Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 224 (2020) 117339

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Advanced approaches for the treatment and amplification of weak spectral signals produced by critical concentrations in white multicomponent systems

Hayam M. Lotfy [a, b], Sarah S. Saleh [c, *], Christine M. El-Maraghy [c]

[a] Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr-El Aini Street, 11562 Cairo, Egypt
[b] Pharmaceutical Chemistry Department, Faculty of Pharmaceutical Sciences & Pharmaceutical Industries, Future University in Egypt, 12311, Cairo, Egypt
[c] Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 11787 6th October, Egypt

ARTICLE INFO

Article history:
Received 26 May 2019
Received in revised form 4 July 2019
Accepted 4 July 2019
Available online 8 July 2019

Keywords:
Metoclopramide
Factorized
Amplitude modulation
H-point
Aspirin

ABSTRACT

An analytical investigation was carried out to study the treatment and amplification of the spectral signals produced by critical concentrations with high accuracy and precision using two advanced approaches. The factorized-spectrum approach was applied through two novel methods which were: absorptivity centering technique via both: factorized zero order absorption spectrum (ACT-FSD$^0_{\Delta A}$) and factorized ratio spectrum (ACT-FSR$_{\Delta P}$). The proposed methods were found to be linear in the ranges of (15−100 μg/mL) and (3−40 μg/mL) for ASP and MTO, respectively. Those methods were compared to the methods following the geometrical standard addition approach: ratio H-point standard addition method (RHPSAM) and geometrical induced amplitude modulation (GIAM). The approaches were applied for the determination of the minor component metoclopramide in its mixture with the major component aspirin in the challengeable ratio of (1,90) respectively in a white multicomponent system. The results obtained from the proposed approaches were statistically compared with each other. The methods were validated according to ICH guidelines where the results were found to be within the acceptable limits. The methods were found to be accurate and reliable for the determination of metoclopramide critical concentration besides aspirin concentration. The results of single factor ANOVA analysis indicated that there is no significant difference among the developed methods. These methods provided simple resolution of this binary combination from synthetic mixtures and pharmaceutical preparation and can be conveniently adopted for routine quality control analysis.

1. Introduction

The spectrophotometric techniques have attracted the attention of researchers all over the world due to its simplicity, reliability and wide applications. The spectrophotometric methods show obvious diversity according to their mathematical background such as derivative calculation [1,2], ratio spectral manipulation [3,4], dual wavelength [5,6] and standard addition [7,8]. The choice of the spectrophotometric method to be applied mainly depends on the spectral nature of the data to be analyzed. Usually for mixtures with partially overlapping spectra, simple methods depending on one mathematical operation could be applied. On the contrary, methods with multi-mathematical operations should be applied for the analysis of complex mixtures with severely or completely overlapping spectra. Then by choosing the suitable method, analysts would be able to solve and analyze simple or complex absorbance matrices.

According to Liang et al. [9] mixtures are classified into white, black and grey multicomponent systems. White systems are labelled with existing spectral information for all the chemical species present in the systems (interferents are well known), black systems include mixtures no data about its chemical composition, while grey systems lies in between white and black where insufficient data of all coexisting chemical interferents where analyte of interest is quantitated in the presence of unknown coexisting interferents. Hyphenated chromatography is the method of choice for black systems, while spectrophotometric analysis could be applied for white and grey systems in the form of univariate and multivariate calibration methods, respectively.

Spectrophotometric analysis has many advantages over other analytical techniques (such as LC-MS or GC−MS…) including reduced analysis time and cost, minimized usage of organic solvents (environment-friendly), availability and ease of application. Unfortunately, the sensitivity of the spectrophotometric methods was always questioned. The reason is the limited concentration range that can be analyzed which is governed by Beer's law [10]. So the application of

* Corresponding author.
E-mail addresses: drsarahsalah@gmail.com, sssaleh@msa.eun.eg (S.S. Saleh).

https://doi.org/10.1016/j.saa.2019.117339
1386-1425/

spectrophotometric analysis was always hindered for samples containing low and critical concentrations due to lack of accuracy and sensitivity. The methods used for solving such a problem should acquire one of two features; either amplifying the weak absorbance signals of the lower concentration by spiking or standard addition followed by subtraction of the known amount added (as proposed in the geometric standard addition approach), or treatment of the weak signals via mathematical manipulations in order to extract the maximum strength of the purest signal of each component solely (as proposed in the factorized spectrum approach).

By spiking samples with pure analyte to amplify the absorbance signal is amplified followed by calculating the difference in concentration which may encounter error. To minimize this error, the solutions were applied to several standard addition steps and the absorbance was measured as a function of the added standard points. This approach was known as H-point standard addition method (HPSAM) [7,11,12]. A further modification of this method was introduced under the name of ratio H-point standard addition method (RHPSAM) [13,14] in which the principles of ratio spectrophotometry and dual wavelength were utilized together with H-point standard addition. Likewise, two methods were recently introduced for the analysis of critical concentrations namely, geometrical amplitude modulation (GAM) and geometrical induced amplitude modulation (GIAM) [15], where the principles of standard addition were merged with isosbestic point in ratio spectra.

Meanwhile, the concept of factorized spectrum was firstly introduced to represent a spectrum with absorbance equals unity at the isoabsorptive point [16,17]. The factorized spectrum enabled the resolution of components using the absorptivity inverse at isoabsorptive point ($1/a_{\lambda iso}$). The factorized spectrum has several advantages over the normalized divisor [17] as it can be generated using the built-in apparatus software and it involves minimal arithmetic manipulations.

In this study, a novel approach utilizing the factorized spectrum was introduced namely, absorptivity centering technique via factorized zero order absorption spectrum (ACT-FSD^0) and factorized ratio spectrum (ACT-FSR). This approach was compared to geometrical standard addition approach applied through: ratio H-point standard addition method (RHPSAM) and geometrical induced amplitude modulation (GIAM). The proposed approaches were applied for the determination of minor component metoclopramide (MTO) in its binary mixture with aspirin (ASP) in the ratio of (1:90) respectively. The proposed methods eliminated the problem of the interference of the spectrum of the major component (ASP) to determine the critical concentration of (MTO) together with (ASP) in their combined pharmaceutical preparation.

Metoclopramide (MTO), 4-Amino−5−chloro-N-[(2−diethylamino) ethyl]-2−methoxybenzamide [18], is an effective antiemetic acting by blocking apomorphine induced vomiting and it increases gastric peristalsis while relaxing the pylorus and the first part of duodenum that leads to speeding gastric emptying [19]. While aspirin (ASP), 2-(Acetyloxy) benzoic acid [18], has analgesic, antipyretic, anti-inflammatory actions [20] and irreversibly inhibits platelet COX-1 so that its antiplatelet effect lasts 8−10 days [19,21]. Both drugs are co-formulated to relieve the pain associated with migraine [22]. Literature survey revealed the reported methods for the simultaneous determination of ASP and MTO using the first derivative spectrophotometric, HPLC [23] and spectrofluorimetric methods [24]. The structural formulae of MTO and ASP are shown in Fig. 1.

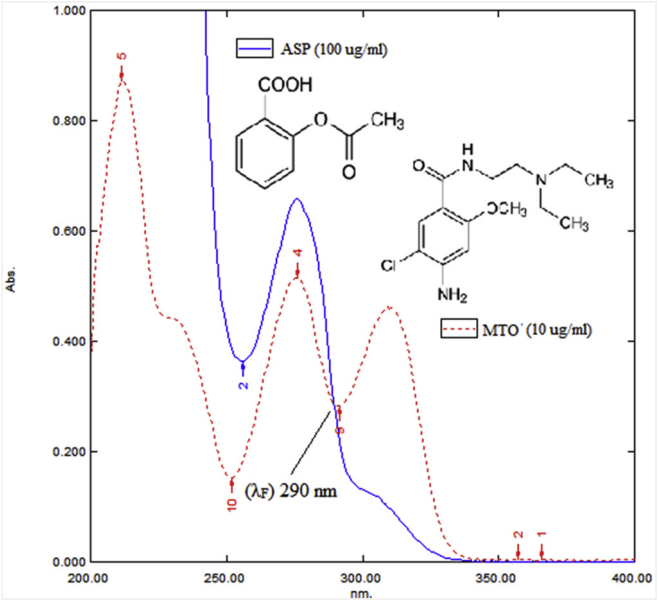

Fig. 1. Chemical structures and zero order absorption spectra (D^0) of Aspirin (ASP) 100 μg/mL and Metoclopramide (MTO) 10,100 μg/ml.

