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An application of derivative and continuous wavelet transforms to the overlapping ratio spectra for the quantitative multiresolution of a ternary mixture of paracetamol, acetylsalicylic acid and caffeine in tablets

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Abstract

Quantitative multiresolution of tablets and ternary mixtures of paracetamol (**PAR**), acetylsalicylic acid (**ASP**) and caffeine (**CAF**) having strongly overlapping spectra was accomplished by two graphical transform methods as ratio spectra first derivative-zero crossing and ratio spectra-continuous wavelet transform-zero crossing (ratio spectra **CWT**-zero crossing) methods. In this study, ratio spectra derivative-zero crossing and ratio spectra **CWT**-zero crossing methods are based on the use of transformed signals of the ratio spectra and their calibration graphs were obtained by measuring the dA/dλ and **CWT** amplitudes of the ratio spectra corresponding to zero crossing points. For the comparison purpose, **PLS** calibration method was applied to predict the content of the same mixtures containing the subject active compounds. The obtained calibrations were tested by using the synthetic mixtures and standard addition technique and they applied to the simultaneous determination of **PAR**, **ASP** and **CAF** in commercial pharmaceutical preparation. The obtained results were statistically compared with each other as well as those obtained by HPLC method and they showed good agreement.

Keywords: Continuous wavelet transform; Overlapping ratio spectra; Determination; Paracetamol; Acetylsalicylic acid; Caffeine

1. Introduction

One of the purposes of the analytical chemistry is to improve the ability of the analytical methods used in various areas such as medicinal chemistry, clinical chemistry, forensic chemistry, and environmental chemistry. In recent years, although the existing modern analytical instruments provide us various advantages such as lower detection limit, the working possibility in the small amount of samples, ease of processes and shorter determination time scale, the analysis methods based on these instruments may not give better resolution of multi-mixtures of analytes. Therefore, analytical chemists try to develop new approaches such as application of new mathematical algorithms to known analytical methods and usage of known combined methods in some cases. Moreover a sophisticated instrumentation combining or improving existing instruments were used in the analytical chemistry and related areas to increase the precision, the accuracy and the reliability of the analysis.

Simultaneous quantitative analysis of pharmaceuticals and biomedical liquids containing multi-active compounds is difficult to perform by classical spectrophotometeric method due to overlapping spectra. In the analysis of multi-mixtures the method needs a separation or extraction procedure. Even if several techniques (HPLC and capillary electrophoresis, etc.) using sophisticated equipments allow the separation and determination of the content of samples, these techniques bring high cost and time consumption. One way of elimination of these drawbacks is application of numerical and graphical techniques to original absorption spectra. The use of spectrophotometric

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techniques combined with mathematical algorithms and wavelet transform has brought a new, fast and and easy to apply methodology for the determination of analytes in samples. Some approaches were explained in the following paragraphs.

Chemometrics techniques known as numerical techniques are useful for the spectrophotometric resolution of complex mixtures of analytes without needing a priory separation or extraction. Although both **PCR** and **PLS** give successful results, they have several disadvantages including abstract mathematical theory and usage of various software.

Berzas Nevado and Guibertau Cabanillas developed ratio spectra derivative-zero crossing method to ternary mixture analysis [1]. This method contains the application of derivative technique to the ratio spectra.

The analytical data started to be investigated with the wavelet transform [2,3]. Recently **CWT** technique with zero crossing point [4,5] are started to use for the simultaneous determination of compounds in pharmaceutical samples. This method is based on the integral transformation of the absorbance data vectors and followed the construction of calibration graphs by measuring **CWT** amplitudes corresponding to zero crossing points. All the applications revealed that these graphical approaches are suitable to the analysis of multi-component mixtures in the presence of the overlapping spectra.

Commercial excedrine migraine tablets containing paracetamol (**PAR**), acetylsalicylic acid (**ASP**) and caffeine (**CAF**) in the theme of this work are used for the temporary relief of pain associated with migraine headaches. In the literature, the simultaneous determination of **PAR**, **ASP** and **CAF** in commercial tablet formulations was carried out by HPLC [6] and HPTLC [7], UV spectrophotometric flow-through multiparameter sensors [8] and a flow-through based on the integration of the retention and UV detection of the analytes on a solid support (C_{18}) bonded beads packed in the flow cell [9].

In this study a new combined method was developed for the resolution of ternary mixture containing **PAR**, **ASP** and **CAF** without any separation step. It contains the application of **CWT** to overlapping ratio spectra data vectors. The calibration graphs were obtained by using the **CWT** amplitudes corresponding to zero crossing points of **CWT** coefficients spectra. This approach was called as ratio spectra **CWT**-zero crossing method. It was observed that ratio spectra **CWT**-zero crossing method successfully provided the simultaneous determination of three active compounds in a simple, fast and inexpensive way. Ratio spectra derivative-zero crossing and **PLS** methods as other different alternatives were applied to the quantitative analysis of the samples of **PAR**, **ASP** and **CAF**. In the applications of these three methods to the synthetic ternary mixtures and pharmaceutical tablets, successful results were obtained. The results of the graphical and numerical methods were valid by using HPLC method.

2. Theoretical aspect

In general case the transformation of original absorption spectra was widely used in the analytical chemistry for solving binary mixtures of analytes. This method, which is called derivative spectrophotometry, is based on the derivation of absorption spectra. Derivative spectrophotometry was applied to the ratio spectra for the resolution of ternary mixtures [1] and it called as “*ratio spectra derivative-zero crossing method*”. In our case, **CWT** method was also subjected to the ratio spectra data vector for the same aim and it expressed as “*ratio spectra **CWT**-zero crossing method*”. The theoretical content of the methods was briefly explained in the following.

2.1. Ratio spectra

If a ternary mixture of three analytes, X, Y and Z is considered and if the absorbance value of this ternary mixture is measured at λ_i, the following equation can be written as

$$\mathrm{Am}_{\lambda_i} = \alpha_{X,\lambda_i} C_X + \beta_{Y,\lambda_i} C_Y + \gamma_{Z,\lambda_i} C_Z \tag{1}$$

where Am_{λ_i} is the absorbance of the ternary mixture at wavelength λ_i, and the coefficients α_{X,λ_i}, β_{Y,λ_i} and γ_{Z,λ_i} are absorptivities of X, Y and Z compounds. C_X, C_Y and C_Z represent the concentrations of compounds.

If Eq. (1) is divided by the spectrum of a standard solution (C_X^0) of one of compounds in ternary mixture, the Eq. (1) becomes:

$$\frac{\mathrm{Am}_{\lambda_i}}{\alpha_{X,\lambda_i} C_X^0} = \frac{\alpha_{\lambda_i} C_X}{\alpha_{X,\lambda_i} C_X^0} + \frac{\beta_{\lambda_i} C_Y}{\alpha_{X,\lambda_i} C_X^0} + \frac{\gamma_{\lambda_i} C_Z}{\alpha_{X,\lambda_i} C_X^0} \tag{2}$$

2.2. Ratio spectra derivative-zero crossing method

If the first derivative of Eq. (2) is taken, then we obtain the following:

$$\frac{\mathrm{d}}{\mathrm{d}\lambda}\left[\frac{\mathrm{Am}_{\lambda_i}}{\alpha_{X,\lambda_i} C_X^0}\right] = \frac{\mathrm{d}}{\mathrm{d}\lambda}\left[\frac{\beta_{\lambda_i}}{\alpha_{X,\lambda_i}}\right]\frac{C_Y}{C_X^0} + \frac{\mathrm{d}}{\mathrm{d}\lambda}\left[\frac{\gamma_{\lambda_i}}{\alpha_{X,\lambda_i}}\right]\frac{C_Z}{C_X^0} \tag{3}$$

