EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH
www.ejpmr.com

A REVIEW ON UV SPECTROPHOTOMETRIC METHODS FOR SIMULTANEOUS MULTICOMPONENT ANALYSIS

Amira H. Kamal*, Samah F. El-Malla and Sherin F. Hammad

Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Tanta University, Egypt

*Author for Correspondence: Dr. Amira H. Kamal

Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Tanta University, Egypt

ABSTRACT

In present era, market is floated with various combinations dosage forms and the number is increased day by day. These multicomponent formulations are gaining interest due to greater patient acceptability, increased potency, multiple action, fewer side effects, and quicker relief. Therefore, it is desired that these formulations meet the entire standards related to their quality, safety, and efficacy. This can only be possible if different analytical techniques are available for their determination. Different UV spectrophotometric methods are used in simultaneous multicomponent analysis. Such methods are based on recording and mathematically processing absorption spectra. This review is mainly focused on simultaneous equation method, difference spectrophotometry, derivative spectrophotometry, absorbance ratio spectra, derivative ratio spectra, double divisor ratio spectra derivative method, successive ratio - derivative spectra, Q-absorbance ratio method, isosbestic point method, absorpitivity factor method, dual wavelength method, ratio subtraction method, mean centering of the ratio spectra, absorption factor method and multivariate methods. An overview of theories and some applications of these methods are presented.

KEYWORDS: spectrophotometric methods, multicomponent analysis, double divisor, successive ratio-derivative, dual wavelength, ratio subtraction, multivariate methods.

INTRODUCTION

Combination drug products occupy a time-honored and important role in therapeutics. When rationally formulated, fixed-combination drugs may produce greater convenience, lower cost, and sometimes greater efficacy and safety.[1]

Analysis of samples with numerous components presents a major challenge in modern analysis.[2] Multi-component analysis has become one of the most appealing topics for analytical chemists in the last few years, in fields as clinical chemistry, drug analysis, pollution control,…. etc.[3]

Different analytical techniques can be applied for multicomponent analysis including; spectrophotometry, chromatography, and electrophoresis. UV spectrophotometric methods for simultaneous determination of drugs are highlighted in this review.

Because most analytes of interest are accompanied in their dosage forms by other compounds absorbing in the same spectral region, classical UV spectral measurements could not be used for their determination.[4] The use of traditional methods like extraction is quite difficult because extraction techniques require large solvent consumption, with accompanying risks of analyte loss or contamination, and possibility of incomplete separation. The procedure may be expensive and time consuming.[2]

UV spectrophotometric techniques are mainly used for multicomponent analysis thus minimizing the cumbersome task of separating interferents and allowing the determination of an increasing number of analytes, consequently reducing analysis time and cost.[5]

Multicomponent UV spectrophotometric methods are based on recording and mathematically processing absorption spectra. They offer the following advantages: [6] avoiding prior separation techniques e.g. extraction, concentration of constituents, and cleanup steps that might be required; spectral data are readily acquired with ease; the process is fast, accurate, and simple; wide applicability to both organic and inorganic systems; typical detection limits of 10^{-4} to 10^{-5} M and moderate to high selectivity.

Different UV spectrophotometric multicomponent analysis methods include
1-Simultaneous equation method
If a sample contains two absorbing drugs (*x* and *y*) each of which absorbs at the λ_{max} of the other, it may be possible to determine both drugs by the technique of simultaneous equation (Vierordt's method) provided that certain criteria apply.

The information required is
- The absorptivities of *x* at λ_1 and λ_2, ax_1 and ax_2 respectively
- The absorptivities of *y* at λ_1 and λ_2, ay_1 and ay_2 respectively
- The absorbance of the diluted samples at λ_1 and λ_2, A_1 and A_2 respectively.

Let C_x and C_y be the concentration of *x* and *y* respectively in the diluted samples. Two equations are constructed based upon the fact that at λ_1, the absorbance of the mixture is the sum of the individual absorbance of *x* and *y*.

$$A_1 = a_{x1}\, b\, C_x + a_{y1}\, b\, C_y \quad \ldots\ldots\ldots (1)$$
$$A_2 = a_{x2}\, b\, C_x + a_{y2}\, b\, C_y \quad \ldots\ldots\ldots (2)$$

For measurements in 1 cm cells, b =1cm. Rearrange Eq.(2)

$$C_y = \frac{(A_2 - a_{x2}\, C_x)}{a_{y2}} \quad \ldots\ldots\ldots (3)$$

Substituting for C_y in eq. (1) and rearranging gives

$$C_x = \frac{(A_2\, a_{y1} - A_1\, a_{y2})}{(a_{x2}\, a_{y1} - a_{x1}\, a_{y2})} \quad \ldots\ldots\ldots (4)$$

$$C_y = \frac{(A_1\, a_{x2} - A_2\, a_{x1})}{(a_{x2}\, a_{y1} - a_{x1}\, a_{y2})} \quad \ldots\ldots\ldots (5)$$

Criteria for obtaining maximum precision, based upon absorbance ratios, have been suggested that place limits on the relative concentrations of the components of the mixture. The criteria are that the ratios $\frac{A2/A1}{a_{x2}/a_{x1}}$ and $\frac{a_{y2}/a_{y1}}{A2/A1}$ should lie outside the range 0.1-2.0 for the precise determination of *y* and *x* respectively. These criteria are satisfied only when the λ_{max} of the two components reasonably dissimilar and if the two components do not interact chemically, thereby negating the initial assumption that the total absorbance is the sum of the individual absorbances. [7] Simultaneous equation method was developed for simultaneous determination of several mixtures, e.g. atenolol and indapamide, [8] and dexibuprofen and paracetamol.[9]

2-Difference spectrophotometry
The selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferents may be markedly improved by the technique of difference spectrophotometry. The essential feature of this method is that the measured value is the absorbance difference (ΔA) between two equimolar solutions of the analyte in different chemical forms which exhibit different spectral characteristics.

The criteria for applying difference spectrophotometry to the assay of the substance in the presence of other absorbing substances are that

1-Reproducible changes may be introduced in the spectrum of the analyte by the addition of one or more reagents.
2-The absorbance of the interfering substances is not altered by that reagent.

The simplest and the most commonly employed techniques for altering the spectral properties of the analyte is the adjustment of the pH by means of aqueous solutions of acids, alkalies or buffers.[7]

3- Derivative spectrophotometry (DS)
DS involves the conversion of a normal spectrum (fundamental, zero-order spectrum) to its first, second or higher derivative spectra by differentiating absorbance of the sample with respect to wavelength(λ).[7] The differentiation of zero-order spectrum can lead to separation of overlapped signals, elimination of background caused by presence of other compounds in a sample, [10] improvement of resolution of mixtures as it enhances the detectability of minor spectral features, and enhancement of sensitivity and specificity. [3]

Derivative spectra yield a more characteristic profile in comparison to the parent one; new maxima and minima appeared and points where derivative spectra cross the *X*-axis. [10]

DS keeps all laws of classical spectrophotometry, e.g. dependence of derivative value on analyte concentration and additivity law. These features allow the determination of several components in a mixture by measuring the amplitude of derivative spectrum of mixture at several wavelengths. If the measured height of derivative peak of analyte is performed at those wavelengths at which spectra of other components undergo zeroing, the measured amplitude is proportional only to concentration of this analyte. This approach of quantitative determination is called "zero-crossing technique".[10] DS has been used for simultaneous determination of different mixtures in pharmaceutical formulation, e.g. loratadine and pseudoephedrine sulfate,[11] aceclofenac and tramadol with paracetamol,[12] tramadol and ibuprofen[13] or dexketoprofen, [14] paracetamol and tapentadol, [15] naproxen and acetaminophen [16] or diphenhydramine,[17] phenylephrine and ketorolac, [18] and amiloride and hydrochlorothiazide with timolol.[19]

4-Absorbace ratio spectra method
Consider a mixture of two compounds *x* and *y*. The absorption spectrum of the mixture "measured in 1 cm cell" is defined by the equation[20]

$$A_M = a_x\, C_x + a_y\, C_y \quad \ldots\ldots\ldots\ldots (6)$$

Where; A_M is the absorbance of the mixture, $a_x\ and\ a_y$ are the molar absorptivities, C_x and C_y are the concentrations of *x* and *y*, respectively. If the absorbance of the mixture is divided by the absorbance

of a standard solution of x (its absorbance $A_x^0 = a_x \, C_x^0$), the following equation results

$$\frac{A_M}{A_X^0} = \frac{C_X}{C_X^0} + \frac{A_y}{A_X^0} \quad \dots\dots\dots\dots\dots\dots\dots(7)$$

