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Non-parametric linear regression of discrete Fourier transform convoluted chromatographic peak responses under non-ideal conditions of internal standard method

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ABSTRACT

This manuscript discusses the application of chemometrics to the handling of HPLC response data using the internal standard method (ISM). This was performed on a model mixture containing terbutaline sulphate, guaiphenesin, bromhexine HCl, sodium benzoate and propylparaben as an internal standard. Derivative treatment of chromatographic response data of analyte and internal standard was followed by convolution of the resulting derivative curves using 8-points $\sin x_i$ polynomials (discrete Fourier functions). The response of each analyte signal, its corresponding derivative and convoluted derivative data were divided by that of the internal standard to obtain the corresponding ratio data. This was found beneficial in eliminating different types of interferences. It was successfully applied to handle some of the most common chromatographic problems and non-ideal conditions, namely: overlapping chromatographic peaks and very low analyte concentrations. For example, a significant change in the correlation coefficient of sodium benzoate, in case of overlapping peaks, went from 0.9975 to 0.9998 on applying normal conventional peak area and first derivative under Fourier functions methods, respectively. Also a significant improvement in the precision and accuracy for the determination of synthetic mixtures and dosage forms in non-ideal cases was achieved. For example, in the case of overlapping peaks guaiphenesin mean recovery% and RSD% went from 91.57, 9.83 to 100.04, 0.78 on applying normal conventional peak area and first derivative under Fourier functions methods, respectively. This work also compares the application of Theil's method, a non-parametric regression method, in handling the response ratio data, with the least squares parametric regression method, which is considered the *de facto* standard method used for regression. Theil's method was found to be superior to the method of least squares as it assumes that errors could occur in both *x*- and *y*-directions and they might not be normally distributed. In addition, it could effectively circumvent any outlier data points. For the purpose of comparison, the results obtained using the above described internal standard method were compared with the external standard method for all types of linearity.

1. Introduction

The basic theory of quantitative analysis in HPLC involves the measurement of peak height or area. To determine the concentration of a compound, the peak area or height is plotted versus the concentration of the substance. For peaks that are well resolved, both peak area and height are proportional to the concentration. Three different calibration methods, each with its own benefits and limitations, can be utilized in quantitative analysis; external standard, internal standard and standard addition method [1].

In this paper, we are dealing with the use of internal standard method (ISM) in the handling of data obtained from HPLC.

The ISM yields the most accurate and precise results. In this method, an equal amount of an internal standard, a component that is not present in the sample, is added to both the sample and the standard solutions. The internal standard selected should be chemically similar to the analyte, have a retention time close to the analyte and derivatise in a similar way to the analyte [1]. Additionally, it is important to ensure that the internal standard is stable and it does not interfere with any of the sample components. The internal standard should be added before any preparation of the sample so that the extraction efficiency can be evaluated. Quantitation is achieved by using ratios of peak height or area of a component to the internal standard. Thus the proportional error will be consequently cancelled [1]. Many examples are present in the literature

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illustrating the use of ISM in HPLC analysis. For example: determination of cocaine in human hair [2], levetiracetam in plasma [3], irinotecan [4], l-thyroxine and eight degradation impurities [5], trazodone and its main active metabolite [6] and econazole nitrate in cream formulations [7].

Korany et al. developed a chemometric method based on non-parametric linear regression of derivative/discrete Fourier transform convoluted high performance liquid chromatographic peak responses in non-ideal conditions in the HPLC analysis using external standard method [8]. It was found that derivative treatment of chromatographic response data followed by convolution of the resulting derivative curves using 8-points $\sin x_i$ polynomials (discrete Fourier functions) was beneficial in eliminating different types of interferences. This was successfully applied to handle some of the most common chromatographic problems and non-ideal conditions, namely: very low analyte concentrations, overlapping chromatographic peaks and baseline drift [8]. This technique was also extended to be used in handling TLC response data and it was found that chemometric techniques could be successfully applied for handling complex chromatograms. This is highly needed in cases where sources of interferences could affect the chromatographic response, e.g., background noise in TLC-densitometric measurements [9].

Other chemometric techniques have been found in the literature. For example, fractional factorial design and central composite design were applied in a new RP-HPLC method for the determination of mycophenolic acid and its metabolite in biological fluids [10]. Also the determination of fexofenadine and pseudoephedrine was developed by employing the partial least squares analysis [11]. Fractional experimental design and multivariate regression analysis were used in the investigation of the HPLC response of NSAIDs [12].