If in addition, $\mathrm{d}/\mathrm{d}\lambda[\beta_{\lambda_i}/\alpha_{X,\lambda_i}]C_Y/C_X^0$ is equal to zero, for a given point corresponding to the wavelength λ_i, then Eq. (3) would be obtained as:

$$\frac{\mathrm{d}}{\mathrm{d}\lambda}\left[\frac{\mathrm{Am}_{\lambda_i}}{\alpha_{X,\lambda_i} C_X^0}\right] = \frac{\mathrm{d}}{\mathrm{d}\lambda}\left[\frac{\gamma_{\lambda_i}}{\alpha_{X,\lambda_i}}\right]\frac{C_Z}{C_X^0} \tag{4}$$

Eq. (4) shows that the derivative of the ratio spectra of ternary mixture is dependent only on the concentration of C_Z and C_X^0 and is independent on the concentrations, C_X and C_Y of other compounds in the ternary mixture. The calibration graphs can be obtained by plotting $\mathrm{d}/\mathrm{d}\lambda[\mathrm{Am}_{\lambda_i}/\alpha_{X,\lambda_i} C_X^0]$ signals versus the concentration of C_Z. This procedure is repeated for other compounds in the ternary mixture.

2.3. Ratio spectra CWT-zero crossing method

A wavelet transform [2,3] involves the decomposition of a signal function or vector into simpler, fixed building blocks at different scales and positions.

$$\Psi_{a,b}(\lambda) = \frac{1}{\sqrt{|a|}}\Psi\left(\frac{\lambda - b}{a}\right) \quad a \neq 0, \quad a, b \in R \tag{5}$$

where a denotes the scale parameter which is a variable used to control the scaling, b represents the translation parameter controlling the translation and R is the domain of real numbers. A mother wavelet $\Psi(\lambda)$ generates the set of functions $\Psi_{a,b}(\lambda)$ by scaling (or dilatation) and shifting (or translation).

In this study we applied Mexican hat function as a continuous wavelet transform [2,3] and its application to the ratio spectra is given below.

If CWT is applied to Eq. (2), the following equation can be obtained as

$$\mathrm{CWT}\left[\frac{\mathrm{Am}_{\lambda_i}}{\alpha_{\mathrm{X},\lambda_i} C_{\mathrm{X}}^0}\right] = \mathrm{CWT}\left[\frac{\beta_{\lambda_i}}{\alpha_{\mathrm{X},\lambda_i}}\right]\frac{C_{\mathrm{Y}}}{C_{\mathrm{X}}^0} + \mathrm{CWT}\left[\frac{\gamma_{\lambda_i}}{\alpha_{\mathrm{X},\lambda_i}}\right]\frac{C_{\mathrm{Z}}}{C_{\mathrm{X}}^0} \tag{6}$$

If $\mathrm{CWT}[\beta_{\lambda_i}/\alpha_{\mathrm{X},\lambda_i}]C_{\mathrm{Y}}/C_{\mathrm{X}}^0$ corresponds to zero point at the wavelength λ_i, Eq. (6) would be obtained in the following form

$$\mathrm{CWT}\left[\frac{\mathrm{Am}_{\lambda_i}}{\alpha_{X,\lambda_i} C_{\mathrm{X}}^0}\right] = \mathrm{CWT}\left[\frac{\gamma_{\lambda_i}}{\alpha_{\mathrm{X},\lambda_i}}\right]\frac{C_Z}{C_{\mathrm{X}}^0} \tag{7}$$

Eq. (7) shows that **CWT** amplitudes of the ratio spectra of ternary mixture are dependent only on the concentration of C_{Z} and C_{X}^0 and are independent on the concentration of other compounds in the ternary mixtures. As before, the calibration graphs can be obtained by plotting $\mathrm{CWT}[\mathrm{Am}_{\lambda_i}/\alpha_{X,\lambda_i}C_{\mathrm{X}}^0]$ signals versus the concentration of C_{Z}. This analogous procedure is repeated for all compounds in the ternary mixture.

2.4. PLS method

The **PLS** calibration method is done by the composition of both concentration and absorbance matrix into latent variables, $A = TP^{\mathrm{T}} + E$ and $C = UQ^{\mathrm{T}} + F$. The vector b is given as $b = W(P^{\mathrm{T}}W)^{-1}Q$, where W is a weight matrix. By using the linear regression $C = a + bA$, the constant a is given by $a = C_{\mathrm{mean}} - A_{\mathrm{mean}}^{\mathrm{T}}b$.

2.5. HPLC method

The chromatograms of three compounds were plotted and stored in the computer. The detector responses were measured in terms of peak area. The data was processed using MassLynx software. Seperation was carried out in room temperature and mobile phase consisted of water and methanol (50:50, v/v). The flow rate was set to 0.6 ml min^{-1} with 10 μl injection volume. The photodiode array detection was performed at 250 nm.

3. Experimental

3.1. Instruments

A Shimadzu UV-160 double beam UV–Vis spectrophotometer possessing a fixed slit width (2 nm) connected to a computer loaded with Shimadzu UVPC software and a LEXMARK E-320 printer were used to record the absorption spectra. Data treatments, regressions and statistical analysis were performed by using the *EXCEL* and **PLS** toolbox 3.0 in Matlab software.

The HPLC method was performed on a Waters HPLC system, equipped with a model Waters Alliance 2695 HPLC pump and 996 photodiode array detector (PDA). The method was conducted using a reversed-phase technique. Samples were eluted with a flow rate of 0.6 mL min^{-1} using a mobile phase consisting of deionized water and methanol (50:50, v/v). The wavelength was set to 250 nm. The mobile phase was prepared daily, filtered through a 0.45 μm membrane filter. A Waters 100 RP-18 column (4.6 mm × 150 mm, 5 μm particle size) was used.

3.2. Commercial tablet formulation

A commercial tablet formulation (EXCEDRINE® MIGRANE coated tablets produced by Bristol–Myers Squibb Co., USA, and batch no. 306532) contains 250 mg **PAR**, 250 mg **ASP** and 65 mg **CAF** per tablet. This pharmaceutical preparation was studied by proposed methods.

All the active compounds, **PAR**, **ASP** and **CAF** were kindly donated from Turkish Pharmaceutical Industrial firms.

3.3. Standard solutions

3.3.1. For spectrophotometry

Stock solutions containing 50 mg/100 mL **PAR**, **ASP** and **CAF** were prepared in 0.1 M HCl. A standard series of the solutions containing 5–25 μg mL^{-1} for **PAR**, **ASP** and 4–28 μg mL^{-1} for **CAF** was obtained from the stock solutions. A training set of 20 standard mixture of solutions consisting of 0–25 μg mL^{-1} **PAR**, **ASP** and 0–28 μg mL^{-1} **CAF** was made form stock solutions. A validation set consisting of 15 synthetic mixture solutions in the concentration range of 5–20 μg mL^{-1} **PAR**, **ASP** and 4–28 μg mL^{-1} **CAF** was prepared by using the same stock solutions. All the solutions were prepared freshly and protected from light.

3.3.2. For HPLC

Stock solutions of 25 mg/100 mL^{-1} of the subject compounds were prepared in methanol–water solvent mixture

(50:50, v/v). The standard solutions and synthetic mixtures were prepared containing 4–22 μg mL^{-1} of **PAR**, **ASP** and 2–20 μg mL^{-1} of **CAF** in the same solvent system. An independent set of 12 synthetic mixture solutions were prepared in the working concentration range for the validation of HPLC method. All prepared solutions were filtered through 0.45 μm membrane filter before injection.