The ratio $\frac{C_X}{C_X^0}$ is a constant value (**Fig. 1**) which can be eliminated by taking the difference in absorbance ratio amplitudes between two wavelengths λ_1 and λ_2 (peak to peak measurement)

$$\left[\frac{A_M}{A_X^0}\right]_{\lambda 1} - \left[\frac{A_M}{A_X^0}\right]_{\lambda 2} = \left[\frac{A_Y}{A_X^0}\right]_{\lambda 1} - \left[\frac{A_Y}{A_X^0}\right]_{\lambda 2} \dots\dots\dots\dots(8)$$

Eq. (8) illustrates that the amplitude difference in the mixture absorbance ratio between two wavelengths λ_1 and λ_2 (termed; peak to peak, peak to trough, maximum to minimum measurement, or ratio difference spectrophotometric method) is equal to the same amplitude difference for compound y after canceling the constant interference due to compound x. The concentration of compound y (C_y) is proportional to the peak to peak amplitudes of its absorbance spectra. A calibration graph is obtained by recording and storing the spectra of solutions of different concentrations of pure y, and the spectrum of a solution of pure x (the divisor x^0). The stored spectra of the solutions of pure y are divided by the standard spectrum of the divisor (x^0). In the generated ratio spectra, the peak to peak amplitudes between the selected wavelengths are measured and plotted against C_y to obtain the calibration graph. By using the calibration graph, the concentration of compound y in the mixture is determined after similar treatment for the mixture solution. The concentration of x in the mixture is determined by an analogous procedure. Ratio spectra method was developed for the simultaneous determination of several binary mixtures e.g., emtricitabine and tenofovir, [22] diclofenac and pantoprazole [21] and ternary mixtures, e.g., omeprazole, tinidazole and clarithromycin. [23]
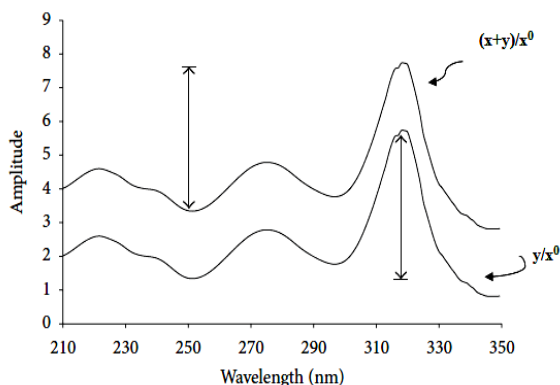

Fig. 1: Ratio spectra of a standard solution of y and a mixture solution (x and y) containing the same concentration of y, using x^0 as a divisor.[21]

5-Derivative ratio spectra method

This simple spectrophotometric method, developed by Salinas et al.,[20] is based on the derivation of the ratio spectra for resolving binary mixtures. It permits the use of the wavelength of highest value of analytical signals with several maxima and minima, which give an opportunity for the determination of active compounds in the presence of other compounds and excipients which could possibly interfere in the assay.[21, 24]

Calculation of the first derivative will remove the constant value due to $\frac{C_X}{C_X^0}$ in Eq. 9, so concentration of y can be easily determined without any interferences from the drug x.

$$\frac{A_M}{A_X^0} = \frac{C_X}{C_X^0} + \frac{A_y}{A_X^0} \quad \dots\dots\dots\dots\dots (9)$$

The difference between the two spectra $\frac{A_M}{A_X^0}$ and $\frac{A_y}{A_X^0}$ (**Fig. 1**) is due to the constant interference value due to compound x ($\frac{C_X}{C_X^0}$). Elimination of such interference can be done by measurement of ratio spectra difference between two wavelengths or calculating derivative of the ratio spectra.[21] Second derivative of the ratio spectra may be also used to improve linearity, mean % recoveries and decrease relative standard deviation.[25] Derivative ratio spectra was modified for the determination of ternary mixtures using the derivative ratio spectra zero-crossing method. This method is realized by measurement of amplitudes at the zero-crossing points in the derivative ratio spectra.[19,26-29]

6-Double divisor ratio spectra derivative method

This method is based on the use of the derivative of the ratio spectrum obtained by dividing the absorption spectrum of the ternary mixture by a standard spectrum of a mixture of two of the three compounds in the mixture, and the measuring at either the maximum or minimum wavelengths. It can only be used for the mixtures that the ratio of the concentrations of two interfering compounds (used as double divisor) is known. In other words, the ratio of the concentrations of two interfering compounds should be the same in calibration, prediction and unknown samples. It is obvious that the ratio of the concentration of the analytes in real samples is always unknown.

Theory

If a mixture of three compounds (x, y and z) is considered, if Beer's law is obeyed for all compounds over the whole wavelength range used and if the path length is 1 cm, the absorption spectrum of the ternary mixture at wavelength λ can be written in form of the equation

$$A_m = a_x \, C_x + a_y \, C_y + a_z \, C_z \dots\dots\dots\dots\dots (10)$$

where A_m is the absorbance of the mixture, a_x, a_y and a_z are the absorptivities of *x*, *y* and *z*, and C_x, C_y and C_z are the concentrations of *x*, *y* and *z*, respectively.

A similar equation for two compounds in the same ternary mixture as in a standard binary mixture can be written as

$$A_m = a_x \ C_x^0 + a_y \ C_y^0 \dots\dots\dots\dots\dots\dots (11)$$

If Eq. (10) is divided by Eq. (11) corresponding to the spectrum of a standard solution of two of the components in the ternary mixture, the ratio spectrum is obtained in the form of Eq. (12)

$$\frac{A_m}{a_x \ C_x^0 + a_y \ C_y^0} = \frac{a_x \ C_x + a_y \ C_y}{a_x \ C_x^0 + a_y \ C_y^0} + \frac{a_z C_z}{a_x \ C_x^0 + a_y \ C_y^0} \dots\dots (12)$$

The ratio of the sum of ($a_x \ C_x + a_y \ C_y$) to the sum of ($a_x \ C_x^0 + a_y \ C_y^0$) is equal to a constant (k) with respect to λ,. If the above constant is replaced in Eq. (12), we obtain Eq. (13)

$$\frac{A_m}{a_x \ C_x^0 + a_y \ C_y^0} = k \ (constant) + \frac{a_z C_z}{a_x \ C_x^0 + a_y \ C_y^0}$$
$$\dots\dots\dots\dots\dots (13)$$

However, if the standard concentrations of C_x^0 and C_y^0 in Eq. (11) are equal or very close to each other, $C_x^0 = C_y^0$ or $C_x^0 \approx C_y^0$, we could write

$$a_x \ C_x^0 + a_y \ C_y^0 = C_x^0 (a_x + a_y) \dots\dots\dots (14)$$

When Eq. (14) is substituted into Eq. (13), Eq. (15) is obtained

$$\frac{A_m}{(a_x + a_y) C_x^0} = k \ (constant) + \frac{a_z C_z}{(a_x + a_y) C_x^0} \dots\dots\dots (15)$$

If the first derivative of Eq. (15) is taken, since the derivative of a constant is zero, Eq. (16) would be obtained

$$\frac{d}{d\lambda} \left[\frac{A_m}{(a_x + a_y) C_x^0} \right] = \frac{d}{d\lambda} \left[\frac{a_z}{(a_x + a_y)} \right] \frac{C_z}{C_x^0} \dots\dots\dots (16)$$

Eq. (16) is the mathematical foundation of multicomponent analysis which permits the determination of the concentration of each of the active compounds in solution without interference from the other components of the ternary system. In practice, Eq. (16) corresponding to the first derivative ratio spectrum of *z* is obtained by dividing the absorption spectrum of the ternary mixture of *x*, *y* and *z* by the standard spectrum of two of the compounds in the ternary mixture. Also, in Eq. (16), the derivative signal of the ratio spectrum of the ternary mixture is dependent only on the concentration values C_Z and C_x^0, but is independent of the concentration values C_X and C_y in the ternary mixture. The concentrations of *x* and *y* are determined by analogous procedures. [27]

7- Successive ratio - derivative spectra method

This method is used for simultaneous determination of the three compounds in ternary mixtures without need to know the ratio of concentration of species. It is based on the successive derivative of ratio spectra in two successive steps.