Chemometric techniques have been widely applied for exploring complex chromatograms. Resolution of overlapping peaks of some pesticide mixtures using HPLC was achieved by the application of partial least squares [13]. A partial least squares chemometric method has been developed to analyze β- and γ-tocopherols separately using RP-HPLC [14]. Parallel factor analysis of HPLC data was used in the separation of overlapping peaks of lidocaine and prilocaine [15]. Determination of overlapped peaks of cortisol and prednisolone was successfully achieved by using HPLC coupled with second-order calibration based on alternating trilinear decomposition algorithm [16].

This work is considered an extension of the authors' previous work which deals with chemometric treatment of HPLC [8] and TLC [9] response data using non-parametric linear regression of discrete Fourier transform convoluted peak responses. In this study, the application of chemometrics in the handling of HPLC response data was investigated using the internal standard method (ISM). As done before in our work [8,9], derivative treatment of chromatographic response data followed by convolution of the resulting derivative curves using 8-points $\sin x_i$ polynomials (discrete Fourier functions) was performed, with the exception that both analytes and internal standard (IS) were treated in the same way. The response of each analyte signal, its corresponding derivative and convoluted derivative data were divided by that of the internal standard with the same mathematical treatment to obtain the corresponding ratio data. This was found beneficial in eliminating different types of interferences. It was successfully applied to handle some of the most common chromatographic problems and non-ideal conditions, namely: overlapping chromatographic peaks and very low analyte concentrations.

This study was carried out using a model mixture, containing terbutaline sulphate (TRB), guaiphenesin (GUA), bromhexine HCl (BRX) and sodium benzoate (SBZ) in presence of propylparaben as an internal standard. It aims at the analysis of TRB, GUA, SBZ and BRX in three different cases, in presence of PPN as internal standard in each case. Since the internal standard method is significantly helpful in eliminating proportional error, improved analytical results were obtained when using this method compared with the external standard method. This was clearly obvious in cases where high incidence of interferences could be found during the analysis e.g. overlapping peaks and very low drug concentrations. The cases studied are; a four component mixture with well-resolved peaks (ideal case I), a three component mixture with overlapping peaks (case II) and one component with very low drug concentration (case III). The chromatographic response data was treated by applying the derivative technique (*D* method) alone and the derivative technique followed by convolution using discrete Fourier functions (*D*/FF method).

The study also presents a comparison between two statistical regression methods for handling data; parametric and non-parametric regression methods [17]. Several examples dealing with the non-parametric treatment of the chromatographic response data were mentioned in our previous work [8,9]. Other reports using the non-parametric methods were also published. For example, Theil's regression method was used for the purpose of comparison between the results obtained from two different methods for quantifying 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate [18]. In the determination of dimethylarginine and other arginine metabolites using HPLC, the group differences were identified with the non-parametric Kruskal–Wallis test [19].

The application of the parametric (least squares) regression method assumes that the data being examined follow normal (Gaussian) distribution. However, the non-parametric regression methods can handle data that may not be normally distributed. Since the central limit theorem of the parametric regression is not really valid for the very small data sets frequently used in analytical work, this makes it of interest to apply non-parametric regression approaches to fitting a straight line to a set of points, the simplest of the non-parametric regression methods is Theil's "incomplete" method, so called to distinguish it from another more complex procedure developed by the same author (the "complete" Theil's method) [17].

2. Theory

2.1. Derivative technique (D method)

Application of derivative techniques to HPLC and HPTLC data and their effect in eliminating different types of errors was successfully studied in the authors' previous works [8,9].

The application of this method depends on the fact that the chromatographic response (R) is a function of time (τ), thus:

If AN stands for the analyte, IS for the internal standard and r for ratio between analyte and internal standard responses.

$$D1_{AN} = \frac{dR_{AN}}{d\tau_{AN}}$$

$$D1_{IS} = \frac{dR_{IS}}{d\tau_{IS}}$$

Then $D1_r = \dfrac{D1_{AN}}{D1_{IS}}$

and

$$D2_{AN} = \frac{d^2R_{AN}}{d\tau_{AN}^2}$$

Table 1
Chromatographic conditions used for cases I–III.