3.4. Tablet analysis

Twenty tablets were weighted and powdered in a mortar. A tablet amount was transferred to a 100 mL^{-1} calibrated flask and dissolved in 100 mL 0.1 M HCl. After dissolution process prepared solutions were filtered with 0.2 μm disposable membrane filter (Sartorious, minisart, $\phi = 0.20$ μm) by using an injector. The final solution was diluted to the working concentration range for application of the three developed methods.

For HPLC method, the same procedure was followed by using methanol–water mixture (50:50, v/v).

4. Results and discussion

Fig. 1 shows that the absorption spectra of **PAR**, **ASP** and **CAF** in ternary mixture are strongly overlapping in the wavelength range of 215–300 nm. The quantitative analysis of the content of ternary mixtures consisting of the above active compounds with overlapping spectra is not always possible by using conventional spectrophotometric methods.

In this study ratio spectra derivative-zero crossing, ratio spectra **CWT**-zero crossing and **PLS** methods were successfully applied to the quantitative determination of three active compounds in ternary mixtures and pharmaceutical tablets.

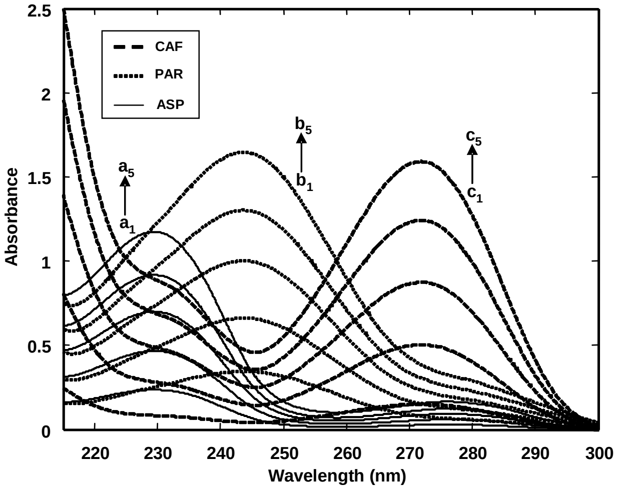

Fig. 1. Absorption spectra of **PAR** (· · ·), **ASP** (—) in the concentration range of ($a_1 = b_1$) 5 μg mL^{-1}, ($a_2 = b_2$) 10 μg mL^{-1}, ($a_3 = b_3$) 15 μg mL^{-1}, ($a_4 = b_4$) 20 μg mL^{-1}, and ($a_5 = b_5$); 25 μg mL^{-1}, and **CAF** (- - - -) in the concentration of (c_1) 4 μg mL^{-1}, (c_2) 10 μg mL^{-1}, (c_3) 16 μg mL^{-1}, (c_4) 22 μg mL^{-1} and (c_5) 28 μg mL^{-1} in 0.1 M HCl.

The main purpose of this study is to show the applicability of ratio spectra **CWT**-zero crossing method to quantitative analysis of the amount of active compounds in samples. The applicability of **CWT** was tested by comparing the results obtained from ratio spectra derivative-zero crossing, **PLS** and also HPLC methods for the same ternary mixtures and pharmaceutical samples.

In order to obtain calibrations, the standard series for two graphical methods was prepared in the concentration range of 5–25 μg mL^{-1} for **PAR** and **ASP**, 4–28 μg mL^{-1} for **CAF** in 0.1 M HCl. The absorption spectra were recorded over the wavelength range of 215–300 nm against a blank (0.1 M HCl). For the validation of these three methods, a recovery set of mixtures consisting of **PAR**, **ASP** and **CAF** were prepared. In this study ratio spectra **CWT**-zero crossing method was developed for the simultaneous analysis of ternary mixtures. This method is a powerful tool for the resolution of overlapping spectra or overlapping ratio spectra in analytical data analysis. In the application of **CWT**, the selection of continuous wavelet transforms is the most important step to get the best signal corresponding to a zero crossing for a given mixture. It may happen that two or more **CWT** methods give satisfactory result, so the most convenient **CWT** method has to be found for the problem. In this paper, we found the optimum transform to be Mexican **CWT** at the scaling factor $a = 125$ (frequency = 0.002). Mexican function was used because it gave the highest sensitivity with big slope values in the linear regression equations for all subjected compounds. In this analytical study, ratio spectra derivative-zero crossing method was used as a comparative method for the quantitation of subject active compounds in the ternary mixtures and pharmaceutical samples. However ratio spectra derivative-zero crossing method gives the small peak amplitudes in respect to the peaks of the original absorption spectra. Therefore, ratio spectra **CWT**-zero crossing method has higher peak amplitudes in comparison to the original spectra. This can be considered an advantage of ratio spectra **CWT**-zero crossing method over ratio spectra derivative-zero crossing method for the resolution of ternary mixtures.

PLS and other chemometric methods can be successfully applied to complex pharmaceutical mixtures. These chemometric methods needs the use of an abstract mathematical content and it is not always easy to be applied.

In the presented study, **CWT** method was developed and applied for the simultaneous determination of **PAR**, **ASP** and **CAF** in the mixtures and commercial pharmaceutical tablet formulation without any separation and any other chemical process. **CWT** based on an integration procedure was compared with ratio spectra derivative-zero crossing method and **PLS** methods regarding the performance of these three methods. Finally, ratio spectra derivative-zero crossing method and **PLS** which is mentioned successful method in the literature were applied to the mixtures of **PAR**, **ASP** and **CAF** with the aim of comparison of the developed **CWT** method. The proposed graphical and numerical methods were valid by HPLC method. We observed that these proposed methods give us the successful results for the determination of active compounds. The procedures of these methods were given in the following sections.

4.1. Ratio spectra CWT-zero crossing method

In the application of this method described in Section 2.3 three different divisors were used for the determination of two pairs of compounds in the ternary mixtures.

To determine **PAR** and **CAF** in their ternary mixtures the absorption spectra of standard series containing **PAR** and **CAF** compounds were divided by the standard spectrum of the solution of 15 μg mL^{-1} **ASP**. As shown in Fig. 2a, their ratio spectra were obtained and transferred as ratio spectra data vectors from EXCEL to wavelet domain. Same procedure was applied to the solutions of samples. The transferred ratio spectra data vectors were processed by Mexican **CWT** at the scaling factor $a = 125$ (frequency = 0.002) and **CWT** spectra were obtained by plotting the **CWT** $C_{a,b}$ coefficients versus wavelengths as seen in Fig. 3a. By using zero crossing technique, **CWT** calibration equations were obtained by measuring the **CWT** amplitudes at 240.8 nm for **CAF** and at 251.0 nm for **PAR** and their statistical results were summarized in Table 1. The **CAF** and **PAR** content in their ternary mixture were determined by the above **CWT** calibration equations.

In the same way for the determination of **CAF** and **ASP** the ratio spectra of **CAF** and **ASP** were obtained by dividing the recorded spectra of standard series of **CAF** and **ASP** to the standard spectrum of the solution of 15 μg mL^{-1} **PAR** (see Fig. 2b). Mexican **CWT** at the scaling factor $a = 125$ (frequency = 0.002) was applied to the ratio spectra data vectors and **CWT** spectra were obtained by plotting the **CWT** $C_{a,b}$ coefficients versus wavelengths as seen in Fig. 3b. The **CWT** amplitudes were measured at 269.3 nm for **CAF** and 262.0 nm for **ASP**. **CWT** signals of the ratio spectra data were drawn as a graph, versus concentrations of **CAF** and **ASP** and two straight lines were obtained and their statistical results were presented in Table 1. By using the calibration graphs, **CAF** and **ASP** were determined in the ternary mixtures.