Theory: consider a mixture of three compounds *x*, *y* and *z*. If Beer's law is obeyed in the whole wavelength range used and by considering the path length as 1 cm, the absorbance of the ternary mixture at each wavelength can be written as

$$A_m = a_x \ C_x + a_y \ C_y + a_z \ C_z \dots\dots\dots\dots (17)$$

Where: A_m is the vector of the absorbance of the mixture, a_x, a_y and a_z are the absorptivity vectors of *x*, *y* and *z*, and C_x, C_y and C_z are the concentrations of *x*, *y* and *z*, respectively. If Eq. (17) is divided by a_z corresponding to the spectrum of a standard solution of z in ternary mixture, the first ratio spectrum is obtained in the form of Eq. (18) (for possibility of dividing operation, the zero values of a_z should not be used in the divisor)

$$B = A_{m/} a_z = a_x \ C_x / a_z + a_y \ C_y / a_z + C_z$$
$$\dots\dots\dots\dots\dots (18)$$

If the first derivative of Eq. (18) is taken, since the derivative of a constant (C_Z) is zero, Eq. (19) would be obtained

$$\frac{dB}{d\lambda} = \frac{d}{d\lambda} [a_x \ C_x / a_z] + \frac{d}{d\lambda} [a_y \ C_y / a_z] \dots(19)$$

By dividing Eq. (19) by $(d/d\lambda)(a_y/a_z)$, corresponding to the derivative of the ratio of the spectra of the standard solutions of *y* and *z*, the second ratio spectrum is obtained as Eq. (20) (for possibility of dividing operation, the zero values of $(d/d\lambda)(a_y/a_z)$ should not be used in the divisor)

$$D = \frac{dB/d\lambda}{(d/d\lambda)(a_y / a_z)} = \frac{(d/d\lambda)[a_x C_x/a_z]}{(d/d\lambda)(a_x/a_z)} + C_y \dots(20)$$

If the first derivative of Eq. (20) is taken since the derivative of a constant (C_y) is zero, Eq. (21) would be obtained

$$\frac{dD}{d\lambda} = \frac{d}{d\lambda} \left[\frac{(d/d\lambda)(a_x C_x/a_z)}{(d/d\lambda)\left(\frac{a_y}{a_z}\right)} \right] \dots\dots\dots\dots\dots (21)$$

Eq. (21) is the mathematical foundation of multicomponent analysis that permits the determination of concentration of each of the active compounds in the solution (*x* in this equation) without interference from

the other compounds of the ternary system (y and z in these equations). As Eq. (21) shows there is a linear relation between the amount of $dD/d\lambda$ and the concentration of *x* in the solution. A calibration curve could be constructed by plotting $dD/d\lambda$ against concentration of *x* in the standard solutions of *x* or in the standard ternary mixtures. For more sensitivity the amount of $dD/d\lambda$ corresponding to maximum or minimum wavelength should be measured. Calibration graphs for y and *z* could be also constructed as described for *x*. [30]

8-Q-absorbance ratio method
This method "also termed **absorption ratio method**" is a modification of the simultaneous equations method. According to this method, the ratio of absorbance at any two wavelengths for a substance, which obeys Beer's law, is a constant value independent of the concentration and path length. This constant is termed as "Hufner's Quotient" or Q-value.   The method involves the measurement of absorbance at two wavelengths, one being the λ_{max} of one of the components (λ_2) and the other being a wavelength of equal absorptivity of the two components (λ_1), called the iso-absorptive point.[7, 9]
The concentration of each component can be calculated by mathematical equations
C_x = $(Q_m-Q_y/Q_x-Q_y) * A/a_1$ .... (22)
C_y = $(Q_m-Q_x/Q_y-Q_x) * A/a_2$ .... (23)
where; C_x and C_y are the concentrations of *x* and *y* respectively, A is absorbance of sample at isoabsorpitive wavelength and a_1 and a_2 are the absorptivity of *x* and **y** respectively at isoabsorpitive wavelength.

$$Q_m = \frac{\text{Absorbance of the sample solution at } \lambda \text{ max of one of the components } (\lambda 2)}{\text{Absorbance of the sample solution at isoabsorpitive wavelength}}$$
......(24)
$$Q_x = \frac{\text{Absorbance of x at } \lambda max \text{ of one of the components } (\lambda 2)}{\text{Absorbance of x at isoabsorpitive wavelength}}$$
.... (25)
$$Q_y = \frac{\text{Absorbance of y at } \lambda max \text{ of one of the components } (\lambda 2)}{\text{Absorbance of y at isoabsorpitive wavelength}}$$
.... (26)

9-Isosbestic " isoabsorptive" point method
Erram and Tipnis [31] developed the isosbestic point method. This technique can be used only if the spectra of the same concentration of the two studied drugs cross at a point called isosbestic or isoabsorptivity point. At the isosbestic point both drugs have equal absorptivities and their mixture acts as a single component and gives the same absorbance as pure drug.

This theory can be confirmed experimentally by recording the absorbance spectra of a certain concentration of the two drugs and the absorbance spectra of a binary mixture containing the same concentration. The absorbance value at the isosbestic points (A_{iso}) was determined, and the total concentration of both drugs was calculated (**Fig. 2**). Since the concentration of one of them in this mixture can be

measured using other spectroscopic method (DS), the concentration of the other could be calculated by subtraction. A linear correlation was obtained between the absorbance values and the corresponding drug concentrations. Consider you have a mixture of two drugs x and y. The absorbance of each drug can be calculated at any wavelength (λ) from the equation
$$A = A_{1cm}^{1\%} \, b \, c \qquad \text{............. (27)}$$

Therefore, for drug *x*: $A_x = A_{x\,1cm}^{1\%} \, b \, c_x$ ......(28)

and for drug *y*: $A_y = A_{y\,1cm}^{1\%} \, b \, c_y$ .... (29)
Where A_x and A_y are the absorbance of *x* and *y*, respectively; C_x and C_y are the concentrations of *x* and *y*, respectively; and $A_{x\,1cm}^{1\%} \, and \, A_{y\,1cm}^{1\%}$ are the absorbtivities when the path length (b) is 1 cm and concentration is 1 g/100 mL for *x* and *y*, respectively. If $C_x = C_y$, and $A_x = A_y$, this λ is called the *isosbestic point*, and at this λ
$$A_{x,\,1cm}^{1\%} = A_{y,\,1cm}^{1\%} \qquad \text{................ (30)}$$
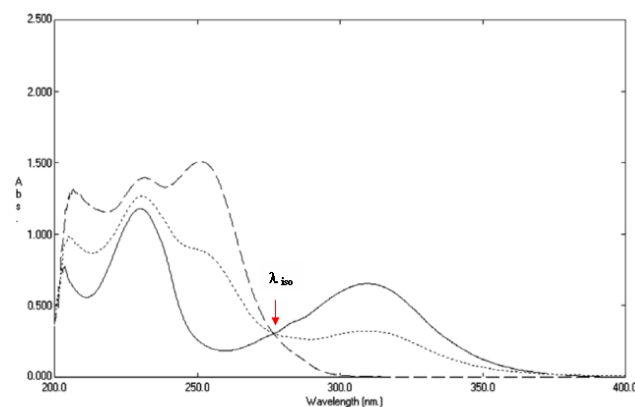
Fig. 2: Zero order absorption spectra of 20 μg. ml⁻¹ of metronidazole (——), 20 μg. ml⁻¹ diloxanide furoate (- - - - -) and (1:1) mixture containing 10 μg. ml⁻¹ of each (.......) using methanol as a blank. [32]

For a mixture of both drugs, the absorbance at this λ can be calculated from the equation
$$A_M = A_{x\,1cm}^{1\%} \, c_{xM} + A_{y\,1cm}^{1\%} \, c_{yM} \quad \text{......(31)}$$
$$A_M = A_{x\,1cm}^{1\%} \, (c_{xM} + c_{yM}) = A_{x\,1cm}^{1\%} \, (\, c_{TM}) \text{..(32)}$$

Where A_M is the absorbance of their mixture at isosbestic point and c_{xM} and c_{yM} are the concentrations of drugs *x* and *y* in the mixture, respectively, and C_{TM} is the concentration of their mixture.