2. Theoretical background

2.1. Factorized-spectrum approach

2.1.1. Absorptivity centering technique via factorized zero order absorption spectrum based on absorbance difference (ACT-FSD$^0{}_{\Delta A}$)

This approach for absorptivity centering [16,25] (ACT) was modified to be applied for resolving binary mixture of X and Y possessing partially or completely overlapped spectra without isoabsorptive point using absorbance difference between two wavelengths, which obeys Beer's law, where interfering substance exhibits an absorbance difference equals to zero (ACT-FSD$^0{}_{\Delta A}$).

The factorized spectrum using absorbance difference between two wavelengths (FSD$^0{}_{\Delta A}$) is achieved through the division of the absorption spectrum of Y, throughout all the measured wavelengths, by the difference of its absorbance values at the two selected wavelengths where component X should show zero difference in its absorbance at these selected wavelengths.

$$\text{Factorized D}^0\text{spectrum of Y via absorbance difference}\left(\text{FSD}^0{}_{\Delta A}\right)$$
$$= A_Y \Big/ \Delta A_{Y'(\lambda s)}$$
$$= a_Y C_Y \Big/ \left[\Delta a_{Y(\lambda s)} C_Y\right]'. \therefore \text{Factorized D}^0\text{spectrum of Y via absorbance difference}\left(\text{FSD}^0{}_{\Delta A}\right)$$
$$= \left[a_Y \Big/ \Delta a_{Y(\lambda s)}\right]'$$

The D^0 of Y can be recovered by multiplying the calculated difference in the absorbance values of the mixture between the two selected wavelengths by its corresponding factorized spectrum.

$$\therefore \text{Recovered D0 of Y (aY CY)} = [\text{aY}/\Delta\ \text{aY}(\lambda s)]' \cdot \Delta\ \text{aY}(\lambda s)\ \text{CY}$$

Finally, The D^0 of component X in the mixture can be recovered through subtracting the recovered D^0 of Y from the D^0 of the corresponding mixture; which is known as spectrum subtraction [26,27].

$$\therefore D^0 \text{of component X(ax Cx)} = [\text{ax Cx} + a_Y C_Y] - a_Y C_Y$$

The concentrations of X or Y in the mixture was separately calculated via corresponding regression equation representing the absorbance value at their maxima versus their corresponding concentrations.

2.1.2. Absorptivity Centering Technique Via Factorized Ratio Spectrum (ACT-FSR$_{\Delta P}$)

This method can be used to resolve binary mixture of X and Y where the two spectra were completely overlapping lacking of two equal absorbance values for the interfering component all over the

spectrum wavelength range which obstructs the application of the novel method using D^0 spectrum via factorized absorbance difference $FSD^0{}_{\Delta A}$. Factorized ratio spectrum using amplitude difference ($FSR_{\Delta P}$) represents a spectrum with unit difference between the amplitudes of two chosen wavelengths where the interfering component is transformed to a constant thus the amplitude difference all over the curve equals to zero. Thus, any two wavelengths in the overlapped region could be used regards the sensitivity and the accuracy. This factorized spectrum achieved through the division of the ratio spectrum of pure Y (throughout all the measured wavelengths) by the difference in the amplitude values at the chosen wavelengths of Y using X as a divisor

$$\text{Factorized ratio spectrum } (FSR_{\Delta P}) = [A_Y/a_X C_X]\Big/\Big[\Delta A_{Y(\lambda s)}\Big]a_{x(\lambda s)}\Big]Cx = [a_Y C_Y/a_X C_X]\Big/\Big[\Delta a_{Y(\lambda s)} C_Y\Big/a_{x(\lambda s)} Cx\Big]$$

$$\therefore \text{Factorized ratio spectrum } (FSR_{\Delta P}) = \Big[A_Y\Big/a_X \cdot a_{X(\lambda s)}\Big/\Delta a_{Y(\lambda s)}\Big]'$$

The ratio spectrum of Y in the mixture can be recovered by multiplying the recorded amplitude difference value of Y [Δ aY(λs) CY/aX(λs)CX] in the mixture by its corresponding factorized spectrum.

$$\text{Recovered ratio spectrum of Y in the mixture } [aYCY/aX\,CX] = [aY/aX \cdot aX(\lambda s)/\Delta\, aY(\lambda s)\ ]'x\ [\Delta aY(\lambda s)CY/aX(\lambda s)CX]$$

The ratio spectrum of the constant X can be recovered by subtracting the recovered ratio spectrum of Y from ratio spectrum of its corresponding mixture then its value is recorded.

∴Recovered ratio spectrum of *X* [axCx/aX CX'] = [axCx/aXCX' + aYCY/aXCX'] − [aY CY/aX CX]

The concentrations of the proposed drugs were calculated via corresponding regression equations representing the amplitude of Y at the maxima and the amplitude of X (constant) versus their corresponding concentrations.

2.2. Geometric standard addition approach

2.2.1. Ratio H-point Standard Addition Method (RHSAM)

This method involves plotting two straight lines representing the amplitude, using the normalized spectrum of (Y) as the divisor, at two selected wavelengths versus the added concentration (X), where the interfering substance (Y) exhibits the same absorbance, where a intercept (RH point) having ($-C_{RH}$; P_{RH}), C_{RH} is the unknown concentration (*Cx*) and P_{RH} is equivalent to the concentration of interfering component (*Cy*) [13,15,28]. The concentrations of X and Y can be calculated mathematically through the following equations:

$$Cx = (B - A)/(M1 - M2)$$

$$Cy = (M1\,B - M2\,A)/(M1 - M2)$$

where M_1 and M_2 are the slopes of standard addition calibration lines at λ_1 and λ_2, respectively; A and B are the intercepts of the two regressions, such that $A = (X/Y')_1 + (Y/Y')$ and $B = (X/Y')_2 + (Y/Y')$.

2.2.2. Geometrical Induced Amplitude Modulation method (GIAM)

Lotfy et al. [15] presented this method in order to determine minor component in mixture (X + Y) showing severely overlapping spectra with no isoabsorptive point. Starting with the calculation of the absorptivity factor for x and y [29,30]. The ratio amplitude at two wavelengths (*Pf and P2*) were calculated using normalized spectrum of Y as a divisor (*Pf* is the absorptivity factor point). The difference of the ratio spectra amplitudes ΔP (P_f-P_2) and amplitudes (*Pf*) were graphically plotted versus the added concentration of the minor component (X) and both straight lines were extrapolated to cut the concentration axis where both minor component (X) and major one (Y) could be recorded. By computing the geometrical extrapolations of each regression line as follows:

$$\text{Extrapolation} = \text{intercept/slope}$$

$$\therefore \Delta P \text{ extrapolation} = A/_{M1} = Cx(\text{minor})$$

$$Pf \text{ extrapolation} = B/_{M2} = [Cx(\text{minor})\,] + \frac{1}{F}[Cy][Cy] = \Big[B/_{M2} - A/_{M1}\Big] * F$$

where (M1) and (M2) are the slopes of each line which is equal to $\left(ax/_{ay}\right)$ and $\left(\Delta ax/_{ay}\right)$, respectively. (A) and (B) are the intercepts of each line which is equal to $\left[\Delta ax/_{ay} \cdot Cx(\text{minor})\right]$ and $\left[ax/_{ay} \cdot Cx(\text{minor}) + \frac{1}{F} Cy\right]$, respectively. (F) is equivalent to the absorptivity factor $ax/_{ay}$.

3. Experimental

3.1. Apparatus and software

Shimadzu (Japan) UV 1800 double beam UV−Visible spectrophotometer and matched quartz cells (1 cm) were used for absorbance measurements. Built-in Shimadzu UV-Probe 2.32 system software was used for data manipulation.