In the similar manner for the quantitative analysis of **PAR** and **ASP**, the ratio spectra of standard series of **PAR** and **ASP** were obtained by using the standard spectrum of the solution of 15 μg mL^{-1} **CAF** as shown in Fig. 2c. In this case, results also yield two straight lines obtained by using the **CWT** amplitudes measured at 248.6 nm for **PAR** and at 258.8 nm for **ASP** shown in Fig. 3c. By means of calibration graphs, the content of **PAR** and **ASP** was determined in the samples. For the calibration graphs the obtained statistical results were given in Table 1. The obtained **CWT** calibration equations were used for the determination of **PAR** and **ASP** in samples.

The power test of **CWT** calibration equations was accomplished by plotting the concentration residuals versus

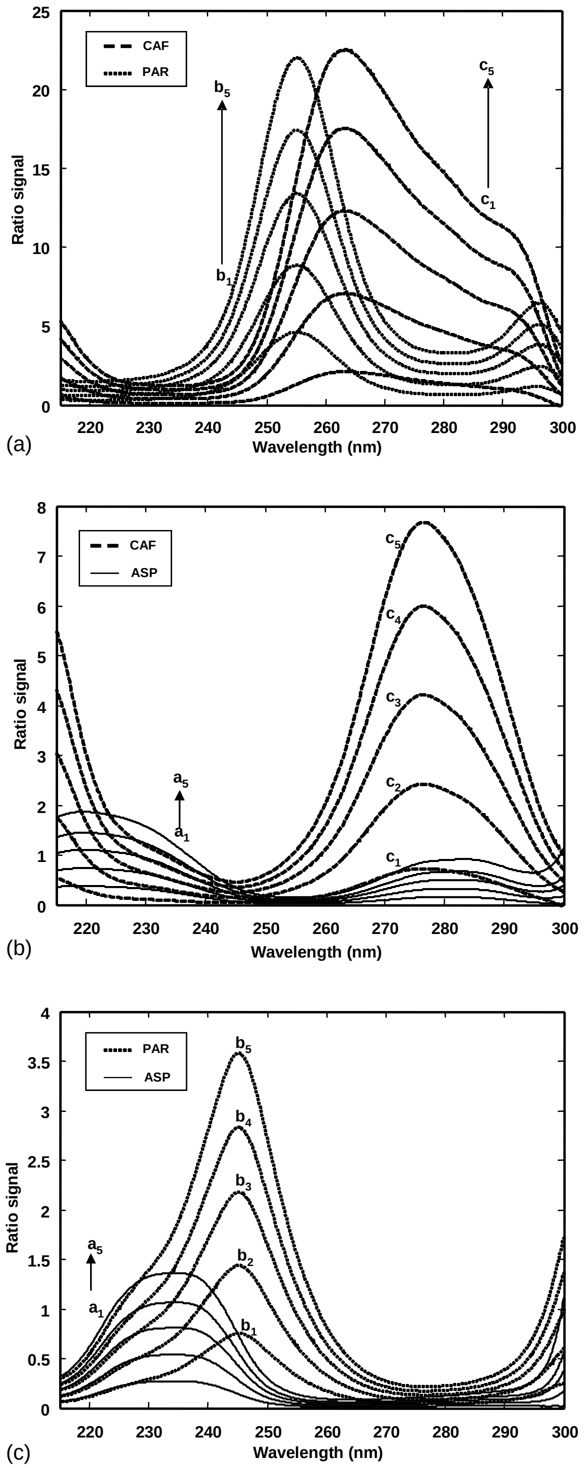

Fig. 2. The ratio spectra of **PAR** (b_1) 5 mg mL^{-1}, (b_2) 10 mg mL^{-1}, (b_3) 15 mg mL^{-1}, (b_4) 20 mg mL^{-1}, (b_5) 25 mg mL^{-1}, **CAF** (c_1) 4 mg mL^{-1}, (c_2) 10 mg mL^{-1}, (c_3) 16 mg mL^{-1}, (c_4) 22 mg mL^{-1}, (c_5) 28 mg mL^{-1} and **ASP** (a_1) 5 mg mL^{-1}, (a_2) 10 mg mL^{-1}, (a_3) 15 mg mL^{-1}, (a_4) 20 mg mL^{-1}, (a_5) 25 mg mL^{-1}. The spectra are divided by 15 mg mL^{-1} of (a) **ASP**, **(b) PAR** and **(c) CAF**.

Table 1
Statistical results of calibration graphs obtained by proposed methods

Method	Divisor	λ	Regression equation	*r*	S.E. (*m*)	S.E. (*n*)	S.E. (*r*)
Ratio spectra derivative-zero crossing	**PAR**	221.4	$A = -0.0184C_{\mathbf{CAF}} - 0.0025$	0.9999	0.0004	0.0000	0.0026
		246.7	$A = -0.2794C_{\mathbf{ASP}} - 0.2028$	0.9998	0.0003	0.0024	0.0003
	CAF	247.1	$A = -0.0044C_{\mathbf{ASP}} - 0.0011$	0.9998	0.0001	0.0007	0.0007
		236.0	$A = 0.0048C_{\mathbf{PAR}} + 0.0014$	0.9998	0.0001	0.0008	0.0008
	ASP	284.0	$A = -0.0166C_{\mathbf{CAF}} + 0.0307$	0.9995	0.0003	0.0054	0.0056
		265.0	$A = -0.0058C_{\mathbf{PAR}} - 0.0017$	0.9997	0.0008	0.0134	0.0128
Ratio spectra CWT-zero crossing	**PAR**	269.3	$A = 1.6320C_{\mathbf{CAF}} - 2.3777$	0.9999	0.0121	0.0121	0.2291
		262.0	$A = -0.0183C_{\mathbf{ASP}} - 0.0025$	0.9988	0.0080	0.1318	0.1257
	CAF	258.8	$A = -0.2537C_{\mathbf{ASP}} - 0.1139$	0.9997	0.0033	0.0546	0.0520
		248.6	$A = 0.9463C_{\mathbf{PAR}} + 0.2996$	0.9998	0.0103	0.1702	0.1623
	ASP	240.8	$A = -3.9112C_{\mathbf{CAF}} + 6.0945$	0.9999	0.0259	0.4686	0.4910
		251.0	$A = 5.8929C_{\mathbf{PAR}} + 2.0027$	0.9998	0.0629	1.0433	0.9998
HPLC method	**PAR**	250.0	$A^{a} = 1233.37C_{\mathbf{PAR}} - 31.23$	0.9999	12.5009	182.8719	167.7167
	CAF	250.0	$A^{a} = 127.75C_{\mathbf{ASP}} - 231.50$	0.9977	6.1127	89.4213	82.0107
	ASP	250.0	$A^{a} = 277.75C_{\mathbf{CAF}} - 126.00$	0.9995	6.2079	79.9827	83.2872

C, concentration (μg mL^{-1}); *A*, amplitudes at selected wavelength for **PAR**, **ASP** and **CAF**; *r*, regression coefficient; S.E. (*r*), standard deviation of linear regression; S.E. (*m*), standard deviation of slope; S.E. (*n*), standard deviation of intercept.

[a] *A*, peak area in the chromatogram.

the predicted concentrations. We observed that the residuals randomly scattered around zero as shown in Fig. 5. The calculated values show a good ability of **CWT** calibration in the determination of the subjected active compounds in the mixture solutions.

4.2. Ratio spectra derivative-zero crossing method

In this method, the acquire spectra of the solutions of the **PAR** and **CAF** compounds, their absorption spectra of solutions were divided by the spectrum of the standard solution of 15 μg mL^{-1} **ASP**. Fig. 2a shows the ratio spectra of these two compounds in the spectral range of 215–300 nm. First derivative of the ratio spectra was calculated by $\Delta\lambda = 4$ nm intervals and resulting spectra were shown in Fig. 4a. In the zero crossing point of each compound, a graph plotted by concentration versus absorption values of other compound yields a straight line that can be used for concentration calculation in synthetic mixtures and tablets. The same method can be use to find the concentration values of other compound. The amounts of **PAR** and **CAF** in the ternary mixture were found to be proportional to the signals at 284.0 nm for **CAF** and 265.0 nm for **PAR** and statistical parameters of their calibration equations summarized in Table 1.