Therefore we can conclude that
$$(c_{xM} + c_{yM}) = (\, c_{TM}) \text{................ (33)}$$
Thus, having the total concentration of both drugs, if the concentration of one of them can be determined separately by any other method, the concentration of the second drug can be calculated by subtraction.[31]This

method has was successfully applied for the simultaneous determination of several binary mixture, e.g., metronidazole and diloxanide furoate,[32] ezetimibe and atorvastatin,[33] and sitagliptin and metformin.[34]

10-Absorpitivity factor method
The absorptivity factor **(modification of the classical isoabsorpitive method)** is applied for the analysis of binary mixture if only there is a large difference in the absorptivity between both drugs, so there is no occurrence of an isoabsorptive point.

In isoabsorptive technique (**Fig. 3**) the spectra of the same concentration of the two studied drugs should cross at a point called isoabsorptivity point at which they have equal absorptivities while in absorptivity factor method the crossing point did not occur at equal concentration. Crossing point is obtained only between different concentrations of the two drugs at which the absorptivities of the two drugs are not equal but they are equal to the inverse of the ratio of the used concentrations.[35]
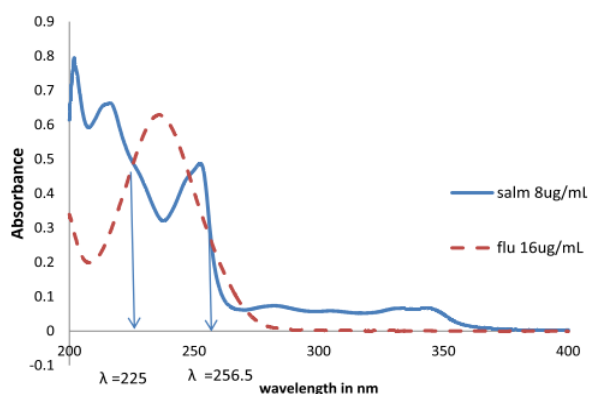

Fig. 3 : Zero order spectra of 8 μg. mL^{-1} salmeterol and 16 μg. mL^{-1} fluticasone showing the absorptivity factor points.[35]

Theory: For two drugs x and y, in the mixture, where concentration of y can be determined by using any of the well established spectrophotometric methods; drug x can be determined by *the absorpitivity factor method*. This method depends on the calculation of the absorptivity factor which is the ratio between the two absorptivities (a_x, a_y) at intersection point with the same absorbance value. This point is called the *absorbtivity factor point (k_F)*. This is summarized as follows[35]

$$A_x = A_y \quad \dots\dots\dots\dots\dots\dots\dots (34)$$
$$a_x \, b_x \, C_x = a_y \, b_y \, C_y \quad \dots\dots\dots\dots (35)$$
Where $b_x = b_y = 1cm$
$$a_x \, C_x = a_y \, C_y \quad \dots\dots\dots\dots\dots (36)$$
$$\frac{a_x}{a_y} = \frac{c_y}{c_x} = F \quad \dots\dots\dots\dots\dots\dots (37)$$
$$a_x = F a_y \quad \dots\dots\dots\dots\dots\dots\dots (38)$$

where, F is the absorptivity factor, a_x and a_y are the absorptivities of x and y respectively.

For mixture of x and y, the total absorbance of x and y at absorptivity factor point k_F can be expressed as follows
$$A_m = A_x + A_y \quad \dots\dots\dots\dots\dots (39)$$
$$A_m = a_x \, b_x \, C_x + a_y \, b_y \, C_y \; (where \; b_x = b_y = 1cm) \dots\dots (40)$$
$$A_m = a_x \, C_x + a_y \, C_y \quad \dots\dots\dots\dots\dots (41)$$

Where A_x, A_y and A_m are the absorbance of x, y and their mixture at k_F respectively, C_x and C_y are the concentrations of x and y respectively, a_x and a_y are the absorptivities of x and y at k_F respectively. When a_x is substituted by Fa_y

$$A_m = a_y \, F \, C_x + a_y \, C_y \quad \dots\dots\dots\dots\dots (42)$$
$$A_m = a_y \, (F \, C_x + C_y) \quad \dots\dots\dots\dots\dots\dots (43)$$

So, the total concentration of the mixture $(FC_x + C_y)$ can be calculated by using a regression equation representing the linear relationship between the absorbance of y and its corresponding concentration at the absorptivity factor point. The concentration of x can be determined after subtraction of concentration of y and multiplication by the inverse of F

$$C_x = [(F \, C_x + C_y) - C_y].1/F \quad \dots\dots\dots\dots (44)$$

Absorpitivity factor method was successfully applied for determination of salmeterol and fluticasone,[35] and sodium cromoglicate and fluorometholone [36] in their dosage forms.

11-Dual wavelength method
Dual wavelength method "also known as two wavelengths method" facilitates analyzing a component in presence of an interfering component by measuring the absorbance difference (ΔA) between two points in the mixture spectrum. In this method **(Fig. 4)**; one of the drugs is considered as a component of interest and the other drug is considered as an interfering component and vice-versa. The basis for such method is the selection of two wavelengths where the interfering component shows the same absorbance (ΔA equals zero) whereas the component of interest shows significant difference in absorbance with concentration. ΔA between two points on the mixture spectra is directly proportional to the concentration of the component of interest independent of interfering component. [37] This method was used for simultaneous determination of different drugs, e.g., atenolol and indapamide, [8] drotaverine and aceclofenac, [37] atorvastatin and ezetimibe,[38] chlorphenir-amine and phenylpropanolamine,[39] dexketoprofen and tramadol.[14]

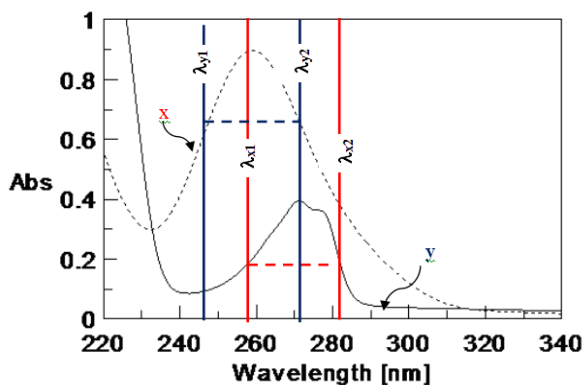**Fig. 4: Selection of wavelengths for dual wavelength method. [14]**

12- Ratio subtraction method (RSM)
If you have a mixture of 2 drugs, *x* and *y* with overlapping spectra, and the spectrum of *y* is extended more than *x*, the determination of *x* can be done by dividing the spectrum of the mixture by a certain concentration of *y* as a divisor (y^0).

The division will give a new curve that represents $\frac{x}{y^0} + constant$. If we subtract this constant, then multiply the new curve obtained after subtraction by y^0, we will obtain the original zero-order D^0 spectrum of *x*. This can be summarized in the following equations

Step1: $\frac{x+y}{y^0} = \frac{x}{y^0} + \frac{y}{y^0} = \frac{x}{y^0} + constant$ ..... (45)

Step2: $\frac{x}{y^0} + constant - constant = \frac{x}{y^0}$ ..... (46)

Step 3: $\frac{x}{y^0} * y^0 = x$ .... (47)

The constant can be determined directly from the curve $\frac{x+y}{y^0}$ by the straight line that is parallel to the wavelength axis in the region where *y* is extended. [40] RSM was successfully applied for determination of multicomponent pharmaceutical products containing e.g., metronidazole and diloxanide,[32] amlodipine and atorvastatin.[41]

To determine the second component (*y*), an extension of the already developed method has been established as a new approach, and known as ***extended ratio subtraction method ERSM***, in which *y* could be determined by dividing the obtained D^0 spectrum of *x* by a known concentration of *x* as a divisor (x^0) to get the value of the constant $\frac{x}{x^0}$. Dividing the spectrum of the mixture (*x* + *y*) by the same divisor (x^0). The division will give a new curve that represents $\frac{x}{x^0} + \frac{y}{x^0}$, where $\frac{x}{x^0}$ is the previously obtained constant. If we subtract this constant, then multiply the obtained curve after subtraction by x^0

(the divisor), therefore we can obtain the zero order absorption spectrum (D^0) of y (original spectrum of *y*).

$\frac{x+y}{x^0} = \frac{x}{x^0} + \frac{y}{x^0} - \frac{x}{x^0} = \frac{y}{x^0} * x^0 = y$ .... (48)

Concentration of *y* is calculated[42] by using the regression equation representing the linear relationship between the absorbance at its λ_{max} versus the corresponding concentration of *y*.