3.2. Materials and reagents

Aspirin (acetyl salicylic acid) standard (ASP) and metoclopramide standard (MTO) were obtained as kind gift samples from Rameda Company and Sunny pharmaceutical company, Cairo, Egypt, respectively. The purities were tested by the official methods [31] and were found to be 99.58 ± 0.56 and 99.78 ± 0.73, respectively.

Market samples. Migramax sachets were purchased from Lloyd's pharmacy, England. Each sachet contains 1620 mg lysine acetylsalicylate (equivalent to 900 mg acetylsalicylic acid), 10 mg metoclopramide hydrochloride and aspartame (E951). It's manufactured by Zentiva Company, United Kingdom.

Solvents. Spectroscopic analytical grade methanol was supplied from (S Merck, Darmstadt, Germany) and distilled water.

3.3. Standard solutions

Stock solutions of ASP and MTO were prepared in a solvent mixture of methanol: water (50,50 v/v), of concentration 1.0 mg/mL. The use of organic solvent was economized to reduce cost and have a positive impact on environment. Working solutions were diluted from the stock solutions using the same solvent mixture to reach a concentration of 100.0 μg/mL for ASP and 50.0 μg/mL for MTO.

3.4. Spectral manipulation and analysis of synthetic mixtures

3.4.1. For factorized-spectrum approach

3.4.1.1. Spectral manipulation. The D^0 absorption spectra were recorded for the working solutions equivalent to (15−100 μg/mL) and (3−40 μg/mL) of ASP and MTO, respectively, prepared separately in the solvent mixture. Calibration curves were constructed for both ASP and MTO by plotting the absorbance of D^0 maxima (ASP at 276.5 nm and MTO at 276.0 nm) against the corresponding concentration and the regression equations were computed. The absorption spectra of both ASP and MTO were divided by ASP

normalized divisor to get the ratio spectra. Calibration curves were constructed for the ratio spectra of both MTO and ASP by plotting the amplitude (MTO $P_{(max)}$ at 270.5 nm and ASP $P_{(constant)}$ at (275.0–295.0 nm) against the corresponding concentration and the regression equations were computed.

Two factorized spectrum were prepared using the software. The factorized spectrum using absorbance difference ($FSD^0{}_{\Delta A}$) of ASP was prepared by dividing the zero-order (D^0) spectrum of any concentration within linearity range by the value of absorbance difference at the two selected points (280.5–309.5 nm). On the other hand, the factorized ratio spectrum using amplitude difference ($FSR_{\Delta P}$) of MTO was prepared where the ratio spectrum of any concentration within linearity range by the value of MTO, generated by using the normalized divisor of ASP, by the value of amplitude difference at the two selected points (270.5–246.5 nm).

3.4.1.2. Analysis of mixtures. The zero-order absorption spectra (D^0) of the synthetic mixtures were reordered. The calculated absorbance difference in the mixtures' absorbance values at (280.5–309.5 nm) was multiplied by the corresponding factorized spectrum to recover the D^0 of ASP present in the mixtures. The full spectrum of MTO was recovered from each mixture by subtracting the obtained D^0 of ASP. The concentrations of ASP and MTO were calculated using the corresponding regression equation of each drug constructed at its maxima.

On the other hand, the ratio spectra of the synthetic mixtures were calculated by dividing their D^0 by the normalized divisor of ASP. The calculated amplitude difference of the mixtures' at (270.5–246.5 nm) was multiplied by the corresponding factorized amplitude difference spectrum of MTO to recover the full ratio spectrum of MTO present in the mixtures. The ratio spectrum of ASP was recovered from the mixtures by subtracting the obtained ratio spectrum of MTO from the ratio spectrum of its corresponding mixture. The concentrations of ASP and MTO are calculated using the corresponding regression equation of each of their amplitudes (MTO $P_{(max)}$ at 270.5 nm and ASP $P_{(constant)}$ at 275.0–295.0 nm).

3.4.2. For geometric standard addition approach

3.4.2.1. Spectral manipulation. Working solutions were prepared in the previously mentioned solvent mixture in the concentration range of (15.0–100.0 μg/mL) and (3.0–40.0 μg/mL) for ASP and MTO, respectively, and their absorption spectra were measured at (200–400 nm) and divided by the normalized ASP spectrum as a divisor. The ratio amplitudes of ASP and MTO were recorded at (226.5, 246.5 nm) for RHPSAM and (290.0, 309.5 nm) for GIAM and then plotted against the corresponding concentrations of each drug where the regression parameters were computed. Nine synthetic mixtures were prepared in solvent mixture using three levels of concentrations of each drug: (200.0, 400.0, 900.0 μg/mL of ASP) and (10.0, 20.0, 30.0 μg/mL of MTO). For standard addition, one milliliter of each mixture was transferred to a set of 10-mL volumetric flasks followed by the addition of different aliquots of MTO (40.0–290.0 μg) and the volumes were completed to the mark with the same solvent.

3.4.2.2. Analysis of mixtures. The mixtures' spectra were divided by the normalized divisor of ASP. For GIAM, two lines were plotted representing the amplitudes 290.0 nm and ΔP (290.0–309.5 nm) against the added concentrations of MTO for each mixture where the extrapolations for each line was calculated. For RHPSAM, two lines were plotted representing the amplitudes at 226.5 and 246.5, nm against the added concentrations of MTO for each mixture.

3.5. Application to pharmaceutical preparation

Five sachets of Migramax® were mixed and a weight equivalent to one sachet (0.177 mg) was transferred to 100 mL volumetric flask, 50 mL of solvent mixture of methanol: water (50:50 v/v) was added, then the contents of the flask were sonicated for 30 min and the solution was filtered into 100 mL volumetric flask. For factorized spectrum approach, the solution was spiked with 400 μg of MTO standard and completed to the mark with the same solvent; but for geometric standard addition approach, no spiking was required and the solution was completed to the mark directly. Further dilution with the same solvent was performed to obtain working standard solutions where the obtained concentrations were within linearity range. The solutions were to be assayed by referring to the previously mentioned procedures. Standard addition technique was carried out where different known concentrations of pure standard of each drug were added to the pharmaceutical dosage form before proceeding in the previously mentioned methods.

4. Results and discussion

This work presented the application of four spectrophotometric methods applied for the analysis of mixtures containing critical concentration which lies outside the Beer's law. The proposed methods were based on either factorized-spectrum approach via zero order absorption spectrum (ACT-$FSD^0{}_{\Delta A}$) and ratio amplitude difference (ACT-$FSR_{\Delta P}$); or geometric standard-addition via induced amplitude modulation (GIAM) and ratio H-point (RHSAM).

4.1. Factorized-spectrum approach

This approach included the application of absorptivity centering technique via two factorized spectra: zero order absorption spectrum (ACT-FSD^0) and ratio spectrum (ACT-FSR). In order to apply (ACT-FSD^0) method, the D^0 absorption spectra of both drugs were scanned, where the spectrum of ASP (Y) showed different absorbance signals all over the working wavelength range, as shown in Fig. 1, so MTO was marked as the interfering component (X) with equal absorbance at 280.5 and 309.5 nm. The factorized spectrum

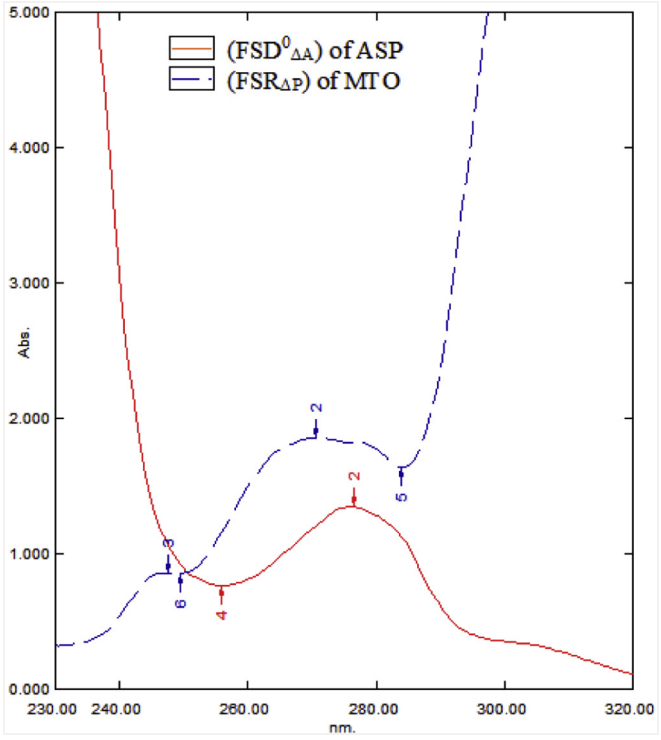

Fig. 2. The absorption factorized spectrum of ASP ($FSD^0{}_{\Delta A}$) at 280.5–309.5 nm and the ratio factorized spectrum of MTO ($FSR_{\Delta P}$) at 270.5–246.5 nm using normalized ASP′ as divisor.