For the determination of the **ASP** and **CAF** concentrations, their absorption spectra of solutions were divided by the spectrum of the standard solution of 15 μg mL^{-1} **PAR** and the ratio spectra was obtained in the region 215–300 nm (Fig. 2b). First derivative of the ratio spectra which was plotted with intervals of $\Delta\lambda = 4$ nm was illustrated in Fig. 4b. The concentrations of **ASP** and **CAF** in the ternary mixture are proportional to the analytical signals at 221.4 nm for **CAF** and 246.7nm for **ASP**. First derivative signals of ratio spectra were plotted as a graph, versus concentrations of **CAF** and **ASP** and two straight lines were obtained and their statistical results were presented in Table 1. The obtained calibration graphs were used for the analysis of **CAF** and **ASP**.

In the same way, the absorption spectra of the solutions of **ASP** and **PAR** in 0.1 M HCI were divided by the spectrum of the standard solution of 15 μg mL^{-1} **CAF** in the same solvent and their ratio spectra were obtained in the spectral region 215–300 nm (Fig. 2c). Fig. 4c shows the first derivative of the ratio spectra which was calculated with intervals of $\Delta\lambda = 4$ nm. The amounts of **ASP** and **PAR** in the ternary mixture were found to be proportional to the signals at 247.1 nm for **ASP** and 236.0 nm for **PAR**. First derivative signals of ratio spectra were plotted as a graph, versus concentrations of **ASP** and **PAR** and two straight lines were obtained and their statistical parameters were indicated in Table 1. By using the calibration graphs the concentrations of **CAF** and **ASP** in their ternary mixture with **PAR** were determined.

The validity of two graphical methods was tested by using various ternary mixtures containing 5–25 μg mL^{-1} **PAR** and **ASP**, and 4–28 μg mL^{-1} for **CAF** in 0.1 M HCl. Mean recoveries and the relative standard deviations were calculated and their results were given in Table 4.

The conditions of instrumental and experimental parameters were optimized to obtain the most distinct curve and higher peak amplitudes corresponding to zero crossing points for the first derivative and **CWT** of the ratio spectra. For selection of an appropriate divisor concentration, some divisor concentrations were tested in the determinations. As a divisor, the standard solution of 15 μg mL^{-1} of each active compound in ternary mixture was found suitable for both graphical methods. In ratio spectra derivative-zero crossing method, the influence of $\Delta\lambda$ for obtaining the first derivative of the ratio spectra was tested and a value of $\Delta\lambda = 4$ nm was founded suitable for determinations of three

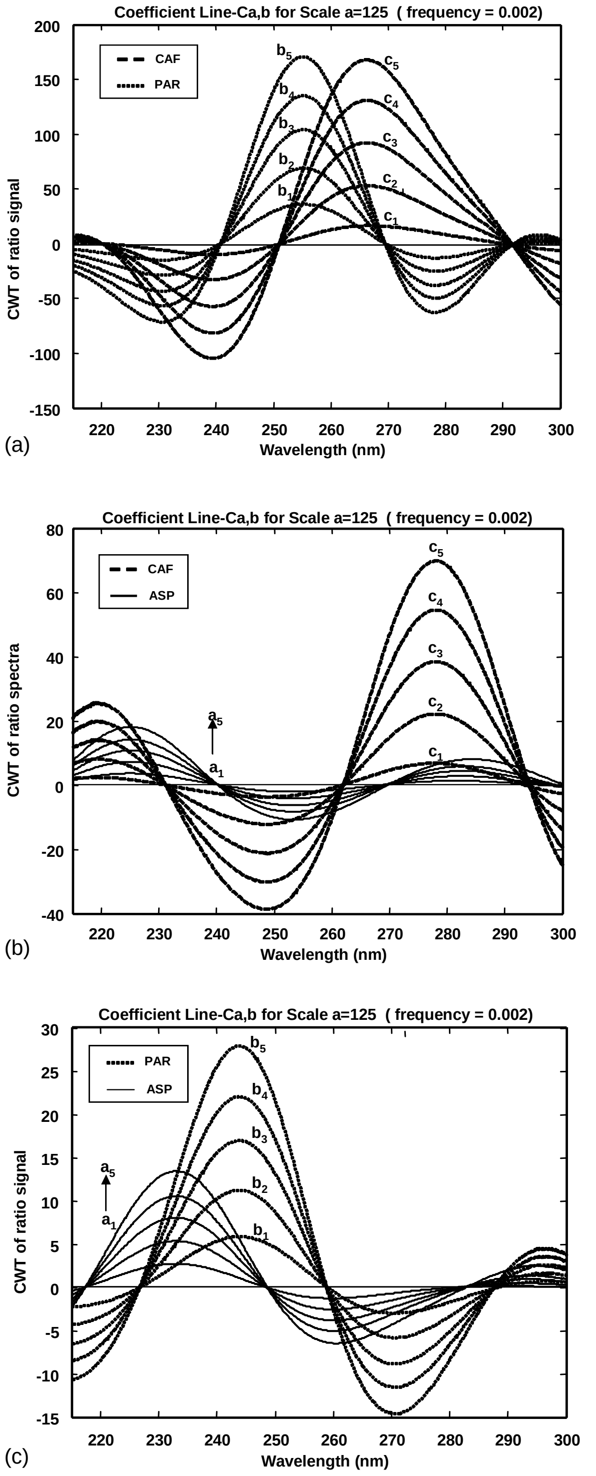

Fig. 3. **CWT** spectra of the ratio spectra of **PAR** (b_1) 5 mg mL^{-1}, (b_2) 10 mg mL^{-1}, (b_3) 15 mg mL^{-1}, (b_4) 20 mg mL^{-1}, (b_5) 25 mg mL^{-1}, **CAF** (c_1) 4 mg mL^{-1}, (c_2) 10 mg mL^{-1}, (c_3) 16 mg mL^{-1}, (c_4) 22 mg mL^{-1}, (c_5) 28 mg mL^{-1} and **ASP** (a_1) 5 mg mL^{-1}, (a_2) 10 mg mL^{-1}, (a_3) 15 mg mL^{-1}, (a_4) 20 mg mL^{-1}, (a_5) 25 mg mL^{-1}. The spectra are divided by 15 mg mL^{-1} of (a) **ASP**, **(b) PAR** and **(c) CAF**.

