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Critical assessment of smart calculation-based spectroscopy versus chemometric-assisted methods: Application to combined antibiotic formulations

Hind Ali Abdullatif [1], Adel Magdy Michael [1,*], Yossra Ahmed Trabik [2] and Miriam Farid Ayad [2]

[1] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ahram Canadian University, Cairo,12573, Egypt*
[2] *Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Ain Shams University, Cairo, 11571, Egypt*

* *Corresponding author at: Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ahram Canadian University, Cairo,12573, Egypt.*
e-mail: adel_magdy_m@yahoo.com (A.M. Michael).

RESEARCH ARTICLE

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ABSTRACT

This work describes a comparative study of two multivariate chemometric and univariate spectrophotometric methods for the determination of a ternary drug mixture containing oxytetracycline HCl, bromhexine HCl, and lidocaine HCl. All methods show high sensitivity and similar linearity range. Meanwhile, the chemometric method has the advantage of higher accuracy, higher specificity and better regression parameters. The two spectrophotometric methods are constant multiplication coupled with spectrum subtraction and successive ratio subtraction coupled with spectrum subtraction while the chemometric method used partial least square and principal component regression models. In addition, a spiking technique was used to increase the concentration of bromhexine HCl in the dosage form, allowing its determination despite its low contribution. Methods were successfully applied in the dosage form Oxyclear® veterinary injection in pure powder as well as in its pharmaceutical formulation. Statistical comparison showed no significant difference between the developed methods and the reference method.

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1. Introduction

Animal health represents pivotal importance to human health and even affects it directly, since dairy products and animal meat is one of the main food humans depend on [1]. Upon that, it is very crucial to maintain animal health and welfare [2]. It has been word widely observed that respiratory system diseases are in constant increase and represents crucial danger nowadays and unfortunately, large animals are majorly affected [3]. Due to this, respiratory system antibiotics have been and still are of great importance. This crucial role of respiratory antibiotics made us very motivated to study them, as they support the safe supply of safe animal products (Milk, meat, and eggs) and defend public health from toxic foodborne pathogens.

Oxyclear® is a veterinary injection used for the treatment of infectious diseases caused by oxytetracycline-sensitive G-ve and G+ve bacteria. It is a ternary mixture composed of oxytetracycline HCl (OXY), bromhexine HCl (BRH), and lidocaine HCl (LID). Lidocaine HCl is the hydrochloride salt of lidocaine, shown in Figure 1, an aminoethylamide and also a prototypical member of amide class anesthetics. Lidocaine interacts with voltage-gated Na^+ channels in the nerve cell membrane, blocks the temporary increase in the permeability of excitable membranes to Na^+, which prevents the generation and also conduction of nerve impulses, which results in a reversible loss of sensation. Lidocaine is a weak base, so in order to be water soluble salt and an injectable form, hydrochloride addition is required [4]. Oxytetracycline HCl (OXY) shown in Figure 1 is the hydrochloride salt form of oxytetracycline, which is a tetracycline derivative produced by *Streptomyces rimosus* exhibiting antimicrobial activity. Oxytetracycline hydrochloride interferes with binding of aminoacyl-*t*RNA to the *m*RNA-ribosome complex, thus preventing peptide elongation and inhibiting protein synthesis [4]. Bromhexine HCl (BRH) shown in Figure 1 is a hydrochloride resulting from the reaction of equimolar amounts of bromhexine and hydrogen chloride. It is used as a mucolytic for the treatments of respiratory disorders associated with productive cough.

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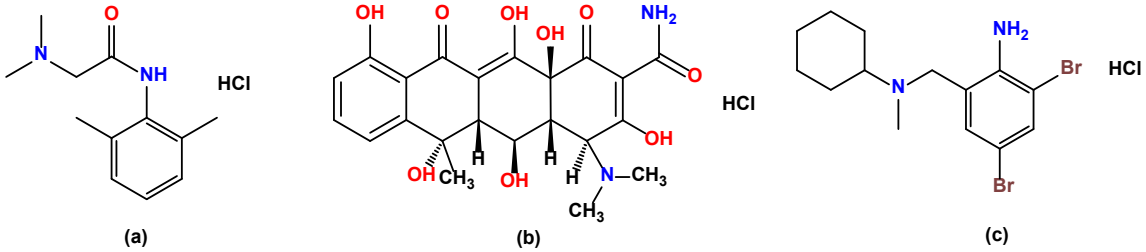

Figure 1. (a) Chemical structure of lidocaine HCl, (b) oxytetracycline HCl, and (c) bromhexine HCl.

Bromhexine HCl is an official drug in British Pharmacopoeia (BP) [5], while lidocaine HCl and oxytetracycline HCl are official drugs in the United States Pharmacopeia (USP) [6].

The literature review revealed several analytical methods for the quantitative determination of bromhexine HCl. These methods include spectrophotometry [7], liquid chromatography [8] and gas chromatography [9], whereas lidocaine HCl analytical methods in the literature include spectrophotometry [10,11], electrochemical methods [12,13], HPLC [14,15], and GLC [16]. Oxytetracycline HCl literature survey revealed several methods including spectrophotometry [17], HPLC [18,19], and electrochemical methods [20,21]. To the best of our knowledge, up to date, no simple spectrophotometric method has been described in the literature for the determination of the three studied drugs simultaneously. The present work aimed to compare the results of this ternary drug assay using multivariate chemometric and univariate spectrophotometric methods and their application to pharmaceutical formulation. The complete stability study was carried out according to the International Conference on Harmonization (ICH) guidelines [22].

2. Experimental

2.1. Instrumentation and software

A double beam UV-Vis spectrophotometer (Model J-760, Jasco, Japan) with 1-cm matched quartz cells was used for the absorbance measurements. The software MATLAB for Windows 7 Mathwork, Inc. 2009 was used for statistical calculations, along with PLS toolbox 2.0 Eigenvector Research Inc. 2005 created by B. M. Wise and N. B. Gallagher for use with MATLAB.

2.2. Materials and reagents

Pure powders of OXY, BRH, and LID were kindly supplied by the Pharma Swede Veterinary Company, Cairo, Egypt. Its purity was found to be 99.50 and 100.30% [6] with respect to OXY and LID, respectively, and 100.34% for BRH [5] according to the official methods. The Oxyclear® injection was supplied by Pharma Swede Veterinary Company, Cairo, Egypt. Each 1 mL claimed to have OXY 50 mg, BRH 3 mg, LID 20 mg.