Case	Elution mode (gradient)	Mobile phase composition		Run time[a] (min)
		Acetonitrile	Aqueous[b] phase	
Ideal case (I)	Start	30	70	0–5
	Transition(linear)			5–8
	End	70	30	8–15
Overlapping peaks (II)	Start	45	55	0–5
	Transition (linear)			5–8
	End	70	30	8–15
very low conc. (III)	Start	30	70	0–5
	Transition(linear)			5–8
	End	70	30	8–15

[a] 1 mL/min flow rate, ambient temperature, detection wavelength is 212 nm (in all cases).
[b] 0.02 M phosphate buffer containing 0.15% (v/v) TEA , pH of the aqueous phase is 3 for all cases.

$$D2_{IS} = \frac{d^2 R_{IS}}{d\tau_{IS}^2}$$

Then $D2_r = D2_{AN}/D2_{IS}$ where $D1_r$ and $D2_r$ are first and second derivative ratios, respectively.

2.2. Derivative technique followed by convolution using Fourier functions (D/FF method)

The basis of harmonic analysis is that a given function, for example, $D1_r$ or $D2_r$ curves of a chromatographic peak, $f(\tau)$ can be expanded in terms of the Fourier series [20,21]. This was previously mentioned in details in our previous work [8,9].

Since this work deals with the ISM, the Fourier function coefficient (t_j) was calculated for both analyte (AN) and internal standard (IS)

$$(t_j)_{AN} = \frac{\sum f(\tau)_{iAN} Tx_i}{\sum (Tx_i)^2}$$

$$(t_j)_{IS} = \frac{\sum f(\tau)_{iIS} Tx_i}{\sum (Tx_i)^2}$$

Then $(t_j)_r = (t_j)_{AN}/(t_j)_{IS}$ where T represents cosine or sine.

The Fourier function coefficients ratios, $(t_j)_r$ are proportional to $f(\tau)$. That is:

$$(t_j)_r = \alpha_j c$$

where α is a constant and c is the concentration of the analyte.

3. Experimental

3.1. Instrumentation

The chromatographic system consisted of Series 200 Vacuum Degasser (Perkin-Elmer™, USA), Series 200 LC pump (Perkin-Elmer™, USA), Series 200 variable-wavelength UV–VIS detector (Perkin-Elmer™, USA) and Series 200 autosampler fitted with a 200 μl sample loop (Perkin-Elmer™, USA). A Perkin-Elmer™ Chromatography Interface 600 Series Link was used. HPLC separations were performed on a Spheri-5 RP C-18 (5 μm) column (250 mm × 4.6 mm). Data were processed using TotalChrom Workstation Chromatography Software (Perkin- Elmer™, USA) on an IBM-compatible PC connected to a Laser printer. The digital chromatographic response data were transferred to a personal computer for subsequent processing using Microsoft Excel 2000 (Microsoft Corp., Richmond, VA, USA).

3.2. Materials and reagents

Terbutaline sulphate (TRB), guaiphenesin (GUA), bromhexine HCl (BRX), sodium benzoate (SBZ) and propylparaben (PPN) were kindly supplied by Borg Pharmaceuticals (Alexandria, Egypt). All solvents were of either HPLC or analytical grade, namely: acetonitrile (Panreac Co., EU), orthophosphoric acid (BDH, Poole, UK) and triethylamine (TEA) (BDH). The water for HPLC was double glass distilled.

3.3. Chromatographic conditions

In all cases, a gradient elution system was used; the mobile phase consisted of acetonitrile and an aqueous phase, which was phosphate buffer (0.02 M Na_2HPO_4 in water) containing TEA solution (0.15%, v/v). The pH of the aqueous phase was adjusted to pH 3.0 by dropwise addition of 0.1 M H_3PO_4 solution. The used chromatographic conditions are summarized in Table 1.

3.4. Preparation of stock and standard solutions

Stock solutions were prepared by dissolving TRB, GUA, BRX, SBZ and PPN (internal standard) in a diluting solvent consisting of a mixture of acetonitrile: buffer pH 3 (30:70) to obtain a concentration of 1 mg mL^{-1}, for each. These stock solutions were further diluted with the diluting solvent to obtain working standard solutions of suitable concentrations as shown in the following cases.