13-Mean centering of the ratio spectra
This method is applied for further improvement of the selectivity to resolve the overlap present between drugs in binary and ternary mixtures. This eliminates the derivative step and therefore the signal-to-noise ratio is enhanced. To explain the mean centering expression, let us consider a three-dimensional vector

$y = \begin{bmatrix} 5 \\ 1 \\ 3 \end{bmatrix}$ .... (49)

We center or mean center (MC) this column by subtracting the mean of three numbers calling

$\bar{y} = \begin{bmatrix} 3 \\ 3 \\ 3 \end{bmatrix}$ .... (50)

$MC(y) = y - \bar{y} = \begin{bmatrix} 5 \\ 1 \\ 3 \end{bmatrix} - \begin{bmatrix} 3 \\ 3 \\ 3 \end{bmatrix} = \begin{bmatrix} +2 \\ -2 \\ 0 \end{bmatrix}$ .... (51)

It could be proved that if the vector *y* is multiplied by *n* (a constant number), the mean centered vector is also multiplied by *n* and also if a constant number is added to the vector *y*, the mean center of this vector is not changed.[43] If there is no interaction among two components of a mixture, that is, x and y, and if Beer's law is obeyed for each compound, it can be expressed as follows

$A_m = a_x \ C_x + a_y \ C_y$ .... (52)

where A_M is the absorbance of the mixture, a_x and a_y are the molar absorpitivies of x and y; and C_x and are C_y the concentrations of x and y, respectively. If Eq. (52) is divided by a_y, the ratio spectrum is obtained in the form of the following equation

$B = \frac{A_m}{a_y} = \frac{a_x C_x}{a_y} + C_y$ .... (53)

Since the mean centering of a constant C_y is zero, mean centering (MC) of eq. (53) would be obtained as

$MC(B) = MC(\frac{a_x C_x}{a_y})$ .... (54)

Eq. (54) illustrates the mathematical explanation for analysis of binary components that permits the determination of concentration of one compound without interference from the other compound of the binary system, and vice versa.[21,38,41,43,44]

14-Absorption factor method (AFM)

This method describes the analysis of a binary mixture where the two components *x* and *y* have overlapped spectra. *Y* shows interference at λ_{max} of *x*, while *x* shows no interference with y at another wavelength (λ_2).
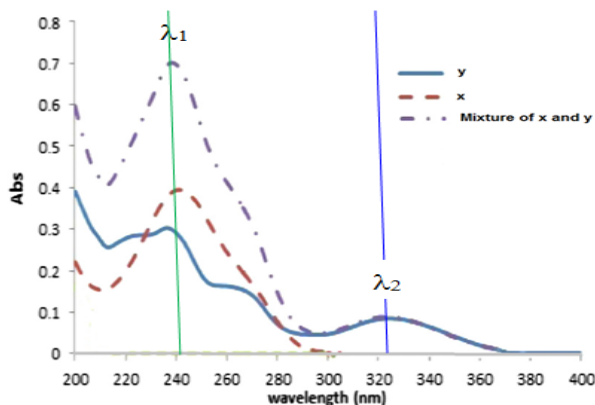

Fig. 5: Zero order spectra of *x* and *y* and their mixture.[36]

As shown in **Fig. 5**, the absorption spectra of *x* and *y* show severe overlap in the wavelength region of 200–300 nm. So, the absorption spectra of the standard solutions of the y with different concentrations were recorded in the wavelength range of 200–400 nm, and the average value of ***absorption factor*** ($\frac{A_{y,\lambda_1}}{A_{y,\lambda_2}}$) was calculated. Since the absorbance of the mixture ($x + y$) at λ_2 nm is equal to that of pure y due to lack of contribution of *x* at this wavelength, the absorption of *x* at λ_1 could be calculated using the following equation:

$$A_{x,\lambda_1} = A_{\lambda_1}(x+y) - \frac{A_{y,\lambda_1}}{A_{y,\lambda_2}} * A_{\lambda_2}(x+y) \ldots\ldots (55)$$

Where; $A_{\lambda_1}(x+y)$ and $A_{\lambda_2}(x+y)$ are the absorbance values of mixture at λ_1 and λ_2 respectively, and $\frac{A_{y,\lambda_1}}{A_{y,\lambda_2}}$ is the absorption factor of pure y.

The concentrations of x and y were calculated from the corresponding regression equation obtained by plotting the absorption values of the zero order spectra, at λ_1 and λ_2, against the corresponding concentrations, respectively. AFM method has been developed for the simultaneous determination of severalmixtures, e.g., ramipril and olmesartan,[45] perindopril and amlodipine,[46] sodium cromoglicate and fluorometholone.[36]

15- Multivariate chemometric methods

Chemometrics, in the most general sense, is the art of processing data with various numerical techniques in order to extract useful information.[47,48] Drug separation, identification, determination and validation have been studied using chemometrics.[49,50] Chemometrics recognizes that it is often better to measure many nonselective signals and then combine them in multivariate model (multivariate analysis), whereby multiple variables are considered simultaneously. A multivariate measurement is defined as one in which multiple measurements are made on a sample of interest. So, more than one variable or response are measured for each sample.[51,52] Multivariate methods include **multiple linear regression (MLR) methods and factor-based methods.**

The nature of the work makes it extremely convenient to organize the data into matrices. In particular, it is useful to organize the dependent and independent variables into separate matrices. In the case of spectroscopy, if the absorbance spectra of a number of samples of known composition are measured, all these spectra are assembled into one matrix called **the absorbance matrix**. All of the concentration values for the components of the sample are also assembled into a separate matrix called **the concentration matrix**. Generally, MLR and PCR techniques employ data organized as matrices of column vectors, while PLS technique employs data organized as matrices of row vectors. [47] The data of matrices are organized into pairs; each absorbance matrix is paired with its corresponding concentration matrix. The pair of matrices comprises a data set. Data sets have different names depending on their origin and purpose. [47] **Training set** is a data set containing measurements on a set of known samples. It is used to develop the calibration which is applied to predict the concentrations of unknown samples. Training set should contain all expected components, span the concentration ranges of interest and contain mutually independent samples. [47,51] **Validation set** is an additional data set containing independent measurements on samples that are independent from the samples used to create the training set. Validation set is used to test the validity of the calibration developed with the training set. The developed calibration is used to predict the concentrations of the components in the validation samples. Then these predicted concentrations are compared to the actual concentrations. [47,51] The absorbance matrix containing the unknown(s) spectra together with the corresponding result matrix containing the predicted concentrations comprise an **unknown set**.

(I) Multiple Linear Regression (MLR) [53]

(a) Classical Least Squares (CLS) or (K-matrix)

Classical least squares (CLS), sometimes known as K-matrix calibration, is so called because, originally, it involved the application of multiple linear regression (MLR) to the classical expression of the Beer-Lambert law of spectroscopy: [47]

$$A = KC \ldots\ldots\ldots\ldots\ldots (56)$$

Computing the calibration

To produce a calibration using classical least squares, a training set consisting of a concentration matrix, C, and an absorbance matrix, A, for known calibration samples

is required. We then solve for the matrix, K. Each column of K will hold the spectrum of one of the pure components. Since the data in C and A contain noise, there will, in general, be no exact solution for Eq. (56). So, the best least squares solution for Eq. (56) has to be found. In other words, K is found such that the sum of the squares of the errors is minimized. The errors as in Eq. (57) are the difference between the measured spectra, A, and the spectra calculated by multiplying K and C.

errors = KC – A……………….....(57)

To solve for K, each side of Eq. (56) is post-multiplied by C^T, the transpose of the concentration matrix.