2.3. Solutions

2.3.1. Standard stock solutions of OXY, BRH, and LID

Prepared separately, in 100 mL volumetric flask, by dissolving an accurately weighed amount (20 mg) of each drug in methanol and then completing until mark with methanol.

2.3.2. Working solutions of OXY, BRH, and LID

The primary stock solutions were diluted with methanol to prepare standard working solutions (100 µg/mL) of each. Different aliquots of working solutions (100 µg/mL) of BRH, LID, and OXY were transferred to a series of 10 mL volumetric flasks and the volume was completed to the mark with methanol. The prepared mixtures contain different ratios of each drug.

2.4. Calibration curves

2.4.1. Spectrophotometric method

For BRH, LID and OXY aliquots, the equivalent to 4-35, 2-12, and 4-28 µg/mL were, respectively, transferred from its working solutions (100 µg/mL) into a set of 10 mL volumetric flasks and the volumes were completed to the mark with methanol. The absorption spectrum of each solution was scanned against a similarly prepared blank. The absorbance was recorded at 248.8, 202.5, and 361.6 nm for BRH, LID, and OXY, respectively, and plotted against the corresponding concentration, and then the regression equation was computed.

2.4.2. Chemometric method

The calibration (training) set was designed with 23 synthetic mixtures of different concentration ratios of BRH, LID, and OXY in the range of 4-35 µg/mL. The solutions were prepared by mixing different volumes of their working solutions into 10 mL volumetric flasks and completing the volume with methanol. The absorption spectra of these prepared mixtures were scanned between 200-400 nm with respect to a blank of methanol. The optimized PLS and PCR models were then constructed [23].

2.4.2.1. Pre-processing the data

The data points of spectra in the range 203-390 nm were transferred to MATLAB for subsequent data analysis and multivariate calibration models were constructed. All spectral data were mean centered before calibration.

2.4.2.2. Construction of the models

The absorbance and concentration matrices of the training set were used together with the PLS-Toolbox 2.0 software for the calculations.

2.4.2.3. Selection of the optimal number of factors to build the PLS and PCR models

The cross-validation method was used, leaving out one sample per time, to select the optimum number of factors. Given a set of 23 calibration samples, the PCR and PLS calibrations were performed on 23 samples and, using this calibration, the concentration of the sample left out was predicted. The predicted concentrations, afterwards, were compared to the known concentrations, and the root mean square error of calibration (RMSEC) was estimated. The RMSEC was calculated in the same manner each time, and a new factor was added to the model. The maximum number of factors used to calculate

the optimum RMSEC was selected to be 12 (half the number of samples plus 1).

2.5. Analysis of synthetic laboratory prepared mixtures

2.5.1. Successive spectrum subtraction coupled with the constant multiplication method (SSS-CM)

The ternary lab mixture (OXY + BRH + LID) was divided by spectrum 6 µg/mL OXY, obtaining a spectrum containing a straight line parallel to the *x*-axis in the extended region 330-400 nm. The constant value obtained is multiplied by the divisor 6 µg/mL OXY to obtain the binary mixture of [BRH + LID]. Finally, this binary mixture spectrum is subtracted from the ternary lab mixture spectrum, resulting in D^0 of OXY, and thus readings at 361.1 nm are substituted in OXY's zero-order regression equation. To find the least extended drug [LID], the binary mixture resulting was divided by the 8 µg/mL BRH spectrum, resulting also in a constant appearing as a straight line parallel to the wavelength axis in the extended region 280-320 nm. When this constant value is multiplied by the divisor 8 µg/mL BRH, [LID] in D^0 was obtained and readings at 202.5 nm could be substituted in the LID zero-order regression equation. To find [BRH], the D^0 LID just obtained is subtracted from binary mixture spectrum, resulting in D^0 BRH, where readings at 248.8 nm could be substituted in BRH's zero order equation [24,25].

2.5.2. Analysis of laboratory prepared mixtures by successive ratio subtraction coupled with spectrum subtraction (SRS-SS)

Starting with the most extended drug OXY, the spectrum of the ternary mixture was divided by the divisor 6 µg/mL OXY. The resulting ratio spectrum was then subtracted by a constant value which was found in the extended region 330-400 nm seen as a straight line parallel to the *x*-axis, then multiplied by the divisor 6 µg/mL OXY, resulting in [BRH + LID], the less extended spectrum. The ternary mixture is subtracted from BRH + LID just obtained, the resulting spectrum is OXY in zero order and readings at 361.6 nm was substituted in OXY's zero-order regression equation [26,27].

To obtain [LID], the binary mixture spectrum BRH and LID obtained was divided by divisor 8 µg/mL BRH. The resulting ratio spectrum was then subtracted by a constant seen as a straight line parallel to the *x*-axis at the extended region 280-320 nm, then multiplied by the divisor 8 µg/mL BRH, resulting in [LID], the least extended spectrum. Finally, the binary mixture of BRH and LID is subtracted from the LID just obtained, resulting in a spectrum of [BRH] in zero order. The readings at 248.8 and 202.5 nm were substituted in the BRH and LID zero-order regression equation, respectively.

2.6. Application to pharmaceutical formulation

In a volumetric flask, 0.25 mL of the mixture was taken and completed until the mark with methanol. The standard addition method is used for BRH, where 3.25 mg of pure BRH was added to the flask before completing the mark with methanol. The claimed amount of BRH in the sample was determined after subtraction which corresponds to the standard added from the total concentration (unknown and added) [28]. Now the claimed amounts are 12.5 mg OXY, 4 mg BRH, and 5 mg LID. The procedures of Sections 2.5.1 and 2.5.2 were followed and the concentration of the drugs was calculated from the regression equations.

2.7. Method validation

2.7.1. Spectrophotometric method

2.7.1.1. Accuracy

The previously mentioned procedures under Sections 2.5.1 and 2.5.2 were repeated for the determination of different concentrations of the three drugs along their linearity range.

2.7.1.2. Precision

2.7.1.2.1 Repeatability

Intraday precision for the drugs studied was evaluated by assaying three freshly prepared solutions (5, 10, 15 µg/mL for OXY and BRH, and 6, 8, 10 µg/mL for LID) in triplicate at certain concentrations on the same day and mean recovery and RSD were calculated.

2.7.1.2.2 Intermediate precision

Interday precision of the proposed methods was evaluated by assaying three freshly prepared solutions (5, 10, 12 µg/mL for OXY, LID, and BRH) in triplicates for three successive days and mean recovery and RSD were calculated.