3.5. Assay of mixtures containing TRB, GUA, SBZ and BRX (ideal case, I)

Accurate volumes of TRB, GUA, SBZ, BRX and PPN stock solutions were transferred into 10-mL volumetric flasks and diluted to volume with the diluting solvent to prepare six standard mixtures within the concentration range of each compound of TRB, GUA, SBZ and BRX (from 4.0 to 200.0 for TRB, 4.0 to 300.0 for GUA, 4.0 to 100.0 for SBZ and 10.0 to 300.0 μg mL^{-1} for BRX, while for PPN a suitable concentration of 40.0 μg mL^{-1} was chosen, Figs. 1 and 2). Triplicate 20-μL injections were made for each mixture solution and were chromatographed under the conditions described for case I (Table 1). For each solution, the peak area and peak height were recorded and their ratio were calculated (peak area or height of the compound to that of the internal standard PPN). The response readings for each peak were recorded at 0.01 min interval (2.3–2.74 min for TRB, 3.18–3.87 min for GUA, 5.06–5.52 min for SBZ, 14.73–15.35 for BRX and 11.2–11.95 min for PPN). The response data were processed using Excel software. Derivative technique (D method) was first applied, first ($D1$) and second ($D2$) derivative data at (0.01 min) time interval were calculated. Then convolutions of the two types of derivative data were made using discrete Fourier functions of

Table 2
Selected points (retention time rang in minutes) of terbutaline sulphate (TRB) , guaiphenesin (GUA) , sodium benzoate (SBZ), bromhexine HCl (BRX) and propylparaben (PPN) for the derivative and convoluted derivative in the ideal and non-ideal cases.

	TRB		GUA		SBZ		BRX		PPN		
	Ideal case I[a]	Non-ideal case II[b]	Ideal case I[a]	Non-ideal case II[b]	Ideal case I[a]	Non-ideal case II[b]	Ideal case I[a]	Non-ideal case III[a]	Ideal case I[b]	Non-Ideal case III[b]	Non-ideal case II[b]
Derivative technique (*D method*)											
First derivative (*D*1)	2.48–2.53	2.12	3.44–3.55	2.51	5.24–5.36	2.95	14.91–15.21	15.88–16.21	11.52	11.62	6.24
Second derivative (*D*2)	2.47–2.51	2.11	3.41–3.50	2.55	5.17–5.30	3.01	14.86–15.06	15.85–16.07	11.56	11.68	6.27
Derivative under Fourier functions (*D/FF method*)											
First derivative under Fourier functions (*D*1/FF)	2.41–2.45	2.13	3.36–3.44	2.51	5.15–5.23	2.95	15.08–15.24	16.06–16.24	11.50	11.60	6.23
Second derivative under Fourier functions (*D*2/FF)	2.42–2.47	2.11	3.41–3.47	2.54	5.19–5.31	2.98	14.89–15.22	15.89–16.22	11.56	11.67	6.27

[a] Measurement of height of analytical response peak to trough amplitude.
[b] Measurement of absolute peak height.

8-points $\sin x_i$ polynomials (*D*/FF method) at (0.01 min) time interval to get convoluted first derivative curves; *D*1/FF and convoluted second derivative curves; *D*2/FF at 0.01 time interval as follows:

$$t = \frac{(0)D0 + (+0.707)D1 + (+1)D2 + (+0.707)D3 + (0)D4 + (-0.707)D5 + (-1)D6 + (-0.707)D7}{4}$$

where *D*0 to *D*7 stand for the eight derivative values; at 0.01 time interval. The numbers in brackets are values of the selected Fourier function. The derivative values (peak to peak or peak to zero) and the convoluted derivative data (peak to peak or peak to zero) were measured at the corresponding time range for each compound as shown in Table 2, Figures 1 and 2. For each type of linearity $D1_r$, $D2_r$, $D1/FF_r$ and $D2/FF_r$, the response data selected for each compound, Table 2, were divided by the response data selected for the internal standard PPN.