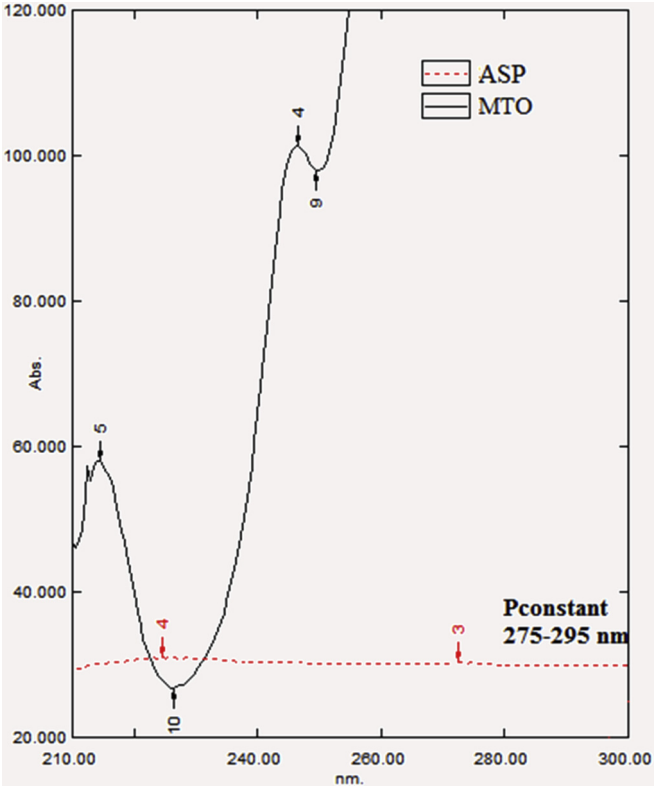

Fig. 3. Ratio spectra of 30 μg/mL of each ASP and MTO using normalized ASP′ as divisor.

of ASP [aY/⊿ aY(λs)]' was prepared using the difference between its absorbance at (280.5–309.5 nm) where MTO absorbance differences equal zero at these selected wavelengths, as shown in Fig. 2. By multiplying the absorbance difference values of each mixture by the ASP factorized spectrum, the zero order (D^0) spectrum of ASP was recovered. While, D^0 spectrum was recovered by subtracting the previously recovered (D^0) spectrum of ASP from the D^0spectrum of the mixture. The concentrations ASP and MTO were calculated using their regression equation representing absorbance at 276.5 nm and 276.0 nm respectively versus their concentrations.

The main advantage of this method (ACT-$FSD^0{}_{\Delta A}$) is the simplicity and minimal manipulation. Complete resolution of binary mixture showing severe overlapped spectra is performed via one manipulation step (multiplying by factorized spectrum). The two components are determined via λmax which increases accuracy, sensitivity and decreases instrumental error. The limiting step for this method is choosing the two wavelengths where the absorbance difference of interfering component equals zero.

On the other hand, if the interfering component exhibits different absorbance signals all over the working wavelength range (absorbance difference does not equal zero) or if determining its absorbance difference is critical, then the application of (ACT-$FSD^0{}_{\Delta A}$) method would be hindered and absorptivity centering technique via factorized ratio spectrum using amplitude difference (ACT-FSR) should be applied. The ASP was marked as interfering substance (X). By dividing the mixtures' spectra by normalized divisor of ASP, in order to eliminate the effect of divisor's choice, the interference of ASP was eliminated by converting it into a constant amplitude all over the wavelength range, as shown in Fig. 3. The ratio amplitude difference of each mixture at the two selected points used in the preparation of the factorized spectrum ($FSR_{\Delta P}$) of MTO (270.5–246.5 nm) were recorded, and then multiplied by ($FSR_{\Delta P}$) of MTO in order to recover the ratio spectrum of MTO present in the mixture, as shown in Fig. 2. By subtracting the obtained ratio spectrum of MTO from the ratio spectrum the mixture, a constant amplitude, equivalent to the concentration of ASP, was obtained all over the wavelength range. The concentrations of ASP and MTO are calculated using the corresponding regression equation of each of their amplitudes $P_{(max)}$ of MTO at 270.5 nm and $P_{(constant)}$ of ASP at (275.0–295.0 nm), as shown in Fig. 3. Nine synthetic mixtures were analyzed using this approach with accepted recovery percentages and standard deviations as listed in Table 1.

Absorptivity centering technique using factorized ratio spectrum (ACT-FSR) could be easily applied for resolving the binary mixtures since the interfering substance was used as a divisor, thus any two points at the ratio spectrum of the drug of interest could be used for getting its factorized ratio spectra without any limitation (unlike ACT-FSD^0) except the contribution of both drugs at the two selected wavelengths. By scanning the D^0 spectra of the drugs of interest, the analyst can decide which method (ACT-FSD^0 or ACT-FSR) to apply depending on the spectrum of the interfering substance.

The factorized –spectrum approach has the advantages of minimum manipulation steps with high accuracy and sensitivity of the recovered spectra of the cited drugs through determination of absorbance or amplitude output signals using λ_{max}. Accordingly, the critical concentrations of MTO could be easily estimated through single standard spiking to the dosage form preparation.

4.2. Geometric standard addition approach

This approach included the application of two methods based on standard addition namely, ratio H-point standard addition method (RHPSAM) and geometrical induced amplitude modulation

Table 1
Experimental results for the analysis of synthetic mixtures using factorized spectrum approach.

Mixture	Taken[a]		(ACT-$FSD^0{}_{\Delta A}$)				(ACT-$FSR_{\Delta P}$)			
			ASP		MTO		ASP		MTO	
	ASP	MTO	Found[a]	R%	Found[a]	R%	Found[a]	R%	Found[a]	R%
1	20	5	19.82	99.10	5.08	101.60	20.05	100.25	4.92	98.40
2	20	10	19.50	97.50	9.96	99.60	20.14	100.70	10.14	101.40
3	20	20	20.30	101.50	20.14	100.70	19.99	99.95	20.14	100.70
4	40	5	39.80	99.50	4.95	99.00	40.80	102.00	5.08	101.60
5	40	10	39.40	98.50	9.98	99.80	39.52	98.80	10.09	100.90
6	40	20	40.09	100.23	19.96	99.80	39.66	99.15	20.12	100.60
7[b]	90	5	91.60	101.78	5.02	100.40	89.85	99.83	5.06	101.20
8	90	10	90.80	100.89	10.12	101.20	90.55	100.61	10.14	101.40
9	90	20	89.52	99.47	20.18	100.90	91.30	101.44	20.19	100.95
				99.83		100.33		100.30		100.79
	Mean ± SD			1.41		0.84		1.02		0.96

[a] In μg/mL, average of three experiments.
[b] Ratio present in pharmaceutical dosage form after spiking with (4 μg/mL) of MTO.