2.7.2. Chemometric method

2.7.2.1. Construction of the validation set

Seven mixtures of OXY, BRH, and LID were prepared by diluting different volumes of their corresponding working solutions into 10 mL volumetric flasks, and completing the volume with methanol. The suggested models were then applied on these mixtures to predict the concentrations of studied drugs.

2.7.2.2. Predictive abilities of the developed models

Several diagnostic tools were used to evaluate the predictive abilities of the suggested chemometric methods, including (i) Predicted versus actual concentration plot (model and sample diagnostic), plot the predicted concentrations of the validation samples against the actual concentration values, (ii) The actual concentration versus the residual concentration, and (iii) Root mean square error of prediction (RMSEP) (Model diagnostic). The RMSEP was calculated for the predicted concentrations of the validation.

3. Results and discussion

Humans rely mainly on large animals for various sources of food like dairy products or meat, which means that animal welfare and health is of major important to us, consumers, because unfortunately, there are diseases that is directly transmitted to human if he consumed a diseased animal [29]. Sad to say, the animal industry often suffers from economic losses caused by diseases from various microorganisms, which makes it hard for poultry industry to expand to meet the demands of consumers [1]. Therefore, antibiotics are of enormous importance as they are often used to treat diseases, prevent infections and as growth promoters in poultry production [3].

Oxyclear®, which is a ternary mixture composed of OXY, BRH, and LID, is one of the respiratory antibiotics, indicated for the treatment of respiratory tract infections caused by oxytetracycline sensitive G-ve and G+ve bacteria as well as *Mycoplasma* and *Chlamydia*, mainly in cases of retained mucoid secretions in large animals.

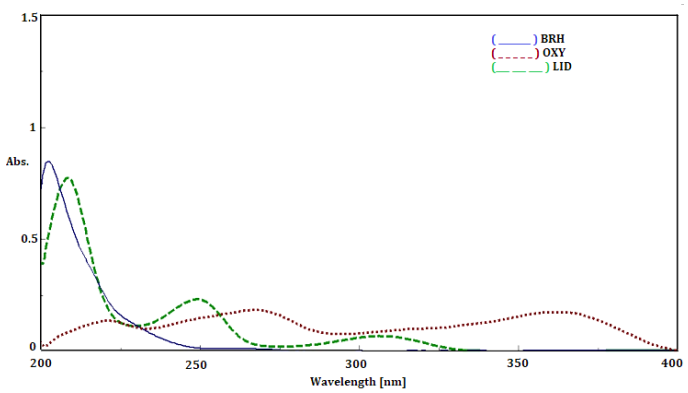

Figure 2. Overlaid zero order absorption spectra of 6 µg/mL oxytetracycline HCl (OXY), 8 µg/mL bromhexine HCl (BRH), and 6 µg/mL lidocaine HCl (LID).

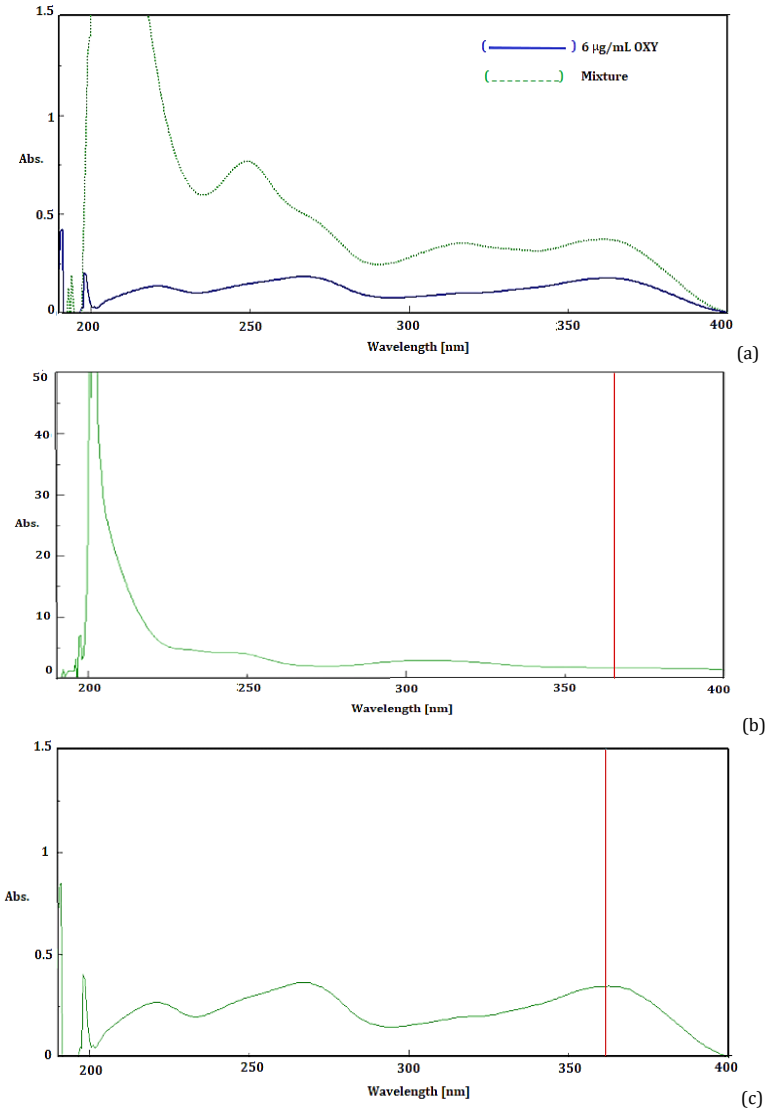

Figure 3. (a) SSS-CM method showing mixture OXY, BRH, and LID overlaid with divisor 6 µg/mL OXY, (b) the constant obtained after division of mixture and divisor, and (c) OXY in zero order after multiplying the constant with the divisor.

There was no method in literature for determining OXY, BRH, and LID together, so the aim of our work was to find an accurate and precise method for the simultaneous analysis of the three drugs, and to compare the results of univariate with multivariate analysis.

It was a rather difficult task to resolve a multi-component spectrum without prior separation, but the methods of resolution were greatly developed. Worth noting that spectrophotometric techniques are generally preferable over other instrumentations like LC-MS or GC-MS which always require optimizing conditions like pH, flow rate, temperature, and more.