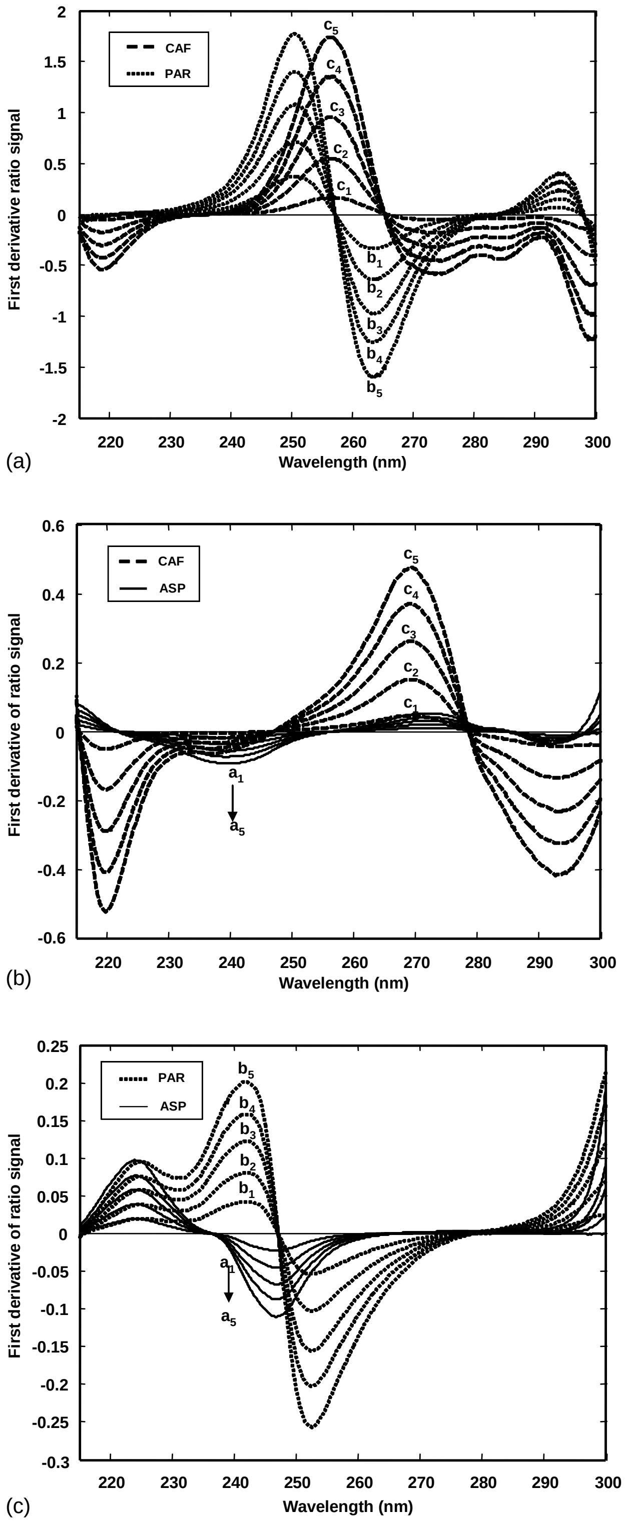

Fig. 4. First derivative ratio spectra of **PAR** (b_1) 5 mg mL^{-1}, (b_2) 10 mg mL^{-1}, (b_3) 15 mg mL^{-1}, (b_4) 20 mg mL^{-1}, (b_5) 25 mg mL^{-1}, **CAF** (c_1) 4 mg mL^{-1}, (c_2) 10 mg mL^{-1}, (c_3) 16 mg mL^{-1}, (c_4) 22 mg mL^{-1}, (c_5) 28 mg mL^{-1} and **ASP** (a_1) 5 mg mL^{-1}, (a_2) 10 mg mL^{-1}, (a_3) 15 mg mL^{-1}, (a_4) 20 mg mL^{-1}, (a_5) 25 mg mL^{-1}. The spectra are divided by 15 mg mL^{-1} of (a) **ASP**, **(b) PAR** and **(c) CAF**.

Table 2
Statistical parameters in the **PLS** calibration step

Parameter	**PAR**	**ASP**	**CAF**
PRESS	0.0173	0.0144	0.0058
SEC	0.1315	0.1198	0.0762
r	0.9999	0.9999	0.9999
n	0.0043	0.0047	0.0008
m	0.9997	0.9997	0.9999

compounds. In case of ratio spectra **CWT**-zero crossing method, the scaling factor $a = 125$ (frequency $= 0.002$) in application of Mexican **CWT** to the overlapping ratio spectra gives us successful results with three divisors.

4.3. PLS method

A concentration set design of the concentration data corresponding to 20 standard mixture of solutions consisting of 0–25 μg mL^{-1} **PAR**, **ASP** and 0–28 μg mL^{-1} **CAF** was randomly organized for the **PLS** calibration. The selection of optimal factor was done by cross-validation procedure by using 20 absorption spectra of samples. This procedure was carried out by **PLS** Toolbox 3.0. According to the cross-validation process, the different factor numbers were tested and the first four factors having minimum values of **PRESS** (prediction residual error sum of squares) were found appropriate for **PLS** calibration. By using actual and predicted concentrations, **PRESS**, **SEC**, correlation coefficient (*r*), intercept (*n*) and slope (*m*) in **PLS** calibration step using the first four factors were calculated and shown in Table 2 (Fig. 5).

In the prediction step, the constructed **PLS** calibration was applied to the synthetic mixtures (Table 3) and the ability of this **PLS** calibration was checked by using the statistical parameters namely **SEP**, correlation coefficient (*r*), intercept (*n*) and slope (*m*) according to the relation between actual and predicted concentrations studied for the constructed **PLS** calibration. The **SEP** values were found as 0.206 for **PAR**, 0.101 for **ASP** and 0.151 for **CAF**. The linear regression analysis between the actual and predicted concentrations of **PAR**, **ASP** and **CAF** was made and their equations are found as $y = 0.9904x + 0.2037$ for **ASP**, $y = 0.8334x + 1.4104$ for **CAF** and $y = 0.9904x + 0.2037$ for **PAR**. Their correlation coefficients are 0.9995 for **PAR**, **ASP** and **CAF**. These values indicate good predictive ability of **PLS** calibration in the prediction of the **PAR**, **ASP** and **CAF** amounts in the mixture solutions. Another analytical test was accomplished by plotting the concentration residuals against the predicted concentrations. We observed that the residuals randomly scattered around zero as shown in Fig. 6.

The **PLS** calibration is used for the estimation of three active compounds in the samples. The applicability of **PLS** method was confirmed by determining **PAR**, **ASP** and **CAF** in the synthetic ternary mixture. The various mixtures consisting of **PAR**, **ASP** and **CAF** in the working concentration range were used for the validation of **PLS** technique. As it can be seen in Table 3, mean recoveries and their relative standard deviations were found to be 100.5 and 1.19%, 100.3 and 0.50% and 100.6 and 2.12% for **PAR**, **ASP** and **CAF**, respectively.

The interference of excipients in the pharmaceutical tablet was studied in detail by ratio spectra **CWT**-zero crossing method, ratio spectra derivative-zero crossing and **PLS** methods. For this reason standard addition method was applied to commercial tablets containing these three compounds. In application of standard addition method to tablets, the mean percentage recoveries and their standard deviation for the proposed methods for six replicate were calculated and shown in Table 4. According to the obtained results a good precision and accuracy was observed for these methods. Consequently the excipients in tablets do not interfere in the analysis of three compounds in tablets.

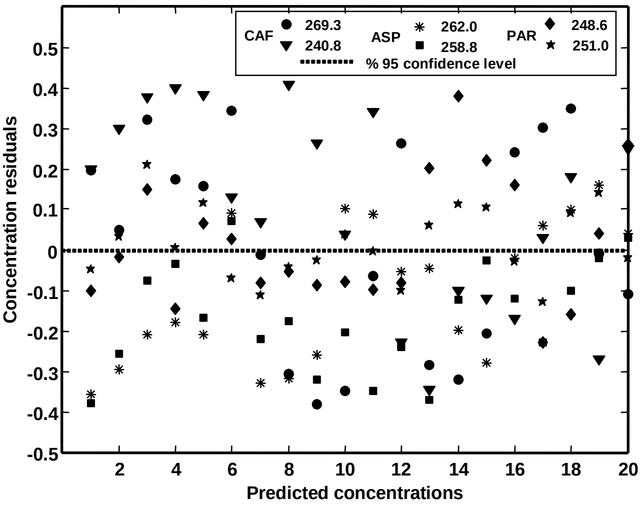

Fig. 5. Concentration residuals for the predicted concentration of **PAR**, **ASP** and **CAF** by using ratio spectra CWT-zero crossing.