3.6. Assay of mixtures of TRB, GUA and SBZ with overlapping peaks (case II)

The same standard mixtures previously prepared in case I were also used in this case, with the exception that other chromatographic conditions were used. Each mixture solution was chromatographed (Table 1) to get six standard chromatograms of the three overlapping peaks with the internal standard PPN, Figs. 3 and 4. For TRB, GUA, SBZ and PPN, the peak area and peak height were recorded and their ratio were calculated as described in Section 3.5. The response data for the three overlapping peaks were recorded at 0.01 min intervals over the range of 2.02–3.07 min. Then they were chemometrically treated using derivative (*D* method) and derivative/Fourier functions (*D*/FF method) as described in Section 3.5. The derivative (*D*) and convoluted derivative (*D*/FF) values (peak to zero) were measured at the selected points shown in Table 2 over the range of 2.02–2.37, 2.37–2.79 and 2.79–3.07 min for *TRB*, *GUA* and *SBZ*, respectively (Fig. 3). For PPN the values of derivative (*D*) and convoluted derivative (*D*/FF) (peak to zero) were also measured at the selected points shown in Table 2 over the range of 5.9–6.68 min (Fig. 4). The response data were recorded at 0.01 min intervals over the range of 2.02–3.07 min. The $D1_r$, $D2_r$, $D1/FF_r$ and $D2/FF_r$ were applied as described under Section 3.5 (Figs. 3 and 4).

3.7. Assay of mixtures containing very low concentration of BRX (case III)

Accurate volumes of BRX stock solutions were transferred into 10-mL volumetric flasks, with the internal standard PPN, and diluted to volume with the diluting solvent to prepare five standard mixtures of very low concentrations (ranging from limit of detection to limit of quantitation, 5.0–8.0 μg mL^{-1} for BRX while for PPN a suitable concentration of 10.0 μg mL^{-1} was chosen) (Figs. 5 and 6). Triplicate 20-μL injections were made for each solution and chromatographed under the conditions described above for case III. The peak area and height were recorded for BRX and PPN and their ratio were calculated as described in Section 3.5. The chromatograms obtained were then chemometrically treated using derivative (*D* method) then derivative/Fourier functions (*D*/FF method) and the $D1_r$, $D2_r$, $D1/FF_r$ and $D2/FF_r$ were applied as described under Section 3.5 (Figs. 5 and 6).

3.8. Assay of pharmaceutical formulation

A volume of 10 mL of the syrup (Allvent® syrup labeled to contain 1.25 mg TRB, 50 mg GUA, 4 mg BRX and 12.5 mg SBZ per 5 mL) was accurately transferred into a 50-mL volumetric flask and completed to volume with the diluting solvent. 1.0 mL of this stock solution was transferred to 10 mL volumetric flask with the internal standard PPN and completed to volume using the diluting solvent, so that a suitable concentration of PPN 40 μg mL^{-1} was obtained. The prepared solution was then chromatographed as under the method described for cases I and II (Fig. 7a and b, Table 1). The data were also processed as described above under Sections 3.5 and 3.6.

4. Results and discussion

4.1. Selection of chromatographic conditions

Practical trials showed that isocratic elution could not be applied for the resolution of the mixture of TRB, GUA, SBZ, BRX and PPN. So a gradient elution was used to resolve this mixture with good chromatographic characteristics (case I). The HPLC method achieved a good resolution of the mixture components (good resolution and selectivity values) within reasonable run time (suitable capacity factors).

4.1.1. Choice of wavelength

Since the response of BRX was favored as it is a weakly absorbing compound compared to the other compounds in the mixture, a wavelength of 212 nm corresponding to λ_{max} of BRX was chosen for the analysis. It was found to be suitable to the other compounds in their ratios present in the syrup.

4.1.2. Choice of internal standard

Under the above selected conditions for case I, several compounds were tried to choose the most suitable internal standard (paracetamol, baclofen, theophylline, metochlorperamide HCl,