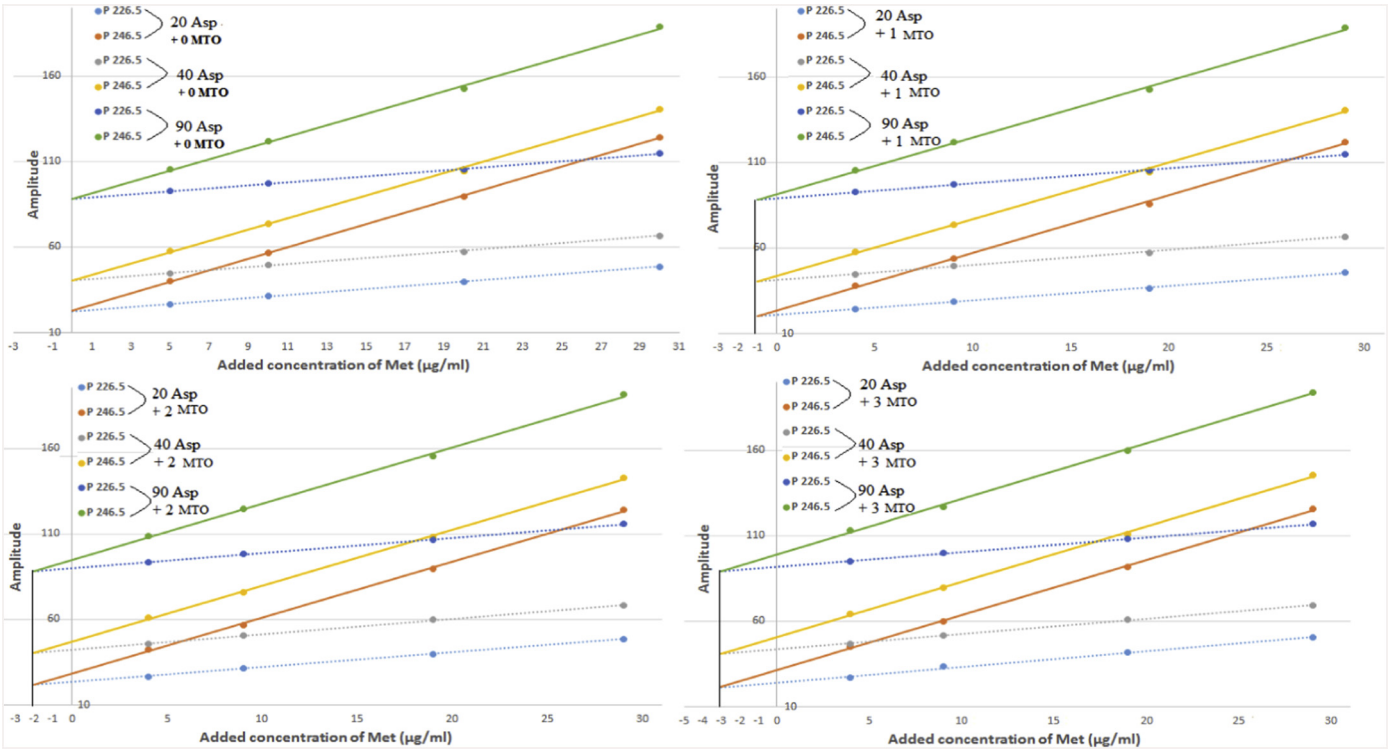

Fig. 4. Plots of ratio H-point standard addition between amplitudes at 226.5 and 246.5 against the added MTO concentrations for sets of 3 synthetic mixtures containing same concentration of MTO but different concentrations of ASP.

(GIAM), where the relation between the standard added and signal response was geometrically presented.

In order to apply the (RHPSAM) method, ASP was marked as the interfering substance due to its relatively higher amplitude signals than MTO which hindered the later accurate quantitation. The constant ratio amplitude, corresponding to ASP, was calculated in the ratio spectra of the mixtures through division by ASP′ normalized divisor, then generating H-point by standard addition of different concentrations of standard MTO to determine the proportionality constants corresponding to the critical concentrations of MTO present in mixtures. The amplitudes at 226.5 and 246.5 nm were selected due to high signal response, and then were plotted against the added concentrations of MTO to obtain two standard addition calibration lines for each mixture, where the two lines were extrapolated to intercept at the RH point ($-C_{RH}$; P_{RH}), as shown in Figs. 4 and 5.The X-coordinate ($-C_{RH}$) is equivalent to MTO critical concentration and Y coordinate (P_{RH}) is the concentration of major component ASP. Accordingly, the concentrations of both components were simultaneously determined using the geometrical computation of both X and Y coordinates through the following equations:

$$C_{MTO} = (B - A)/M1 - M2$$

$$C_{ASP} = M1B - M2A/(M1 - M2)$$

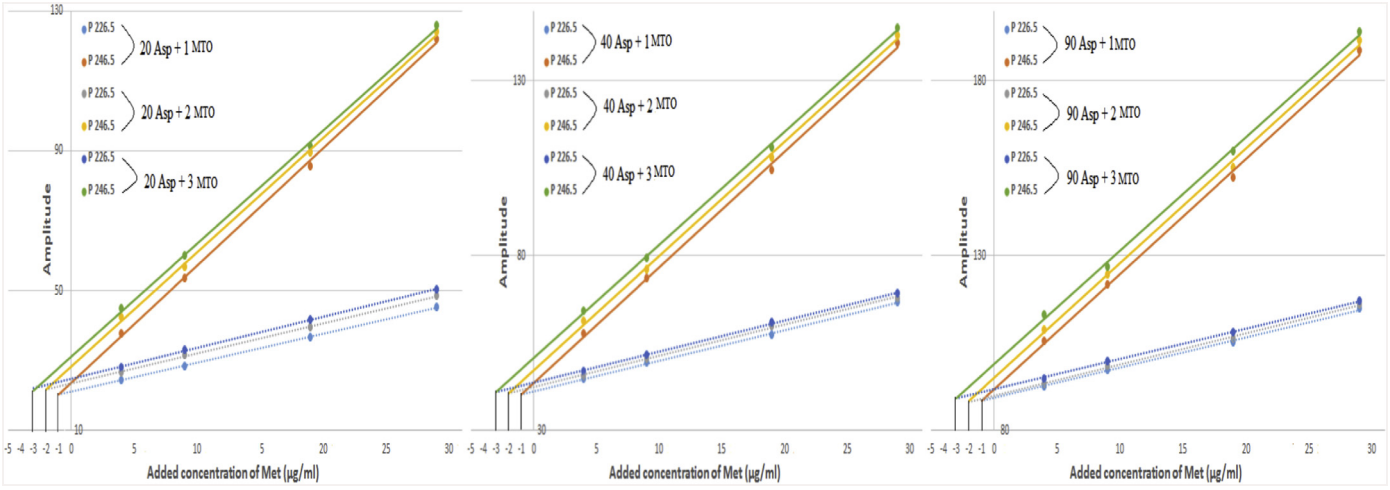

Fig. 5. Plots of ratio H-point standard addition between amplitudes at 226.5 and 246.5 against the added MTO concentrations for sets of 3 synthetic mixtures containing same concentration of ASP but different concentrations of MTO.

Table 2
Experimental results for the analysis of synthetic mixtures using geometric standard addition approach.