$A\,C^T = KC\,C^T$……………………..(58)
$A\,C^T\,[CC^T]^{-1} = K\,[CC^T]\,[CC^T]^{-1}$……..…………….. (59)
$A\,C^T\,[CC^T]^{-1} = K$ ……..…………….(60)

Predicting unknowns

$A_{unk} = K\,C_{unk}$ …………………..(61)
$K^T\,A_{unk} = K^T\,K\,C_{unk}$ …………..…..(62)
$[K^TK]^{-1}\,K^T\,A_{unk} = [K^TK]^{-1}\,[K^TK]\,C_{unk}$ ………(63)
$[K^TK]^{-1}\,K^T\,A_{unk} = C_{unk}$ …………………. (64)
$K_{cal} = [K^TK]^{-1}\,K^T$ ……………..(65)
$C_{unk} = K_{cal}\,A_{unk}$ ……………………… (66)

K_{cal} is called the calibration matrix or the regression matrix. It contains the calibration, or regression, coefficients which are used to predict the concentrations of an unknown from its spectrum. CLS can work quite well under the right conditions. In particular, it is important that the concentration values for all of the components present in the training samples are provided. [47,51,54]

(b) Inverse Least Squares (ILS) or (P-matrix)

Inverse least squares (ILS), sometimes known as P-matrix calibration, is so called because, originally, it involved the application of multiple linear regression (MLR) to the inverse expression of the Beer-Lambert law of spectroscopy: [47]

$C = PA$ …………………….(67)

Computing the calibration

P matrix will contain one row of coefficients for each component being predicted. Each row will have one coefficient for each spectral wavelength. Thus, P will have as many columns as there are spectral wavelengths. Since the data in C and A contain noise, there will, in general, be no exact solution for Eq. (67), so, the best least squares solution for Eq. (67) has to be found. In other words, P is found such that the sum of the squares of the errors is minimized. The errors are the difference between the measured concentrations, C, and the concentrations calculated by multiplying P and A:

errors = PA – C ……………..(68)

To solve for P, each side of Eq. (67) is post-multiplied by A^T, the transpose of the absorbance matrix.

$C\,A^T = P\,AA^T$ ……..……………….(69)
$C\,A^T\,[AA^T]^{-1} = P\,[AA^T]\,[AA^T]^{-1}$ ……………….(70)

$C\,A^T\,[AA^T]^{-1} = P$ …………………….(71)

Predicting unknowns [47]
$C_{unk} = P\,A_{unk}$ ………………(72)

(II) Factor-based methods

A factor space is nothing more than a particular coordinate system that offers certain advantages. When we operate in a factor space, instead of the native data space, we are simply mapping our data into a new coordinate system. We are not actually changing the data itself. [47] Principal component regression (PCR) is sometimes described as performing a least squares regression of the projections of the data onto the basis vectors of a factor space using inverse least squares.[47,20,55,56] Partial least squares (PLS) method is a multivariate calibration method based on factor analysis. The basic concept of PLS regression was originally developed by Wold. [57] A detailed description of the mathematical principles of the PLS algorithm was reported by Martens and Naes.[58] PLS method involves simultaneously the independent and the dependent variables in the data compression and decomposition operations.[47,48] There are several reasons to use the coordinate system of a factor space rather than the native space comprised of physically meaningful coordinates. These reasons are numerical conditioning, reduced assumptions, noise rejection, new insights into the data and data compression.[47]

Any pair of axes lying in the plane which holds the spectra comprises a factor space for that data. Each axis is a factor of the data space. These are usually called abstract factors because they usually do not have an easily interpretable physical meaning.   Instead of specifying each spectrum in terms of all its wavelengths, it will be specified in terms of its projections onto the factors. These projections are often called the scores. The projection of a spectrum onto the factors is nothing more than the distance of that spectrum along the direction of each factor. We could find as many factors as there are wavelengths in the spectra. Each factor will capture the maximum variance of the data that was not yet spanned by the earlier factors and it must be mutual orthogonal to all the factors that precede it.[47] The first eigenvector spans the maximum variance of the data that can be spanned with a single vector. That is why this eigenvector is also called the first principal component of the data set. The second eigenvector of the data will span the maximum possible amount of the remaining variance that was not spanned by the first factor. Each eigenvector has an eigenvalue associated with it. The eigenvalue of an eigenvector is equal to the sum of the squares of the projections of the data onto the eigenvector [47] as shown in **Fig. 6**. These calibrations involve multiple steps.   First, a set of mutually orthogonal vectors (factors) is found that spans the data space. Next, it is decided how many factors should be kept to use in the calibration.   Finally, calibration

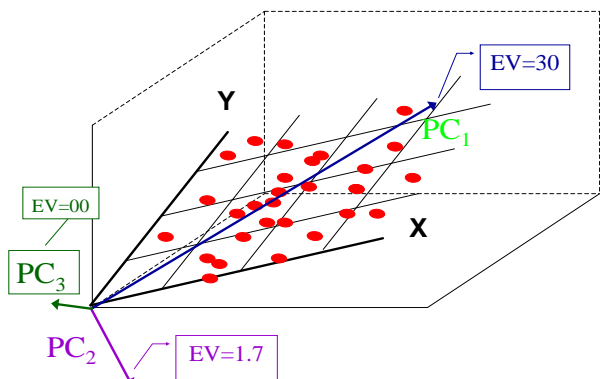matrices and/or coefficients are generated using the selected number of factors.[53]

Fig. 6: Mapping of the data of a binary mixture into a new coordinate system then, plotting of the first principal component (PC_1) of highest eigenvalue, the second principal component (PC_2) perpendicular to the first one (PC_1) and the third principal component (PC_3) perpendicular to both (PC_1) and (PC_2) for data of a binary mixture. [47]

(a) Principal Component Regression (PCR)

Principal component regression is sometimes described as performing a least squares regression of the projections of the data onto the basis vectors of a factor space using ILS. [47]

Generating the abstract factors (eigenvectors)

All of the factors for the data matrix can be calculated using a number of different algorithms. Strictly speaking, it is not generally needed to calculate all of the factors. The first N factors need only to be calculated where N is large enough to determine how many factors should be included in the basis space. [47]

Keeping the significant factors

: if the number of factors retained is more than the required, more noise will be added to the calibration and errors may occur. On the other hand, if the number retained is too small, meaningful data that could be necessary for the calibration might be discarded.[47,52,53] Several indicators or functions offered by Chemometrics TOOLBOX for use with MATLAB [53] could be used for determining the optimum number of factors (rank). For example, PCAPRESS indicator which generates a calibration for every possible rank (number of factors retained). Then, use each calibration to predict the concentrations for a set of independently measured, independent validation samples. The predicted residual error sum-of-squares, or PRESS, for each calibration, is calculated and the calibration that provides the best results is selected. The number of factors used in that calibration is the optimal rank for that system [47,51,59] as shown in **Fig. 7**.
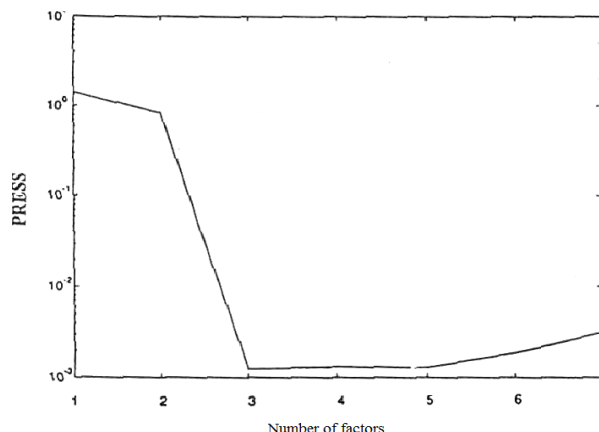

Fig. 7: PRESS versus number of factors for a PCR model.[47]

Computing the calibration [47]

$$A_{proj} = Vc^T A \quad\quad\quad\quad\quad\quad\quad (73)$$

where: A_{proj} is the matrix containing the new coordinates (the projections), A is the original training set absorbance matrix, Vc is the matrix containing the basis vectors, one column for each factor retained. Substitute A_{proj} into Eq. (67) in place of A; and P matrix here in PCR is called F matrix

$$C = F A_{proj} \quad\quad\quad\quad\quad (74)$$
$$C A^T_{proj} = F A_{proj} A^T_{proj} \quad\quad\quad\quad (75)$$
$$CA^T_{proj}[A_{proj} \ A^T_{proj}]^{-1} = F \ A_{proj}A^T_{proj}[A_{proj} \ A^T_{proj}]^{-1} \quad\quad\quad\quad (76)$$
$$C A^T_{proj} [A_{proj} A^T_{proj}]^{-1} = F \quad\quad\quad\quad (77)$$

Predicting unknowns [47]

$$C_{unk} = F Vc^T A_{unk} \quad\quad\quad\quad (78)$$
$$C_{unk} = F_{cal} A_{unk} \quad\quad\quad\quad (79)$$

Where: F_{cal} (PCR calibration matrix) = the quantity Vc^T F pre-calculated at calibration time.