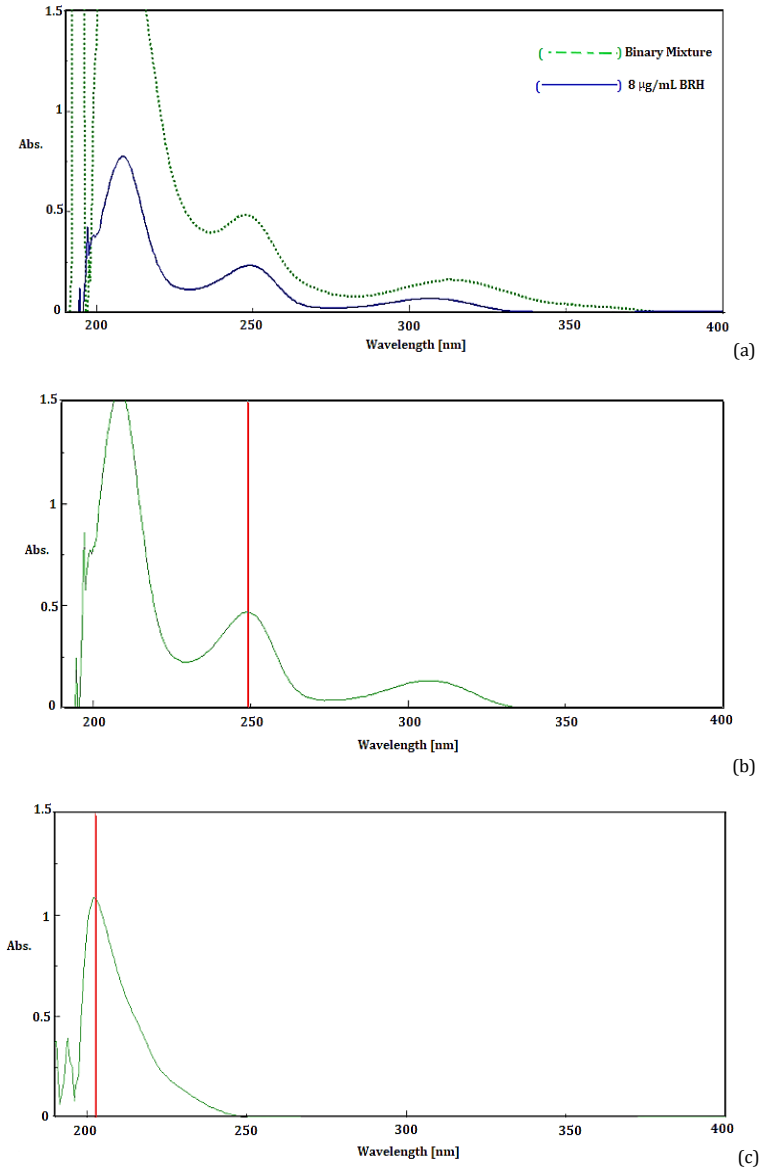

Figure 4. (a) SSS-CM method showing binary mixture after subtraction of the OXY spectrum to obtain the two less extended drugs, overlaid with 8 µg/mL BRH, (b) BRH in zero order after dividing the previous overlaid spectrums and multiplying the constant with the divisor, and (c) LID after subtracting the obtained BRH spectrum from the binary mixture spectrum.

The methods used in this study are also very easy to use, are fast, and are yet very sensitive and cheap to apply for the analysis of this ternary mixture. These methods were mainly focused on mathematical steps or a combination of approaches together with traditional techniques. Two methods were applied for the analysis of ternary mixture as SSS-CM and SRS-SS.

First, when choosing the best solvent, distilled water, acetonitrile, and methanol were tested for the best dissolution and sharp spectrums, and methanol was found to give the best results. In OXY, there was a desirable bathochromic shift than distilled water which helped in resolving the severely over-lapped drugs in zero order spectrum. In the LID, the sharpest peak was found with methanol. Regarding the best peak value, it was found that between the two peaks of OXY (267.0 and 361.6 nm), and BRH (208.5, 248.8 and 306.0 nm), 361.6 and 248.8 nm for OXY and BRH, respectively; were found to be the optimal with respect to accuracy and sensitivity.

The SSS-CM method depended on having an extended drug [OXY] more than the second [BRH], which in return extended more than the third [LID] in the zero-order spectrum, as shown in Figure 2. By dividing the laboratory mixture spectrum by 6 µg/mL OXY, we obtained a spectrum containing a straight line parallel to the wavelength axis in the extended region 330-400 nm. The constant value obtained is multiplied by the divisor 6 µg/mL OXY to obtain the binary mixture of [BRH+LID]. Finally, this binary mixture spectrum is subtracted from the ternary lab mixture spectrum, resulting in OXY at D^0, and thus readings at 361.1 nm are substituted in OXY's zero-order regression equation. To find our least extended drug LID, the binary mixture that resulted was divided by the 8 µg/mL BRH spectrum, which also results in a constant appearing as a straight line parallel to the wavelength axis. When this constant value is multiplied by the divisor 8 µg/mL BRH, the LID in D^0 was obtained and the readings at 202.5 nm could be substituted in the LID zero-order regression equation. To find BRH, the D^0 LID just obtained is subtracted from binary mixture spectrum, resulting in D^0 BRH, where readings at 248.8 nm could be substituted in BRH's zero order equation. The method was illustrated in Figures 3 and 4.

