.4	-0.2813	291	0.09818	203.4	0.01427
290.8	-0.2695	203.2	-0.2711	290.8	0.09746	203.2	0.01426
290.6	-0.1945	203	-0.3703	290.6	0.09867	203	0.01393
290.4	-0.428	202.8	-0.2551	290.4	0.09806	202.8	0.01522
290.2	-0.0367	202.6	-0.2423	290.2	0.09714	202.6	0.01482
290	-0.0729	202.4	-0.3976	290	0.1008	202.4	0.01512
289.8	-0.5665	202.2	-0.3484	289.8	0.09776	202.2	0.01541
289.6	0.0192	202	-0.3942	289.6	0.09947	202	0.01589
289.4	0.0196	201.8	-0.2628	289.4	0.09859	201.8	0.01624
289.2	-0.303	201.6	-0.2169	289.2	0.10049	201.6	0.01658
289	-0.294	201.4	-0.3924	289	0.09972	201.4	0.01662

Spiramycin				Metronidazole			
Constant			1.9872	Constant			-0.10806
Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients	Wavelength (nm)	Coefficients
288.8	0.0879	201.2	-0.4849	288.8	0.10126	201.2	0.01589
288.6	-0.1716	201	-0.319	288.6	0.09964	201	0.01661
288.4	-0.1917	200.8	-0.24	288.4	0.10002	200.8	0.01708
288.2	0.0397	200.6	-0.2039	288.2	0.10083	200.6	0.01737
288	-0.2248	200.4	-0.3448	288	0.10074	200.4	0.01636
287.8	0.2595	200.2	-0.1785	287.8	0.10202	200.2	0.01802
287.6	-0.2114	200	-0.2127	287.6	0.10005	200	0.01774

Appendix 4: Results of the principal components coefficients of spiramycin and metronidazole for PCR model

Mixture No.	Spiramycin (µg.mL-1)	Metronidazole (µg.mL-1)	Z1	Z2	Z3	Z4	Z5	Z6
1	6	3	2.904751	0.861167	-0.95514	0.005998	-0.04566	0.036491
2	6	4	4.383452	1.055472	-1.08221	0.094658	-0.10594	0.03599
3	6	5	3.767696	1.797477	-1.30157	-0.02871	-0.0526	0.026516
4	6	10	6.642342	3.86842	-1.72534	-0.05038	-0.07198	0.030051
5	6	15	10.32765	5.356814	-1.21659	0.080231	-0.10041	0.058633
6	8	3	4.437799	0.324071	-0.97061	0.046532	-0.10358	0.034389
7	8	4	3.889186	1.062043	-1.15322	-0.04648	-0.04422	0.024189
8	8	5	5.59638	1.250275	-1.19275	0.104213	-0.12601	0.0275
9	8	10	8.216259	3.200389	-1.38221	0.085868	-0.12065	0.040269
10	8	15	10.92303	4.968453	-1.1457	0.040713	-0.11304	0.020399
11	10	3	5.093358	0.053879	-0.97867	0.062186	-0.1103	0.01803
12	10	4	5.672962	0.415186	-1.11074	0.068954	-0.10446	0.026149
13	10	5	6.159482	0.882334	-1.19804	0.079614	-0.1109	0.018985
14	10	10	8.891183	2.905377	-1.33163	0.056411	-0.10448	0.006389
15	10	15	11.60769	4.701049	-1.11292	0.01505	-0.10163	0.017472
16	20	3	8.144827	-1.4597	-1.07863	0.035536	-0.10982	0.025445
17	20	4	8.741952	-1.1789	-1.1984	0.00631	-0.11217	0.033052
18	20	5	9.202273	-0.63679	-1.24777	0.011633	-0.10516	0.013541
19	20	10	12.02508	1.182775	-1.24851	-0.02998	-0.09126	0.021146
20	20	15	14.56901	3.050437	-0.94988	-0.09878	-0.08726	0.021075
21	30	3	11.13894	-3.00978	-1.33762	-0.03321	-0.10178	0.027082
22	30	4	11.64927	-2.61915	-1.36719	-0.02559	-0.10212	0.017412
23	30	5	12.27386	-2.20543	-1.39659	-0.03445	-0.1071	0.041141
24	30	10	15.52309	-0.35393	-1.2164	0.38915	-0.05042	0.023145
25	30	15	17.70143	1.471453	-0.88865	-0.10952	-0.09143	0.034342