(b) Partial Least Squares (PLS)

Like PCR, PLS involves the generation of abstract factors. However, instead of generating factors for the spectral information only, it also generates factors for the concentration information. Then, it rotates the spectral and concentration factors towards each other in order to optimize the regression between the spectral data and the concentration data. [47,53] Since the spectral noise is independent from the concentration noise, a perfectly linear relationship is no longer possible. So, the best which can be done is to restore optimum congruence in the least squares sense. The main advantage of the PLS method is based on the interrelated decomposition of the concentration matrix C and the absorbance matrix A, so that with this algorithm the most robust calibration at present can be obtained. PLS calibration can be used for very complex mixtures since only knowledge of constituents of interest is required. [47,48,51]

Computing the calibration: [20, 47,48]

absorbance values obtained over a preset wavelength range are used to construct an absorbance matrix A of n x m dimensions (where n is the number of mixtures and m that of

wavelengths). Analyte concentrations are also arranged in a matrix C of n x p dimensions (where p is the number of mixture components). Both A and C are resolved into two smaller matrices S and L (the scores and loadings matrices, respectively) plus an error matrix (E)

$$A = S_A L_A + E_A \qquad (80)$$
$$C = S_C L_C + E_C \qquad (81)$$

where S_A is the absorbance score matrix, L_A is the absorbance loading matrix, S_C is the concentration score matrix, L_C is the concentration loading matrix; E_A and E_C are the error matrices, the dimensions of which coincide with those of the original absorbance matrix (n x m) and concentration matrix (n x p), respectively. Matrices L_A and L_C can be related via a diagonal matrix

$$L_C = L_A V + E_e \qquad (82)$$

where E_e is an error matrix.

Predicting unknowns [47,48]

Matrix V is used to estimate unknown concentrations from a spectrum of absorbance A_o:

$$C_o = A_o \ (S_C A)^{-1} \ V L_C \qquad (83)$$

Where S_C, L_C and A can be obtained from the calibration process.

Multivariate calibration methods have been applied to the resolution of overlapping spectra for the determination of active compounds in samples containing two or more compounds.[25] Also, multivariate methods can be applied to chromatographic data for simultaneous determination of some drugs in a multicomponent dosage form.[60,61,62] Chemometric techniques present a wide array of modeling and processing tools that provide evolving of chemistry into a discipline gathering both experimental and modeling.

REFERENCES

1. Crout JR. (Fixed combination prescription drugs: FDA policy). J Clin Pharmacol, 1974; 14(5-6): 249-254.
2. Bozdoğan A, Acar AM, Kunt GK. (Simultaneous determination of acetaminophen and caffeine in tablet preparations by partial least-squares multivariate spectrophotometric calibration). Talanta, 1992; 39(8): 977-979.
3. Ojeda CB,Rojas FS. (Recent developments in derivative ultraviolet/visible absorption spectrophotometry). Anal Chim Acta, 2004; 518(1): 1-24.
4. Korany MA, Wahbi AM, Mandour S, Elsayed MA. (Determination of certain drugs in multicomponent formulations by first derivative ultraviolet sspectrophotometry). Anal Lett, 1985; 18: 21-34.
5. Saldanha TC,de Araújo MU,Neto BB,Chame HC. (Simultaneous analysis of Co^{2+}, Cu^{2+} Mn^{2+}, Ni^{2+} and Zn^{2+} in the ultraviolet region Using 4-(pyridil-2-azo) resorcinol and multivariate calibration). Anal Lett, 2000; 33(6): 1187-1202.

6. Skoog DA,Holler FJ,Crouch SR. Principles of Instrumental Analysis. 6th. ed., Canada; Thomson Corporation: 2007.
7. Beckett AH,Stenlake JB. Practical Pharmaceutical Chemistry. part II, 4th. ed., London; Bloomsbury Publishing: 2001.
8. Fernandes N,Nimdeo MS,Choudhari VP,Kulkarni RR,Pande VV,Nikalje AG. (Dual wavelength and simultaneous equation spectrophotometric methods for estimation of atenolol and indapamide in their combined dosage form). Int J Chem Sci, 2008; 6(1): 29-35.
9. Chitlange SS,Soni R,Wankhede SB,Kulkarni AA. (Spectrophotometric methods for simultaneous estimation of dexibuprofen and paracetamol). Asian J Res Chem, 2009; 2(1): 30-33.
10. Patel KN,Patel JK,Rajput GC,Rajgor NB. (Derivative spectrometry method for chemical analysis: A review). Der Pharmacia Lettre, 2010; 2(2): 139-150.
11. Mabrouk MM,el-Fatatry HM,Hammad SF,Wahbi AA. (Simultaneous determination of loratadine and pseudoephedrine sulfate in pharmaceutical formulation by RP-LC and derivative spectrophotometry). J Pharm Biomed Anal, 2003; 33(4): 597-604.
12. Srinivasan K,Alex J,Shirwaikar A,Jacob S,Sunil Kumar M,Prabu S. (Simultaneous derivative spectrophotometric estimation of aceclofenac and tramadol with paracetamol in combination solid dosage forms.). Indian J Pharm Sci, 2007; 69(4): 540-545.
13. Thomas A,Dumbre N,Nanda R,Kothapalli L,Chaudhari A,Deshpande A. (Simultaneous determination of tramadol and ibuprofen in pharmaceutical preparations by first order derivative spectrophotometric and LC methods). Chromatographia, 2008; 68(9-10): 843-847.
14. Mabrouk MM,Hammad SF,El-Fatatry HM,El-Malla SF. (Spectroscopic methods for determination of dexketoprofen trometamol and tramadol HCl). Inventi Impact: Pharm Analysis & Quality Assurance, 2014; 2014(4): 276-282.
15. Desai SD,Patel BA,Parmar SJ,Champaneri NN. (Development and validation of first order derivative spectrophotometric method for simultaneous estimation of paracetamol and tapentadol hydrochloride in tablet dosage form). Asian J Pharmaceut Res Health Care, 2013; 5(1): 8-15.
16. Medina JR, LÓpez-tableros CA,Altamirano GH,Ángeles GA,Hurtado M,DomÍnguez-ramÍrez AM. (Simultaneous determination of naproxen sodium and acetaminophen in fixed dose combinations formulations by first order derivative spectroscopy: application to dissolution studies). Int J Pharm Pharmaceut Sci, 2015; 7(5): 183-188.

17. Mabrouk MM, Hammad SF,Mansour FR,El-Khateeb BZ. (Simultaneous determination of naproxen and diphenhydramine by reversed phase

liquid chromatography and derivative spectrophotometry). Der Pharma Chemica, 2015; 7(12): 181-192.

18. Mabrouk MM, Hammad SF,Mansour FR,Michael MA. (Reversed phase high performance liquid chromatographic method for simultaneous determination of phenylephrine hydrochloride and ketorolac tromethamine). World J Pharm Sci, 2015; 3(10): 2152-2159.

19. Abdel-Hay MH,Gazy AA,Hassan EM,Belal TS. (Derivative and derivative ratio spectrophotometric analysis of antihypertensive ternary mixture of amiloride hydrochloride, hydrochlorothiazide and timolol maleate). J Chin Chem Soc 2008; 55(5): 971-978.

20. Salinas F,Nevado BJ,Mansilla EA. (A new spectrophotometric method for quantitative multicomponent analysis resolution of mixtures of salicylic and salicyluric acids). Talanta, 1990; 37(3): 347-351.

21. Bhatt NM,Chavada VD,Sanyal M,Shrivastav PS. (Manipulating ratio spectra for the spectrophotometric analysis of diclofenac sodium and pantoprazole sodium in laboratory mixtures and tablet formulation). Sci World J, 2014; 2014(1): 1-10.

22. Ashour HK,Belal TS. (New simple spectrophotometric method for determination of the antiviral mixture of emtricitabine and tenofovir disoproxil fumarate). Arabian J Chem, 2013; DOI: 10.1016/j.arabj.

23. Lotfy HM,Hagazy MA. (Comparative study of novel spectrophotometric methods manipulating ratio spectra: An application on pharmaceutical ternary mixture of omeprazole, tinidazole and clarithromycin). Spectrochim Acta Part A, 2012; 96: 259-270.

24. Samir A,Salem H,Abdelkawy M. (Simultaneous determination of salmeterol xinafoate and fluticasone propionate in bulk powder and Seritide®; diskus using high performance liquid chromatographic and spectrophotometric method). Pharmaceut Anal Acta, 2012; 3(8): 1-7.