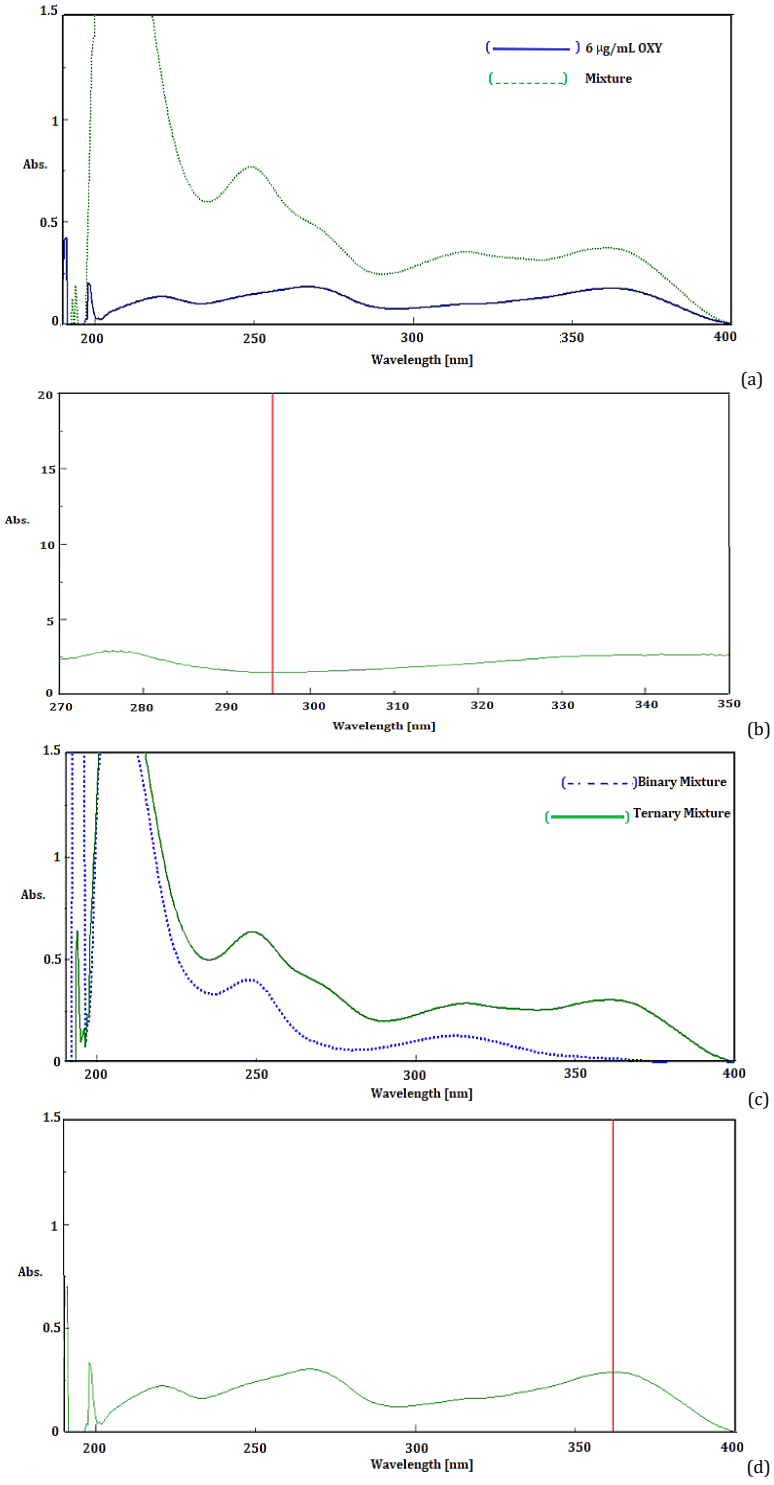

Figure 5. (a) SRS-SS method showing the ternary mixture OXY, BRH, and LID overlaid with divisor 6 µg/mL OXY, (b) the constant obtained after division of mixture and divisor, (c) the binary (less extended drugs) spectrum obtained after subtracting the constant as a number and multiplying with the divisor, overlaid with the ternary mixture, and (d) OXY (more extended drug) in zero order obtained by subtracting ternary spectrum from binary spectrum.

The SRS-SS method also depended on having an extended drug more than the second, which in return extended more than the third. Starting with OXY, the most extended drug, the spectrum of the ternary mixture was divided by the divisor 6 µg/mL OXY, and the spectrum of the resulting ratio was then subtracted by a constant value that was found in the extended region 330-400 nm seen as a straight line parallel to the wavelength axis, then multiplied by the divisor 6 µg/mL OXY, resulting in [BRH + LID], the less extended spectrum. The ternary lab mixture is subtracted from [BRH+LID] just obtained, resulting in a D^0 OXY spectrum and readings at 361.6 nm were substituted into the OXY zero-order regression equation.

Table 1. Validation parameters for simultaneous determination of OXY, BRH, and LID using SSS-CM and SRS-SS spectrophotometric results.

Parameter	OXY		BH		LID	
	SSS-CM	SRS-SS	SSS-CM	SRS-SS	SSS-CM	SRS-SS
Range (µg/mL)	4.00-28.00		4.00-35.00		2.00-12.00	
Slope	0.029		0.029		0.090	
Intercept	-0.01		-0.01		-0.84	
R^2	0.9998		0.9999		0.9998	
Mean ±SD	99.54±1.027		99.72±0.718		100.15±0.896	
Intraday Precision (RSD *)	±0.278		±0.835		0.896	
Interday Precision (RSD *)	±0.687		±0.577		±0.325	
Selectivity	99.65±0.944	99.88±0.637	100.52±1.314	100.55±0.966	100.34±0.866	100.53±1.504

* RSD: The intraday (n = 3) relative standard deviation of concentrations 5, 10, 15 µg/mL; the interday, respectively, relative standard deviation of concentrations 10 µg/mL for OXY, while the intraday (n = 3) relative standard deviation of concentrations 5, 10, 15 µg/mL; the interday, respectively, relative standard deviation of concentrations 10 µg/mL for LID, and intraday (n = 3) relative standard deviation of concentrations 6, 8, 10 µg/mL; the interday, respectively, relative standard deviation of concentrations 12 µg/mL for BRH.

Table 2. Validation parameters for simultaneous determination of OXY, BRH, and LID using optimized PLS and PCR models and dosage form results.

Parameter	PCR			PLS		
	OXY	BRH	LID	OXY	BRH	LID
Intercept	-0.0258	0.1275	0.1115	0.0035	-0.0148	0.0478
Slope	1.0029	0.9986	0.9847	1.0017	1.0014	0.9912
R^2	0.9999	0.9998	0.9997	0.9999	0.9999	0.9996

Then for the determination of LID, the binary mixture spectrum [BRH + LID] obtained was divided by the divisor 8 µg/mL BRH. The resulting ratio spectrum was then subtracted by a constant seen as a straight line parallel to the wavelength axis at the extended region 280-320 nm, then multiplied by the divisor 8 µg/mL BRH, resulting in [LID], the least extended spectrum. Finally, the binary mixture [BRH + LID] is subtracted from [LID] just obtained, resulting in a spectrum of D^0 BRH. Readings at 248.8 and 202.5 nm were substituted into the zero-order regression equation of BRH and LID, respectively. The method was illustrated in Figure 5.

There was an issue in the dosage form that was that due to BRH's relatively small amount found in the dosage form, it was very hard to detect. In consequence, standard addition enrichment technique had to be applied in order to delete the deviation from Beer's law which results in cases of very low concentrations, that occur when transmittance values are almost close to 100% where incident light approaches transmitted one. The enrichment technique had to be applied to the mixtures, as the choice of the optimum concentration range depended on the spectral characteristics of the compound, the absorptivity and the ratio without changing its concentration in the mixture, which may allow deviations from the Beer law due to electrostatic attraction between the ions.