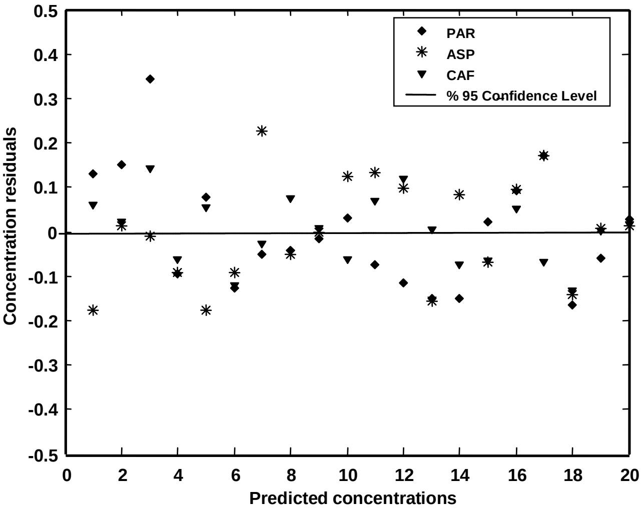

Fig. 6. Concentration residuals for the predicted concentration of **PAR**, **ASP** and **CAF** by using **PLS** calibration

Table 3
Recoveries of **PAR**, **ASP** and **CAF** in various synthetic mixtures by the proposed methods

	Method			Recovery (%)														
	Divisor			Ratio spectra CWT-zero crossing						Ratio spectra derivative-zero crossing						PLS calibration		
	Added ($\mu g\,mL^{-1}$)			PAR (15 $\mu g\,mL^{-1}$)		CAF (15 $\mu g\,mL^{-1}$)		ASP (15 $\mu g\,mL^{-1}$)		PAR (15 $\mu g\,mL^{-1}$)		CAF (15 $\mu g\,mL^{-1}$)		ASP (15 $\mu g\,mL^{-1}$)				
	PAR	**ASP**	**CAF**	**CAF** 269.3 nm	**ASP** 262.0 nm	**PAR** 248.6 nm	**ASP** 258.8 nm	**CAF** 240.8 nm	**PAR** 251.0 nm	**CAF** 221.1 nm	**ASP** 246.7 nm	**PAR** 236.0 nm	**ASP** 247.1 nm	**CAF** 284.0 nm	**PAR** 265.0 nm	**PAR**	**ASP**	**CAF**
1	5.0	20.0	5.2	99.9	98.2	98.0	98.1	103.8	99.0	108.4	99.2	102.3	99.3	105.2	100.5	102.0	99.8	98.0
2	10.0	20.0	5.2	100.9	98.5	99.8	98.7	105.7	100.3	107.7	96.6	101.6	100.8	97.0	101.0	101.6	100.4	100.6
3	15.0	20.0	5.2	106.2	98.9	101.0	99.6	106.3	101.4	102.0	103.6	100.2	102.9	100.7	102.1	102.1	101.4	102.9
4	20.0	20.0	5.2	102.9	91.1	99.3	95.8	105.7	100.0	106.0	98.0	100.1	97.4	103.6	100.2	99.8	100.4	102.7
5	25.0	20.0	5.2	105.0	99.0	100.3	99.2	104.4	100.5	105.1	105.7	99.7	105.3	107.4	100.8	100.7	100.5	103.7
6	20.0	5.0	5.2	106.6	100.3	100.1	101.4	98.6	99.7	96.4	102.3	99.5	100.0	97.7	100.5	100.8	100.2	104.2
7	20.0	10.0	5.2	102.2	96.7	99.6	97.8	101.3	99.4	108.9	102.0	99.3	100.9	99.0	100.2	100.3	100.8	101.2
8	20.0	15.0	5.2	105.1	97.9	99.7	98.8	104.7	99.8	102.1	101.7	99.7	100.9	103.9	100.4	100.4	100.9	101.7
9	20.0	20.0	5.2	107.4	97.2	99.6	98.4	105.0	99.9	103.7	101.0	101.7	100.4	105.3	100.2	100.3	100.2	100.6
10	20.0	25.0	5.2	106.7	95.6	99.6	98.0	100.7	100.2	106.7	99.3	101.5	98.9	105.6	100.4	100.2	100.1	98.8
11	20.0	20.0	4.0	106.6	95.7	99.5	98.3	108.5	100.0	101.9	100.5	100.7	100.0	102.6	100.5	100.3	100.5	98.1
12	20.0	20.0	10.0	105.6	94.7	99.6	96.8	95.7	99.5	96.8	100.1	99.9	99.6	96.9	100.6	100.0	99.6	97.9
13	20.0	20.0	16.0	98.2	94.8	101.0	96.1	95.2	100.3	99.6	98.4	99.6	97.9	99.6	102.1	100.7	99.6	99.0
14	20.0	20.0	22.0	98.5	96.5	101.9	96.4	98.2	100.6	96.9	99.4	99.3	99.0	96.1	102.2	100.7	100.0	98.6
15	20.0	20.0	28.0	95.7	97.1	102.6	94.9	96.0	100.5	96.3	99.4	99.8	99.0	94.4	102.9	96.9	99.8	100.5
Mean				103.2	96.8	100.1	97.9	102.0	100.1	102.6	100.5	100.3	100.2	101.0	101.0	100.5	100.3	100.6
R.S.D.				3.62	2.34	1.13	1.71	4.26	0.58	4.43	2.31	0.98	1.94	4.05	0.88	1.19	0.50	2.12

R.S.D.: relative standard deviation.

4.4. HPLC method

A reversed-phase HPLC method was used for the validation of proposed graphical and numerical methods. Some mobile phase and wavelengths were tested for the separation and determination of **PAR**, **ASP** and **CAF** in the ternary mixture. The optimized experimental conditions were explained in Section 2.5. In the optimized conditions the retention times of **ASP**, **PAR** and **CAF** were found as 1.10, 1.54 and 1.74 min, respectively. Fig. 7 shows a typical chromatogram obtained from the analysis of standard series of solutions and inset figure also shows the tablet mixture chromatogram using the proposed method.

The calibration curves for active compounds were constructed by plotting concentration versus peak area and showed good linearity in the concentration range pronounced in previous section. The representative linear equations and other parameters were shown in Table 1.

In application of HPLC method to the ternary synthetic mixtures, the mean percentage recoveries with relative standard deviations were calculated as 99.9 and 1.57 for **CAF**, 98.6 and 1.14 for **PAR** and 102.9 and 4.66 for **ASP**.

The validity of the HPLC method was assessed by applying the standard addition technique for six replicate. The results were presented in Table 4. In this table, the results indicate that there is no interference from the excipients used in the formulation of the tablets.

4.5. Analysis of tablet content

The obtained results by applying ratio spectra **CWT**-zero crossing method, ratio spectra derivative-zero crossing and **PLS** methods to the commercial tablet preparation were shown in Table 5. A good coincidence was observed

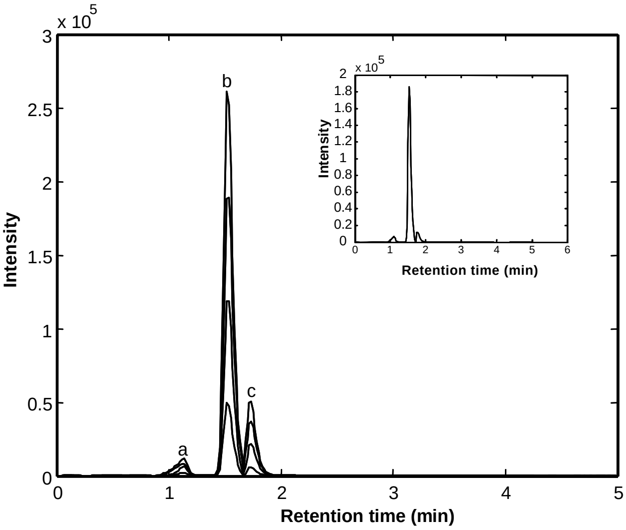

Fig. 7. HPLC chromatograms of (a) 4–22 µg/ml **ASP**, (b) 4–22 µg/ml **PAR** and (c) 2–20 µg/ml **CAF** in methanol and water mixture (50:50, v/v).