25. Wahbi AM,Mabrouk MM,Moneeb MS,Kamal AH. (Simultaneous determination of the two non-steroidal anti-inflammatory drugs "diflunisal and naproxen" in their tablets by chemometric spectrophotometry and HPLC). Pakistan J Pharm Sci, 2009; 22(1): 8-17.

26. Nevado BJ,Cabanillas CG,Salinas F. (Spectrophotometric resolution of ternary mixtures of salicylaldehyde, 3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde by the derivative ratio spectrum-zero crossing method). Talanta, 1992; 39(5): 547-553.

27. Dinç E,Onur F. (Application of a new spectrophotometric method for the analysis of a ternary mixture containing metamizol, paracetamol and caffeine in tablets). Anal Chim Acta, 1998; 359(1–2): 93-106.

28. Gallego JM,Arroyo JP. (Spectrophotometric resolution of ternary mixtures of Dexamethasone, Polymyxin B and Trimethoprim in synthetic and pharmaceutical formulations). Anal Chim Acta, 2001; 437(2): 247-257.

29. El-Fatatry HM,Mabrouk MM,Hammad SF,El-Malla SF. (Simultaneous determination of tapentadol HCl and paracetamol by ratio-spectra derivative spectrophotometry). World J Pharmaceut Sci, 2015; 3(7): 1290-1297.

30. Afkhami A,Bahram M. (Successive ratio-derivative spectra as a new spectrophotometric method for the analysis of ternary mixtures). Spectrochim Acta Part A, 2005; 61(5): 869-877.

31. Erram SV,Tipnis HP. (Simple spectrometric analysis of propranolol hydrochloride and hydrochlorothiazide from combined pharmaceutical dosages). Indian Drugs, 1994; 31: 65-72.

32. El-Ghobashy MR,Abo-Talib NF. (Spectrophotometric methods for the simultaneous determination of binary mixture of metronidazole and diloxanide furoate without prior separation). J Adv Res, 2010; 1(4): 323-329.

33. Baghdady YZ,Al-Ghobashy MA,Abdel Aleem AE,Weshahy SA. (Spectrophotometric and TLC-densitometric methods for the simultaneous determination of ezetimibe and atorvastatin calcium). J Adv Res, 2013; 4(1): 51-59.

34. Riad SM,Rezk MR,Mahmoud GY,AbdelAleem AE. (Spectrophotometric determination of sitagliptin and metformin in their pharmaceutical formulation). Int J Compr Pharm, 2013; 3(5): 1-4.

35. Samir A,Salem H,Abdelkawy M. (New developed spectrophotometric method for simultaneous determination of salmeterol xinafoate and fluticasone propionate in bulk powder and Seritide diskus inhalation). Bull Fac Pharm Cairo Univ, 2012; 50:121-126.

36. Saleh SS,Lotfy HM,Hassan NY,Elgizawy SM. (A comparative study of validated spectrophotometric and TLC-spectrodensitometric methods for the determination of sodium cromoglicate and fluorometholone in ophthalmic solution). Saudi Pharm J, 2013; 21(4): 411-421.

37. Jain J,Patadia R,Vanparia D,Chauhan R,Shah S. (Dual wavelength spectrophotometric method for simultaneous estimation of drotaverine hydrochloride and aceclofenac in their combined tablet dosage form). Int J Pharm Pharm Sci, 2010; 2(4): 76-79.

38. Abdelwahab NS,El-Zeiny BA,Tohamy SI. (Two spectrophotometric methods for simultaneous determination of some antihyperlipidemic drugs). J Pharm Anal, 2012; 2(4): 279-284.

39. Kaura AK,Gupta V,Kaura M,Roy G,Bansal P. (Spectrophotometric determination of chlorpheniramine maleate and phenylpropanolamine hydrochloride by two wavelengths method). J Pharm Res, 2013; 7: 404-408.

40. El-Bardicy MG,Lotfy HM,El-Sayed MA,El-Tarras MF. (Smart stability-indicating spectrophotometric methods for determination of binary mixtures without prior separation). J AOAC Int, 2008; 9(2): 299-310.

41. Darwish HW,Hassan SA,Salem MY,El-Zeiny BA. (Three different spectrophotometric methods manipulating ratio spectra for determination of binary mixture of Amlodipine and Atorvastatin). Spectrochim Acta Part A, 2011; 83:140-148.

42. Hayam Mahmoud Lotfy ,Hagazy MA-M. (Comparative study of novel spectrophotometric methods manipulating ratio spectra: An application on pharmaceutical ternary mixture of omeprazole, tinidazole and clarithromycin). Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2012; 96: 259-270.

43. Afkhami A,Bahram M. (Mean centering of ratio spectra as a new spectrophotometric method for the analysis of binary and ternary mixtures). Talanta, 2005; 66: 712-720.

44. Saleh SS,Lotfy HM,Hassan NY,Elgizawy SM. (A comparative study of validated spectrophotometric and TLC- spectrodensitometric methods for the determination of sodium cromoglicate and fluorometholone in ophthalmic solution). Saudi Pharmaceut J, 2013; 21(4): 411-421.

45. Patel CV,Khandhar AP,Captain AD,Patel KT. (Validated absorption factor spectrophotometric and reversed-phase high-performance liquid chromatographic methods for the determination of ramipril and olmesartan medoxomil in pharmaceutical formulations). Eurasian J Anal Chem, 2007; 2(3): 159-171.

46. Prajapati J,Patel M,Prajapati R,Prajapati N. (Simultaneous determination of perindopril erbumine and amlodipine besylate by absorption factor method). Int J Appl Biology Pharmaceut Technol, 2011; 2(3): 230-233.

47. Kramer R. Chemometric techniques for quantitative analysis. ed., New York; CRC Press: 1998.

48. Otto M. Chemometrics: statistics and computer application in analytical chemistry. ed., Weinheim; New York Wiley-VCH: 1999.

49. Meloun M,Militky J,Forina M. Chemometrics for analytical chemistry. ed., New York; Ellis Horwood: 1992.

50. Lilley K,Wheat T. (Drug identification in biological matrices using capillary electrophoresis and chemometric software). J Chromatogr B: Biomed Sci Appl, 1996; 683(1): 67-76.

51. Kenneth R,Randy J,Seasholtz M. Chemometrics: A practical guide. ed., New York; John Willey and Sons Inc.,: 1998.

52. Ni Y,Gong X. (Simultaneous spectrophotometric determination of mixtures of food colorants). Anal Chimica Acta, 1997; 354(1): 163-171.

53. Kramer R. Chemometrics TOOLBOX for use with MATLAB, The Math Works. ed., Natick Inc.: 1995.

54. Thomas EV,Haaland DM. (Comparison of multivariate calibration methods for quantitative spectral analysis). Anal Chem, 1990; 62(10): 1091-1099.

55. Dinç E,Baleanu D. (Spectrophotometric quantitative determination of cilazapril and hydrochlorothiazide in tablets by chemometric methods). J Pharmaceut Biomed Anal, 2002; 30(3): 715-723.

56. Dinç E,Băleanu D,Onur F. (Chemometric quantitative analysis of pyridoxine HCl and thiamine HCl in a vitamin combination by principal component analysis, classical least squares, and inverse least squares techniques). Spectro Lett, 2001; 34(3): 279-288.

57. Wold H. Research Papers in Statistics. ed., New York; John Wiley& Sons, Inc.: 1966.

58. Martens H,Naes T. Multivariate calibration. ed., John Wiley & Sons: 1992.

59. Haaland DM, Thomas EV. (Partial least-squares methods for spectral analyses. Relation to other quantitative calibration methods and the extraction of qualitative information). Analytical Chemistry, 1988; 60(11): 1193-1202.

60. Kamal AH, Mabrouk MM, Hammad SF, El-Fatatry HM. (Chemometric approach to simultaneous chromatographic determination of ibuprofen and famotidine in tablets). Inventi Impact: Pharm Analysis & Quality Assurance, 2015; 2014: 66-75

61. Kamal AH, Mabrouk MM, Hammad SF, El-Fatatry HM. (Simultaneous determination of esomeprazole magenisum trihydrate and naproxen by combined HPLC- chemometric techniques and RP-HPLC method). Int J Pharm, 2015; 5: 107-121.

62. Kamal AH, Mabrouk MM,El-Fatatry HM,Hammad SF. (Determination of enantiomeric composition of ofloxacin in tablets by chemometric techniques applied to overlapped chromatograms). Der Pharma Chemica, 2015; 7: 117-126.