Concerning multivariate analysis, recently, quantitative spectroscopy has been greatly improved by the use of variety of multivariate statistical methods. Multivariate calibrations are significantly beneficial in spectral analysis since the simultaneous inclusion of multiple spectral points (or wavelengths) may improve outstandingly in terms of the precision and applicability of quantitative analysis in pharmaceutical preparations and biological samples [30,31]. Among the various regression methods which exists for multivariate calibration, the factor analysis based on partial least squares (PLS) and principal component regression (PCR) regression have attracted much attention in chemometrics. In the cases where only partial knowledge of the components is present, PCR and PLS can give satisfactory results. PCR presupposes that the error is solely in the instrumental response and that the concentration matrix is error-free, while PLS implies that the error is equally distributed between the concentration and instrumental response (spectral) matrix. Therefore, PLS produces a more robust model because it removes noise from both concentration and absorbance data.

3.1. Validation

The method validation was carried out according to the ICH guidelines [22] as follows:

3.1.1. Linearity

Linearity of the methods was examined by constructing multiple calibration graphs on three diverse days. The calibration graphs were constructed within the concentration ranges which was selected on the basis of the expected drugs concentration during the assay of the dosage form. Each concentration was tested three times. The concentration ranges, calibration equations, and other statistical parameters are illustrated in Table 1.

3.1.2. Accuracy

Methodologies to examine the linearity of cited drugs were repeated three times to determine nine different concentrations of pure OXY, BRH, and LID. The accuracy, expressed as recovery %, was presented in Table 1.

3.1.3. Precision

The intraday and interday precision was investigated by analyzing three different concentrations of the mentioned drugs within the linearity range, three times for three pure samples of the drugs on a single day, and also on three consecutive days, the results expressed as RSD are illustrated in Table 1.

3.1.4. Specificity

Specificity was proven by analyzing different mixtures having the drugs in various ratios all within the linearity range. Acceptable percentage recoveries with low standard deviation among the other methods were obtained and listed in Table 1.

The actual concentrations were plotted against the predicted concentrations, showing that the slope was almost one as recorded in Table 2. Residual concentration plots are shown in Figure 6. These were used to determine whether the model accounted for the concentration variations in the validation set [32].

The validation parameters for both univariate and multivariate are shown in Tables 1 and 2. Both methods showed successful applications to the analysis of laboratory prepared mixtures with high selectivity.

3.2. Application to pharmaceutical formulations

The methods were also applied for the assay of OXY, BRH, and LID in injection veterinary formulation as presented in Table 3.

Table 3. Determination of OXY, BRH, and LID in pharmaceutical formulation by the proposed methods.

Pharmaceutical formulation [Oxyclear® Injection]	Found %											
	Spectrophotometric methods						Chemometric methods					
	SSS-CM			SRS-SS			PCR			PLS		
	OXY	BRH *	LID	OXY	BRH*	LID	OXY	BRH *	LID	OXY	BRH *	LID
Mean	99.34	99.913	102.00	99.40	99.90	102.00	100.10	98.99	99.77	100.35	98.47	98.89
SD	±0.71	±0.92	±0.87	±0.91	±0.71	±0.32	±0.55	±0.69	±0.55	±0.12	±0.26	±0.88

*After subtraction of 3.25 mg/mL BRH for enrichment.

Table 4. Five-level three factorial experimental design shown as OXY, BRH, and LID concentration in calibration and validation sets in µg/mL using PCR and PLS models.

Mixture	Concentration			Mixture	Concentration		
	OXY	BRH	LID		OXY	BRH	LID
1 *	4	4	12	13 *	14	30	6
2	4	6	8	14	14	35	12
3	4	18	12	15	20	4	8
4	4	30	4	16	20	18	12
5	4	35	6	17 *	20	30	6
6	6	4	6	18 *	20	35	12
7 *	6	6	12	19	28	4	12
8	6	18	4	20	28	6	12
9	6	30	12	21	28	16	8
10	6	35	8	22	28	30	8
11	14	6	6	23 *	28	35	4
12 *	14	18	8				

* Validation set.

Table 5. Statistical comparison between the results obtained by the proposed methods and the reference methods for the determination of OXY, BRH, and LID.

Drug	OXY					BRH					LID				
Methods	SRS-SS	SSS-CM	PLS	PCR	USP [a]	SRS-SS	SSS-CM	PLS	PCR	BP [b]	SRS-SS	SSS-CM	PLS	PCR	BP [c]
Mean	99.88	99.78	100.18	99.97	99.48	100.55	100.52	100.04	0.98	100.34	100.53	100.34	99.77	99.712	100.34
SD	0.70	0.77	0.40	0.43	1.42	0.97	1.32	1.31	2.00	0.77	1.51	0.87	1.27	1.37	0.87
Variance	0.83	0.88	0.64	0.66	1.19	0.99	1.15	1.44	1.41	0.88	1.23	0.93	1.13	1.17	0.76
n	6	6	7	7	6	6	6	7	7	6	6	6	7	7	6
Student's t-test	0.44	0.61	1.31	0.98	-	0.39	0.28	0.55	1.79	-	0.27	0.03	1.01	1.04	-
F-value test	1.43	1.36	1.87	1.82	-	1.12	1.31	1.30	1.61	-	1.61	1.22	1.48	1.53	-

[a] Direct spectrophotometric determination: measuring the maximum absorbance at 353 nm [6].
[b] Titration method [5].
[c] Titration method [5].