Table 4
Results of standard addition method applied to commercial tablet preparation by the proposed methods

Recovery	Ratio spectra (CWT-zero crossing)						Ratio spectra (derivative-zero crossing)						PLS; calibration			HPLC; method		
	PAR (15 µg mL^{-1})		**CAF** (15 µg mL^{-1})		**ASP** (15 µg mL^{-1})		**PAR** (15 µg mL^{-1})		**CAF** (15 µg mL^{-1})		**ASP** (15 µg mL^{-1})							
	CAF 269.3 nm	**ASP** 262.0 nm	**PAR** 248.6 nm	**ASP** 258.8 nm	**CAF** 240.8 nm	**PAR** 251.0 nm	**CAF** 221.1 nm	**ASP** 246.7 nm	**PAR** 236.0 nm	**ASP** 247.1 nm	**CAF** 284.0 nm	**PAR** 265.0 nm	**PAR**	**ASP**	**CAF**	**PAR**	**ASP**	**CAF**
Mean	102.3	98.1	97.4	95.9	103.9	98.4	103.7	100.0	97.4	99.4	100.4	99.2	98.7	100.9	99.9	99.6	96.6	103.3
S.D.	2.32	2.21	1.69	1.35	1.24	1.82	3.82	1.36	0.55	1.35	3.49	0.52	0.28	0.42	0.81	2.28	0.74	3.23
R.S.D.	2.27	2.25	1.74	1.41	1.19	1.85	3.68	1.36	0.57	1.36	3.48	0.52	0.28	0.42	0.81	2.29	0.77	3.13

Lable claim (mg): 250 mg **PAR**, 250 mg **ASP** and 65 mg **CAF** per tablet; S.D., standard deviation.

Table 5
Results of the commercial Excedrin tablet preparations by the proposed methods

	Ratio spectra CWT-zero crossing (mg per tablet)						Ratio spectra derivative-zero crossing (mg per tablet)						PLS calibration (mg per tablet)			HPLC method (mg per tablet)		
	PAR (15 μg mL^{-1})		**CAF** (15 μg mL^{-1})		**ASP** (15 μg mL^{-1})		**PAR** (15 μg mL^{-1})		**CAF** (15 μg mL^{-1})		**ASP** (15 μg mL^{-1})							
	CAF 269.3 nm	**ASP** 262.0 nm	**PAR** 248.6 nm	**ASP** 258.8 nm	**CAF** 240.8 nm	**PAR** 251.0 nm	**CAF** 221.1 nm	**ASP** 246.7 nm	**PAR** 236.0 nm	**ASP** 247.1 nm	**CAF** 284.0 nm	**PAR** 265.0 nm	**PAR**	**ASP**	**CAF**	**PAR**	**ASP**	**CAF**
Mean	65.0	246.3	243.3	243.1	67.7	245.5	67.6	247.7	244.6	246.2	68.0	245.4	243.7	254.7	63.3	243.4	246.5	67.5
S.D.	1.92	5.46	2.69	2.58	1.09	2.81	1.05	3.85	6.32	3.86	1.70	3.59	3.64	2.92	0.91	1.85	2.58	2.78
R.S.D.	2.95	2.22	1.11	1.06	1.61	1.15	1.55	1.56	2.58	1.57	2.50	1.46	1.49	1.15	1.44	0.76	1.05	4.12
S.E.	0.61	1.73	0.85	0.81	0.34	0.89	0.33	1.22	2	1.22	0.54	1.14	1.21	0.97	0.30	0.83	1.15	1.24
CL ($P = 0.05$)	1.19	3.38	1.67	1.60	0.67	1.74	0.65	2.39	3.91	2.39	1.05	2.23	2.38	1.91	0.59	1.63	2.26	2.44
$t_{\text{calculated}}$	1.14	0.23	0.02	1.23	0.89	0.41	0.79	0.13	0.10	0.37	0.86	0.32	0.05	1.29	2.23			
$t_{\text{theoretical}}$	2.26	2.26	2.26	2.26	2.26	2.26	2.26	2.26	2.26	2.26	2.26	2.26	2.26	2.26	2.26			
$F_{\text{calculated}}$	1.11	2.33	0.49	0.52	0.36	0.53	2.74	1.16	2.69	1.16	0.87	1.15	1.12	0.67	1.10			
$F_{\text{theoretical}}$	2.98	2.98	2.98	2.98	2.98	2.98	2.98	2.98	2.98	2.98	2.98	2.98	2.98	2.98	2.98			

Lable claim (mg): 250 mg **PAR**, 250 mg **ASP** and 65 mg **CAF** per tablet; results obtained are average of 10 replicate for each method.; S.E., standard error; CL, confidential limit.

between the experimental results and label claim of the commercial tablet formulation in this study.

The numerical values of all statistical parameters calculated in Table 5 are in acceptable determination limits in application of two methods to the tablets. The tablet experimental results of ratio spectra **CWT**-zero crossing method, ratio spectra derivative-zero crossing and **PLS** methods were valid by using HPLC method. The HPLC method's results are given in the Table 5.

This table summarize the statistical comparison of the results obtained by applying the proposed graphical and HPLC methods. In comparison of proposed graphical methods with HPLC method, the calculated *t*- and *F*-test values with 95% of confidential limit indicate that there is no significant difference between all the applied methods.

5. Conclusion

Simultaneous determination of **PAR**, **ASP** and **CAF** in the mixtures and tablets was successfully achieved by ratio spectra **CWT**-zero crossing, ratio spectra derivative-zero crossing and **PLS** methods in spite of the overlapping spectra of the three compounds in the spectral range of 215–300 nm. The fundamental purpose of our study is a new application of ratio spectra **CWT**-zero crossing method to the ternary mixtures and tablets consisting of subject compounds. Ratio spectra derivative-zero crossing and **PLS** methods were used for the comparison of the results of new approach "ratio spectra **CWT**-zero crossing"

The validation of experimental results obtained from graphical and numerical methods was carried out by HPLC method. When all the proposed methods were compared with each other, a good agreement was observed. We believe that the proposed graphical and numerical methods are useful, rapid, very cheap and yet easy to apply for the analysis of **PAR**, **ASP** and **CAF** with respect to the **HPLC** method.

PLS is using a mixture design for calibration and it requires many concentration sets. **PLS** calibration contains matrix calculations, so in some cases the calculations lead us to some undefined operations from the mathematical point of view. In other words, **PLS** requires a selection of the wavelength interval or wavelength point in order to obtain good calibration and prediction. **CWT** is using the linear concentration range for preparation of the calibration curve. Standard series in the linear concentration range does not require any repletion process.

CWT calibration obeys the Lambert-Beer law and it does not need the selection of the wavelength and it uses **CWT** amplitudes corresponding to the maximum and minimum points of **CWT** coefficients spectra. The peak amplitude intensities are controlled by two parameters (dilation and translation parameters) of the **CWT** transformation. These parameters are used for the increasing of the sensitivity of the calculated calibration graphs.

Consequently a numerical and three graphical methods can be proposed and applied for the quality control and routine analysis of these three compounds in pharmaceutical dosage forms.

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