Mixture no.	Taken[a]		RHPSAM				GIAM			
	ASP	MTO	Regression equation[b,c]	r	Found[a] ASP	Found[a] MTO	Regression equation[b]	r	Found[a] ASP	Found[a] MTO
1	20	1	$P_{226.5} = 0.8364x + 21.035$ $P_{246.5} = 3.3578x + 23.564$	0.9993 0.9989	20.19	1.00	$P_{290} = 10.623C + 32.277$ $\Delta P = -33.142C - 33.903$	0.9991 0.9989	20.15	1.02
2	20	2	$P_{226.5} = 0.8394x + 21.803$ $P_{246.5} = 3.3597x + 26.88$	0.9993 0.9988	20.11	2.01	$P_{290} = 11.027C + 44.586$ $\Delta P = -33.024C - 66.888$	0.9977 0.9987	20.18	2.02
3	20	3	$P_{226.5} = 0.8295x + 22.901$ $P_{246.5} = -33.142C - 33.903$	0.9987 0.9992	20.51	2.98	$P_{290} = 11.315C + 56.942$ $\Delta P = -32.376C - 96.512$	0.9987 0.9990	20.51	2.98
4	40	1	$P_{226.5} = 0.8699x + 41.3$ $P_{246.5} = 3.3046x + 43.721$	0.9988 0.9984	40.43	0.99	$P_{290} = 10.491C + 52.657$ $\Delta P = -33.381C - 32.709$	0.9994 0.9988	40.39	0.98
5	40	2	$P_{226.5} = 0.8986x + 42.321$ $P_{246.5} = 3.2753x + 47.102$	0.9990 0.9980	40.5	2.051	$P_{290} = 10.671C + 64.264$ $\Delta P = -33.099C - 67.749$	0.9998 0.9996	39.75	2.05
6	40	3	$P_{226.5} = 0.8922x + 43.594$ $P_{246.5} = 3.2397x + 50.67$	0.9990 0.9995	40.9	3.01	$P_{290} = 10.746C + 74.627$ $\Delta P = 33.271C - 98.864$	0.9999 0.9996	39.73	2.97
7[d]	90	1	$P_{226.5} = 0.8718x + 89.284$ $P_{246.5} = 3.3057x + 91.732$	0.9988 0.9986	88.40	1.00	$P_{290} = 10.284C + 104.36$ $\Delta P = = -33.279C - 32.717$	0.9987 0.9985	91.65	0.98
8	90	2	$P_{226.5} = 0.8844x + 90.088$ $P_{246.5} = 3.2924x + 95.007$	0.9979 0.9982	88.28	2.04	$P_{290} = 10.461C + 116.97$ $\Delta P = -33.136C - 65.932$	0.9994 0.9977	91.92	1.99
9	90	3	$P_{226.5} = 0.8682x + 91.089$ $P_{246.5} = 3.3108x + 98.401$	0.9986 0.9990	88.49	2.99	$P_{290} = 10.78C + 130.61$ $\Delta P = -32.915C - 100.54$	0.9988 0.9976	90.61	3.05
Mean R% ± SD					100.36 ± 1.73	100.38 ± 1.18			100.95 ± 1.12	100.11 ± 1.72

[a] In μg/mL, average of three experiments.
[b] In regression equations 'C' is the added concentration of MTO and ΔP is difference in amplitudes at (290−30.9.5) nm.
[c] Ratio present in pharmaceutical dosage form.

where M_1 and M_2 are the slopes of the standard addition calibration lines obtained on applying the RHPSAM at 226.5 and 246.5 nm, respectively; A and B are the intercepts of the two calibration lines, respectively. The calculations for each synthetic mixture were listed in Table 2.

The RHPSAM could be applied for the simultaneous determination of both components (major and minor) at maximum amplitude signals in the absence of any constrains in choosing the two specific wavelengths, as the constant values generated in the ratio curves are extended along the ratio spectra.

The geometrical induced amplitude modulation (GIAM) method could be applied for a mixture of a severely overlapping spectra of two components which exhibit large difference in their absorptivities and no isoabsorpative point. To apply this method, absorptivity factor point (λ_F) should be selected; where pairs of different concentrations of the two components show a crossing

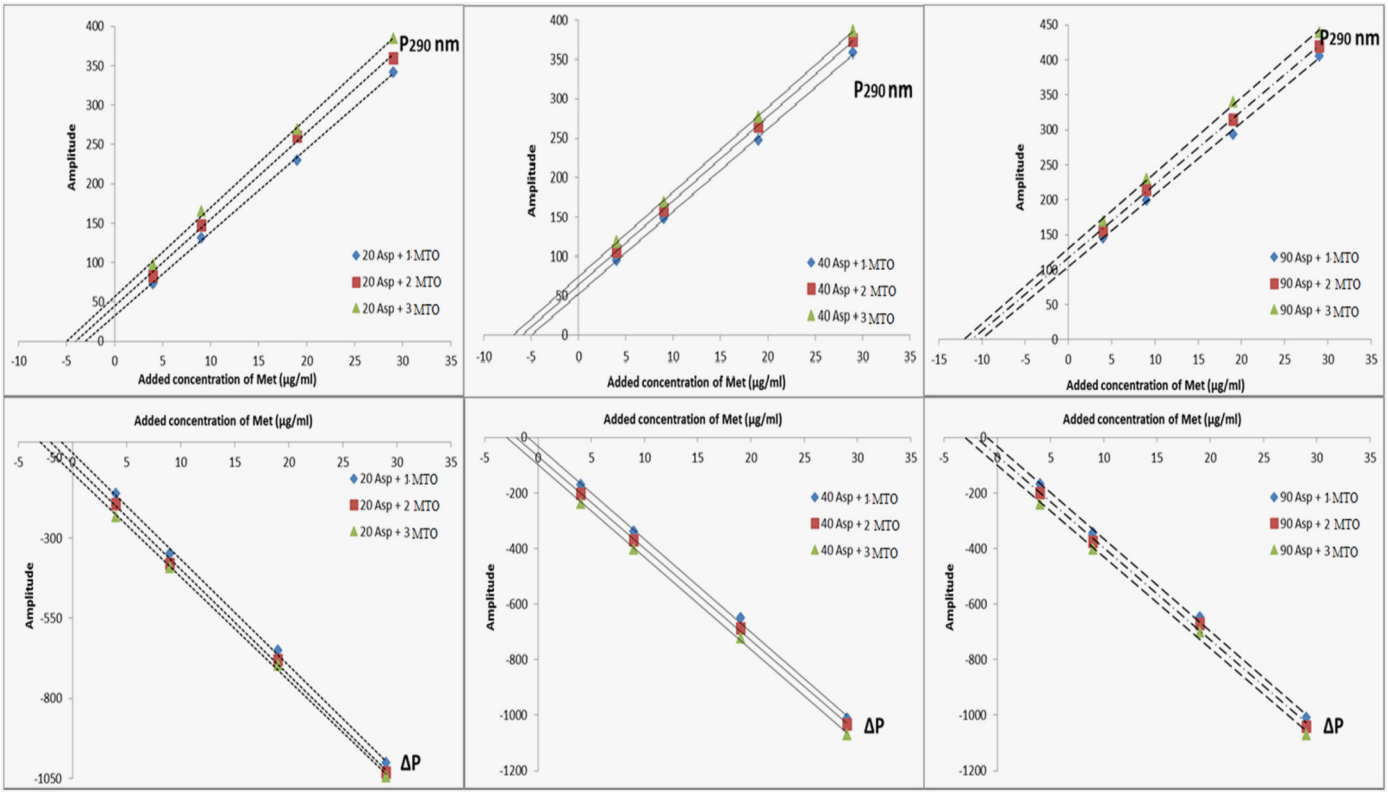

Fig. 6. Plots of geometrical induced amplitude modulation (GIAM) between amplitudes at absorptivity factor point (P_{290}) and amplitude differences ($\Delta P_{290-309.5}$) against the added MTO concentrations for different concentrations of synthetic mixtures [ASP + MTO].

Table 3
Application of the proposed spectrophotometric approaches for the analysis of the pharmaceutical formulation.

Methods	ASP		MTO	
	Found[c] Recovery% ± S.D.	Pure added[d] Recovery% ± S.D.	Found[c] Recovery% ± S.D.	Pure added[d] Recovery% ± S.D.
ACT-FSD$^{0}_{\Delta A}$[a]	100.52 ± 0.85	100.23 ± 0.66	101.25 ± 1.75	99.63 ± 1.63
ACT-FSR$_{\Delta P}$[a]	101.23 ± 0.77	100.56 ± 0.91	101.55 ± 1.64	100.22 ± 1.22
RHPSAM[b]	101.15 ± 0.66	99.25 ± 0.43	100.55 ± 0.84	99.66 ± 0.67
GIAM[b]	100.98 ± 0.79	100.25 ± 0.52	100.25 ± 0.656	99.63 ± 0.82

[a] ASP claimed to be 90 μg/mL and MTO to be 1 μg/mL (after subtraction of spiked MTO concentration equivalent to 4 μg/mL).
[b] ASP claimed to be 90 μg/mL and MTO to be 1 μg/mL.
[c] Average of six experiments.
[d] Average of three experiments (pure added equivalent to 20,35,50 μg/mL of MTO and 5, 8, 10 μg/mL of ASP).

point where absorptivities are not equal and absorptivity factor (F) is calculated to be the inverse of the ratio of the used concentrations. The absorptivity factor point (λ_F) for ASP and MTO was selected at 290.0 nm where the absorptivity factor (F) was calculated to be equal to 10 ($a_{ASP}/a_{MTO=}$ F = 10) as shown in Fig. 1.