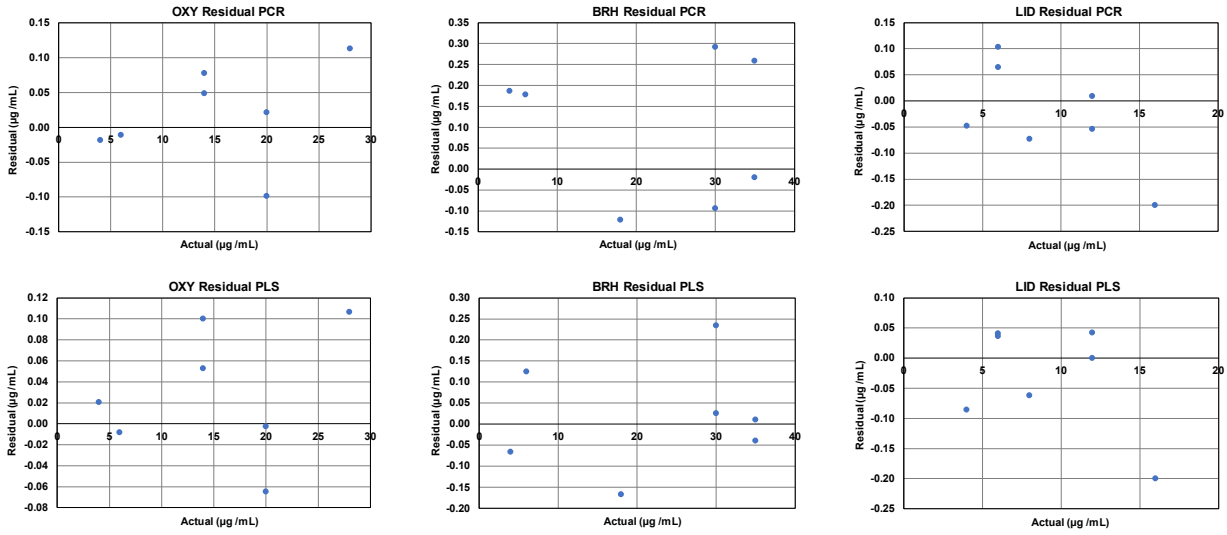

Figure 6. PCR and PLS residual concentrations.

Concerning the multivariate analysis, the prediction ability of the suggested PLS and PCR models, was validated, they were used to predict the concentration of Oxyclear® in validation samples containing different percentages of the three components. Table 4 shows the different mixtures used for the calibration and validation sets.

3.3. Statistical analysis

The proposed methods and official methods for OXY, BRH and LID were statistically compared with respect to t and F values, indicating similar accuracy and precision, and the results were recorded in Table 5. Furthermore, statistical comparison did not show significant differences between the developed methods and the official methods [5,6] as shown in Table 5.

4. Conclusions

The work introduced simple, precise, and sensitive univariate and multivariate spectrophotometric methods for the determination of the ternary mixture. They do not require a

special program or expensive instruments and could be easily applied, especially after optimizing the conditions for a successful and accurate simultaneous determination of the three drugs. Chemometric methods showed better resolution, regression parameters and selectivity than univariate methods. Spectrophotometric methods have an advantage of being simple and cost effective than other analytical techniques. Both proposed techniques could be used in quality control laboratories for routine analysis of the studied drugs.

Disclosure statement

Conflict of interest: The authors declare that they have no conflict of interest.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.

CRediT authorship contribution statement

Conceptualization: Adel Magdy Michael; Methodology: Adel Magdy Michael; Software: Hind Ali Abdullatif, Adel Magdy Michael; Validation: Hind Ali Abdullatif, Adel Magdy Michael; Formal Analysis: Hind Ali Abdullatif, Adel Magdy Michael; Investigation: Hind Ali Abdullatif; Resources: Hind Ali Abdullatif; Data Curation: Hind Ali Abdullatif, Adel Magdy Michael; Writing - Original Draft: Hind Ali Abdullatif; Writing - Review and Editing: Yossra Ahmed Trabik, Adel Magdy Michael, Miriam Farid Ayad; Visualization: Adel Magdy Michael; Supervision: Yossra Ahmed Trabik, Adel Magdy Michael, Miriam Farid Ayad; Project Administration: Yossra Ahmed Trabik, Adel Magdy Michael, Miriam Farid Ayad.

ORCID and Email

Hind Ali Abdullatif
hanoood7@hotmail.com
https://orcid.org/0000-0002-2224-6357
Adel Magdy Michael
adel_magdy_m@yahoo.com
adel.m@acu.edu.eg
https://orcid.org/0000-0002-9875-6003
Yossra Ahmed Trabik
ytrabik@pharma.asu.edu.eg
https://orcid.org/0000-0002-9084-4551
Miriam Farid Ayad
dr.miriamayad@pharma.asu.edu.eg
https://orcid.org/0000-0002-7748-4248

References

[1]. He, Y.; Yang, X.; Xia, J.; Zhao, L.; Yang, Y. Consumption of meat and dairy products in China: a review. *Proc. Nutr. Soc.* **2016**, *75*, 385–391.
[2]. Perry, B. D.; Randolph, T. F.; McDermott, J. J.; Sones, K. R.; Thornton, P. K. Investing in animal health research to alleviate poverty. Nairobi: ILRI, 2002.
[3]. Krčméry, V.; Grúnertová, H. On the mechanism of action of tetracycline antibiotics: V. The effect of oxytetracycline on the reduction of triphenyltetrazolium by staphylococci and some other microorganisms. *Folia Microbiol. (Praha)* **1964**, *9*, 222–231.
[4]. Wheeler, D. L.; Barrett, T.; Benson, D. A.; Bryant, S. H.; Canese, K.; Chetvernin, V.; Church, D. M.; DiCuccio, M.; Edgar, R.; Federhen, S.; Feolo, M.; Geer, L. Y.; Helmberg, W.; Kapustin, Y.; Khovayko, O.; Landsman, D.; Lipman, D. J.; Madden, T. L.; Maglott, D. R.; Miller, V.; Ostell, J.; Pruitt, K. D.; Schuler, G. D.; Shumway, M.; Sequeira, E.; Sherry, S. T.; Sirotkin, K.; Souvorov, A.; Starchenko, G.; Tatusov, R. L.; Tatusova, T. A.; Wagner, L.; Yaschenko, E. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* **2007**, *36*, D13–D21.
[5]. Cartwright, A. *The British pharmacopoeia, 1864 to 2014 medicines, international standards and the state*; Routledge: London, England, 2016.
[6]. Williams, R. L. Official USP Reference Standards: Metrology concepts, overview, and scientific issues and opportunities. *J. Pharm. Biomed. Anal.* **2006**, *40*, 3–15.
[7]. Donmez, O. A.; Bozdogan, A.; Kunt, G.; Div, Y. Spectrophotometric multicomponent analysis of a mixture of chlorhexidine hydrochloride and lidocaine hydrochloride in pharmaceutical formulation using derivative spectrophotometry and partial least-squares multivariate calibration. *J. Anal. Chem.* **2010**, *65*, 30–35.
[8]. El-Naem, O. A.; Saleh, S. S. Eco-friendly UPLC-MS/MS analysis of possible add-on therapy for COVID-19 in human plasma: Insights of greenness assessment. *Microchem. J.* **2021**, *166*, 106234.
[9]. Yang L. L.; Yuan Y. S.; Tu X. D. Determination of bromhexine in plasma by gas chromatography-electron capture detection and pharmacokinetic studies. *Se Pu* **2000**, *18*, 543–545.
[10]. Omer, L. S.; Ali, R. J. Extraction-Spectrophotometric Determination of Lidocaine Hydrochloride in Pharmaceuticals. *Int. J. Chem.* **2017**, *9*, 49.
[11]. Merey, H. A.; Ramadan, N. K.; Diab, S. S.; Moustafa, A. A. Green spectrophotometric methods for the determination of a binary mixture of lidocaine hydrochloride and cetylpyridinium chloride in the presence of dimethylaniline. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2020**, *242*, 118743.
[12]. Rahbar, N.; Ramezani, Z.; Ghanavati, J. CuO-nanoparticles modified carbon paste electrode for square wave voltammetric determination of lidocaine: Comparing classical and Box–Behnken optimization methodologies. *Chin. Chem. Lett.* **2016**, *27*, 837–842.
[13]. Saad, A. S.; Alamein, A. M. A. A.; Galal, M. M.; Zaazaa, H. E. Novel green potentiometric method for the determination of lidocaine hydrochloride and its metabolite 2, 6-dimethylaniline; Application to pharmaceutical dosage form and milk. *Electroanalysis* **2018**, *30*, 1689–1695.
[14]. Furwanti, C.; Hendrajaya, K.; Indrayanto, G. Simultaneous HPLC Determination of Lidocaine Hydrochloride and Hexachlorophene in a Suppository Product. *Media Pharm. Indones. (MPI)* **2020**, *3*, 27–36.
[15]. Abdelwahab, N. S.; Ali, N. W.; Abdelkawy, M.; Emam, A. A. Validated RP-HPLC and TLC-densitometric methods for analysis of ternary mixture of cetylpyridinium chloride, chlorocresol and lidocaine in oral antiseptic formulation. *J. Chromatogr. Sci.* **2015**, 54 (3), 318–325.
[16]. Marakkarakath, H. C.; Bannimath, G.; Raikar, P. P. Simultaneous estimation of lidocaine and prilocaine in topical cream by green gas chromatography. *J. Appl. Pharm. Sci.* **2019**, *9*, 66–72.
[17]. Toral, M. I.; Sabay, T.; Orellana, S. L.; Richter, P. Determination of oxytetracycline from salmon muscle and skin by derivative spectrophotometry. *J. AOAC Int.* **2015**, *98*, 559–565.
[18]. Gajda, A.; Jablonski, A.; Bladek, T.; Posyniak, A. Oral fluid as a biological material for antemortem detection of oxytetracycline in pigs by liquid chromatography–tandem mass spectrometry. *J. Agric. Food Chem.* **2017**, *65*, 494–500.
[19]. Ghorbel-Abid, I.; Belhassen, H. Development of a high- performance liquid chromatography with diode- array detection method using monolithic column for simultaneous determination of five tetracyclines residues in fish muscles. *J. Food Nutr. Disord.* **2016**, *5*, 5.
[20]. Yuan, F.; Zhao, H.; Wang, X.; Quan, X. Determination of oxytetracycline by a graphene—gold nanoparticle-based colorimetric aptamer sensor. *Anal. Lett.* **2017**, *50*, 544–553.
[21]. Ghodsi, J.; Rafati, A. A.; Shoja, Y. First report on electrocatalytic oxidation of oxytetracycline by horse radish peroxidase: Application in developing a biosensor to oxytetracycline determination. *Sens. Actuators B Chem.* **2016**, *224*, 692–699.
[22]. International Conference on Harmonization, ICH; Validation of analytical procedures: Text and methodology Q2 (R1), 2005.
[23]. Martens, H.; Nas, T. *Multivariate Calibration*; John Wiley & Sons: Chichester, England, 1991.
[24]. Lotfy, H. M.; Hegazy, M. A.; Mowaka, S.; Mohamed, E. H. Novel spectrophotometric methods for simultaneous determination of Amlodipine, Valsartan and Hydrochlorothiazide in their ternary mixture. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2015**, *140*, 495–508.
[25]. Lotfy, H. M.; Fayez, Y. M.; Tawakkol, S. M.; Fahmy, N. M.; Shehata, M. A. E.-A. Spectrophotometric resolution of the severely overlapped spectra of clotrimazole with dexamethasone in cream dosage form by mathematical manipulation steps. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2018**, *202*, 115–122.
[26]. Lotfy, H. M.; Tawakkol, S. M.; Fahmy, N. M.; Shehata, M. A. Successive spectrophotometric resolution as a novel technique for the analysis of ternary mixtures of pharmaceuticals. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2014**, *121*, 313–323.
[27]. Lotfy, H. M.; Tawakkol, S. M.; Fahmy, N. M.; Shehata, M. A. A comparative study of novel spectrophotometric resolution techniques applied for pharmaceutical mixtures with partially or severely overlapped spectra. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2015**, *136*, 937–952.
[28]. Lotfy, H. M.; Saleh, S. S.; Hassan, N. Y.; Salem, H. Computation of geometric representation of novel spectrophotometric methods used for the analysis of minor components in pharmaceutical preparations. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **2015**, *151*, 628–643.
[29]. Newell, D. G.; Koopmans, M.; Verhoef, L.; Duizer, E.; Aidara-Kane, A.; Sprong, H.; Opsteegh, M.; Langelaar, M.; Threfall, J.; Scheutz, F.; van der Giessen, J.; Kruse, H. Food-borne diseases — The challenges of 20years ago still persist while new ones continue to emerge. *Int. J. Food Microbiol.* **2010**, *139*, S3–S15.
[30]. Michael, A. M.; Rezk, M. R.; Lotfy, H. M.; Shehata, M. A. Comparative Study of Multivariate and Univariate Determination of Zolmitriptan in

the Presence of its Degradation products. *Anal. Chem. Lett.* **2015**, *5*, 73–84.
[31]. Hassan, N. A comparative study of spectrophotometric methods versus chemometric methods; An application on a pharmaceutical binary mixture of ofloxacin and dexamethasone. *Int. Res. J. Pure Appl. Chem.* **2013**, *3*, 90–110.
[32]. Brereton, R. G. *Chemometrics: Data analysis for the laboratory and chemical plant*; John Wiley & Sons: Chichester, England, 2003.