The ratio spectra obtained from the standard addition procedure and spectral manipulation procedure of RHPSAM were used. The amplitudes at absorptivity factor point (P_F) 290.0 nm and the amplitude differences ΔP (290.0–309.5 nm) were plotted against the added concentrations of MTO for each mixture where the extrapolation for each line was calculated, as shown in Fig. 6. By computing the geometrical extrapolations, the concentrations of MTO and ASP can be directly calculated through the following equations:

$$C_{MTO} = A/M1$$

$$C_{ASP} = \left[B/M2\right] - \left[A/M1\right]*10$$

where A, B are the intercepts; M_1 and M_2 are the slopes of the standard addition calibration lines obtained at ($\Delta P_{290.0-309.5}$) and ($P_{f\ 290}$), respectively. The calculations for each synthetic mixture were listed in Table 2. This method can be applied as an alternative to RHPSAM as it requires the same manipulation but with much simpler geometric computation.

Regarding the geometric standard-addition approach, it was found that the normalized divisor used in the previous methods simplified the signal/noise ratio and eliminated the factor of the divisor's choice where the recorded amplitudes were directly modulated to concentrations. This approach suffers from multiple manipulation steps (practical preparations and calculations), but it reflected the importance of applying standard addition, and discarding the spiking process of a single sample (as performed in the first approach) which positively affected the obtained results. The proposed approaches were successfully applied for the determination of the laboratory prepared mixtures as shown in Tables 1 and 2.

Both approaches were applied for the analysis of the pharmaceutical dosage form. For factorized spectrum approach, spiking with standard MTO was required to reach linearity range of the proposed methods, where the final concentrations of ASP and MTO of the prepared working solutions where the concentration were 90.0 μg/mL and 5.0 μg/mL respectively, after spiking with an amount of 4.0 μg/mL of standard MTO. On the other hand, there was no need for spiking in the geometric standard addition approach, so the final concentrations of ASP and MTO of the prepared working solutions where the concentration were 90.0 μg/mL and 1.0 μg/mL respectively. The results for the analysis of pharmaceutical preparation and standard addition techniques is listed in Table 3, which proves the accuracy of the proposed methods and the elimination of the interference by excipients.

5. Method validation and statistical analysis

The two proposed approaches were validated in compliance with the ICH guideline [32] including: linearity, range, accuracy, repeatability, inter-day precision, specificity and robustness. The data shown in Tables 4 proved that the approaches were accurate, precise and robust over the mentioned linearity range, while Tables 1 and 2 proved the specificity of the proposed approaches.

Table 5 showed the calculated F values for the statistical comparison of the results obtained by the proposed methods and official ones. Table 6 showed one-way ANOVA statistical comparison of the results obtained by the proposed approaches when applied to synthetic mixtures with different concentrations. The results from both tables showed that there was no significant difference

Table 4
Assay parameters and validation sheet for the proposed spectrophotometric approaches.

Parameters		ASP					MTO					
Wavelength (nm)	D_0 276.5	P const 275–295	P 226.5	P 246.5	P290	P 309.5	D_0 276	P 270.5	P 226.5	P 246.5	P 290	P 309.5
Range[a] (μg/mL)	15–100				20–100		3–40					
Slope	0.0066	1.0096	0.9986	1.0036	1.0254	1.0321	0.0487	6.9728	1.1201	3.2753	9.4579	41.042
Intercept	0.0015	−0.0608	−0.8025	−0.0214	−0.0341	0.4123	0.0063	10.425	2.4263	3.9188	6.4475	11.299
Correlation coeff. (r)	0.9997	0.9997	0.9996	0.9998	0.9997	0.9995	0.9998	0.9998	0.9998	0.9998	0.9997	0.9995
Mean[a]	100.20	99.97	100.94	100.96	100.80	100.70	99.94	99.91	99.91	99.73	99.70	99.65
RSD%	0.796	0.716	0.716	0.759	0.953	1.023	1.227	0.758	0.950	1.459	1.774	1.626
Accuracy[a,b]	101.13 ± 0.98	99.98 ± 1.02	99.87 ± 1.32	99.65 ± 0.69	100.25 ± 0.98	100.99 ± 0.85	100.98 ± 1.03	99.74 ± 0.72	99.06 ± 0.81	100.11 ± 0.86	100.93 ± 1.35	99.96 ± 1.32
Repeatability[a,c]	0.887	1.147	1.154	0.789	0.887	0.841	0.685	0.531	0.633	0.752	1.124	1.120
Inter-day precision [a,c]	0.963	1.229	1.235	1.023	1.102	0.963	0.778	0.741	0.619	0.888	1.241	1.230
Robustness[a,c,d]	1.012	1.003	1.663	0.996	1.003	0.852	0.736	0.635	0.790	0.761	0.687	0.932

[a] Average of three experiment.
[b] Mean ± standard deviation of 3 concentrations of each drug.
[c] Relative standard deviation of 3 concentrations of each drug.
[d] Robustness were checked by testing the effect of solvent (45, 55, 60% methanol).

Table 5
Statistical comparison between the results obtained by the proposed method and the official BP methods for the determination of ASP and MTO in pure powder form.

	ASP							MTO						
	Factorized spectrum		Geometric standard addition				Official method[a]	Factorized spectrum		Geometric standard addition				Official method[a]
	D_0 276.5	P const 275–295	P 226.5	P 246.5	P 290	P 309.5		D_0 276	P 270.5	P 226.5	P 246.5	P 290	P 309.5	
Mean %	100.20	99.97	100.94	100.96	100.80	100.70	99.58	99.94	99.91	99.91	99.73	99.70	99.65	99.78
Variance	0.6334	0.5132	0.5125	0.5764	0.9081	1.0465	0.3170	1.5061	0.5746	0.9016	2.1285	3.1456	2.6430	0.5270
SEM[b]	0.325	0.271	0.271	0.310	0.389	0.418	0.252	0.464	0.309	0.388	0.596	0.724	0.664	0.325
n	6	7	7	6	6	6	5	7	6	6	6	6	6	5
F value[c]	1.998	1.619	1.617	1.818	2.865	3.301		2.858	1.090	1.711	4.039	5.969	5.015	

[a] BP methods for ASP is acid-base titration method, while for MTO it is potentiometric non-aqueous titration method.
[b] SEM: Standard error of mean.
[c] The corresponding tabulated values of *F* equals to 6.256 (for $n = 6$) and 6.163 (for $n = 7$) at $P = 0.05$.

Table 6
Results of one-way ANOVA for comparison of the proposed methods for the determination of ASP and MTO in synthetic mixtures.

Source of variation		Degree of freedom	Sum of squares	Mean square	*F* value[a]	*P* value[a]	*F* critical[a]
ASP	Between columns	3	5.654	1.885	1.038	0.389	2.015
	Within columns	32	58.123	1.816			
	Total	35	63.777				
MTO	Between columns	3	2.196	0.732			
	Within columns	32	47.821	1.494	0.490	0.692	2.015
	Total	35	50.017				

[a] There was no significance difference between the methods using one-way ANOVA at $P < 0.05$.

between the proposed approaches with respect to accuracy and precision.

6. Conclusion

This work presented two spectrophotometric approaches for the treatment and amplification of the spectral signals exhibited by the critical concentrations of MTO in its pharmaceutical preparation with ASP. A novel approach utilizing the factorized spectrum was introduced namely, absorptivity centering technique via factorized zero order absorption spectrum (ACT-FSD$^0{}_{\Delta A}$) and via factorized ratio spectrum (ACT-FSR$_{\Delta P}$). The obtained results were compared to those of the methods based on geometrical standard-addition approach which were: ratio H-point standard addition method (RHPSAM) and geometrical induced amplitude modulation (GIAM). All the proposed methods showed acceptable results regarding accuracy and precision. The advantages and limitations of the proposed approaches were discussed where the factorized spectrum approach transcended through its simplicity.

References

[1] H. Trabelsi, F. Raouafi, M. Limam, K. Bouzouita, Derivative spectrophotometric determination of droperidol in presence of parabens, J. Pharm. Biomed. Anal. 29 (2002) 239–245.
[2] E. Dinc, A comparative study of the ratio spectra derivative spectrophotometry, Vierordt's method and high-performance liquid chromatography applied to the simultaneous analysis of caffeine and paracetamol in tablets, J. Pharm. Biomed. Anal. 21 (1999) 723–730.
[3] H.M. Lotfy, S.S. Saleh, N.Y. Hassan, S.M. Elgizawy, A comparative study of the novel ratio difference method versus conventional spectrophotometric techniques for the analysis of binary mixture with overlapped spectra, Am. J. Anal. Chem. 3 (2012) 761–769.
[4] H. Salem, H.M. Lotfy, N.Y. Hassan, M.B. El-Zeiny, S.S. Saleh, A comparative study of different aspects of manipulating ratio spectra applied for ternary mixtures: derivative spectrophotometry versus wavelet transform, Spectrochim. Acta, Part A 135 (2015) 1002–1010.
[5] H.W. Darwish, S.A. Hassan, M.Y. Salem, B.A. El-Zeany, Three different methods for determination of binary mixture of amlodipine and atorvastatin using dual wavelength spectrophotometry, Spectrochim. Acta, Part A 104 (2013) 70–76.
[6] H.M. Lotfy, S.S. Saleh, N.Y. Hassan, H. Salem, Novel two wavelength spectrophotometric methods for simultaneous determination of binary mixtures with severely overlapping spectra, Spectrochim. Acta, Part A 136 (2015) 1786–1796.
[7] M. Givianrad, M. Saber-Tehrani, P. Aberoomand-Azar, M. Mohagheghian, H-point standard additions method for simultaneous determination of sulfamethoxazole and trimethoprim in pharmaceutical formulations and biological fluids with simultaneous addition of two analytes, Spectrochim. Acta, Part A 78 (2011) 1196–1200.
[8] E. Hund, D.L. Massart, J. Smeyers-Verbeke, Evaluation of the H-point standard additions method (HPSAM) and the generalized H-point standard additions method (GHPSAM) for the UV-analysis of two-component mixtures, J. Pharm. Biomed. Anal. 21 (1999) 23–42.
[9] Y.-Z. Liang, O.M. Kvalheim, R. Manne, White, grey and black multicomponent systems: a classification of mixture problems and methods for their quantitative analysis, Chemom. Intell. Lab. Syst. 18 (1993) 235–250.
[10] D. Harvey, Modern Analytical Chemistry, McGraw-Hill, United States of America, 2000.
[11] P. Campins-Falco, J. Verdu-Andres, F. Bosch-Reig, H-point standard additions method for resolution of binary mixtures with simultaneous addition of both analytes, Anal. Chim. Acta 315 (1995) 267–278.
[12] H. Abdollahi, Simultaneous spectrophotometric determination of chromium(VI) and iron(III) with chromogenic mixed reagents by H-point standard addition method and partial least squares regression, Anal. Chim. Acta 442 (2001) 327–336.
[13] A.M. Yehia, Development and validation of new spectrophotometric ratio H-point standard addition method and application to gastrointestinal acting drugs mixtures, Spectrochim. Acta, Part A 109 (2013) 193–200.
[14] N.T. Lamie, A.M. Yehia, Development of normalized spectra manipulating spectrophotometric methods for simultaneous determination of Dimenhydrinate and Cinnarizine binary mixture, Spectrochim. Acta, Part A 150 (2015) 142–150.
[15] H.M. Lotfy, S.S. Saleh, N.Y. Hassan, H. Salem, Computation of geometric representation of novel spectrophotometric methods used for the analysis of minor components in pharmaceutical preparations, Spectrochim. Acta, Part A 151 (2015) 628–643.
[16] H.M. Lotfy, Y.R. Omran, Novel absorptivity centering method utilizing normalized and factorized spectra for analysis of mixtures with overlapping spectra in different matrices using built-in spectrophotometer software, Spectrochim. Acta, Part A 200 (2018) 167–178.
[17] H.M. Lotfy, S.S. Saleh, Investigating advanced approaches based on Iso-absorptivity coefficient in unresolved spectral signals of binary mixtures, J Anal Methods Chem 2019 (2019).
[18] E.G. Clarke, A.C. Moffat, Clarke's Isolation and Identification of Drugs: In Pharmaceuticals, Body Fluids and Post Mortem Material, 2nd ed., Pharmaceutical Press, London, 1986.
[19] B.G. Katzung, A.J. Trevor, Basic & Clinical Pharmacology, McGraw-Hill, United States, 2009.
[20] K.D. Tripathi, Essentials of Medical Pharmacology, 6th ed., Jaypee Brothers, New Delhi, 2008.
[21] L. Brunton, K. Parker, D. Blumenthal, L. Buxton, Goodman &Gilman's: Manual of Pharmacology and Therapeutics, the McGraw-Hill Inc., USA, 2008.
[22] S.C. Sweetman, Martindale" the Complete Drug Reference", 34 ed.,

Pharmaceutical Press, London, 2005.
[23] F.F. Belal, M.K.S. El-Din, M.M. Tolba, H. Elmansi, Derivative spectrophotometric and liquid chromatographic methods for the simultaneous determination of metoclopramide hydrochloride and aspirin in pharmaceuticals, J. Chromatogr. Sci. 52 (2014) 1224−1232.
[24] H. Elmansi, S.A.E.A. Mohamed, M.E. Fathy, Simultaneous determination of metoclopramide and aspirin by spectrofluorimetric technique: application to pharmaceutical formulations and human plasma, Anal. Methods 8 (2016) 1281−1292.
[25] H.M. Lotfy, S.S. Saleh, Investigating advanced approaches based on Iso-absorptivity coefficient in unresolved spectral signals of binary mixtures, J Anal Methods Chem 2019 (2019) 1−10.
[26] A. Samir, H.M. Lotfy, H. Salem, M. Abdelkawy, Development and validation of simultaneous spectrophotometric and TLC-spectrodensitometric methods for determination of beclomethasone dipropionate and salbutamol in combined dosage form, Spectrochim. Acta, Part A 128 (2014) 127−136.
[27] H.M. Lotfy, S.S. Saleh, Testing the purity of spectral profiles: finger-print resolution of complex matrices and extraction of absorbance signals, Spectrochim. Acta, Part A 205 (2018) 160−169.
[28] A.M. Yehia, M.K. Abd El-Rahman, Application of normalized spectra in resolving a challenging Orphenadrine and paracetamol binary mixture, Spectrochim. Acta, Part A 138 (2015) 21−30.
[29] A. Samir, H. Salem, M. Abdelkawy, New developed spectrophotometric method for simultaneous determination of salmeterol xinafoate and fluticasone propionate in bulk powder and Seritide diskus inhalation, Bull. Fac. Pharm. Cairo Uni. 50 (2012) 121−126.
[30] S.S. Saleh, H.M. Lotfy, N.Y. Hassan, S.M. Elgizawy, A comparative study of validated spectrophotometric and TLC- spectrodensitometric methods for the determination of sodium cromoglicate and fluorometholone in ophthalmic solution, Saudi Pharm. J. 21 (2013) 411−421.
[31] British Pharmacopoeia vol. Volume I & II, 2013. London.
[32] Q.B. International Conference on Harmonization (ICH), Validation of Analytical Procedures: Methodology, Federal Register vol. 62, 1997.