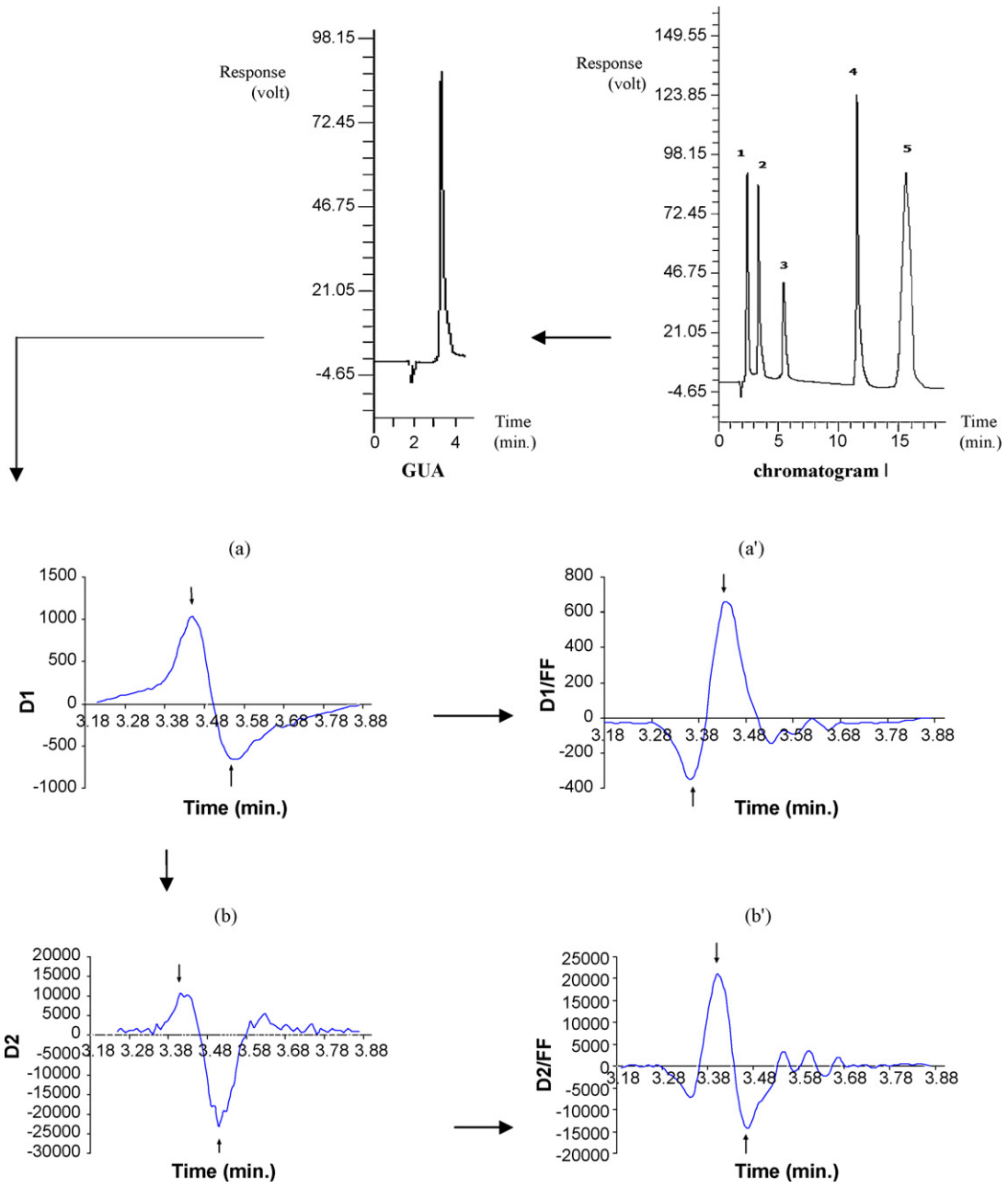

Fig. 1. First derivative (a) and second derivative (b) curves of 50 μg mL^{-1} guaiphenesin (GUA) and their corresponding convoluted Fourier function curves (a′) and (b′), in the ideal case I, derived from chromatogram I, which represent a synthetic mixture of (1) 50 μg mL^{-1} terbutaline sulphate, (2) 50 μg mL^{-1} guaiphenesin, (3) 20 μg mL^{-1} sodium benzoate, (5) 100 μg mL^{-1} bromhexine HCl with (4) internal standard (propylparaben) 40 μg mL^{-1} in the ideal case I.

propyphenazone and propylparaben). Among all, propylparaben (PPN) was selected since it had reasonable retention time since it was eluted between the peaks of the studied compounds with good peak shape. A concentration of 40 μg mL^{-1} was chosen since it gave a moderate response.

4.1.3. Choice of the mobile phase

As done in our previous work [8,9], mobile phase composition was dependant on the intended outcome to be studied. In case II, it was meant to be a non-ideal chromatogram by varying the percentage of acetonitrile.

4.1.3.1. Effect of the organic modifier. The mobile phase used was 0.02 M phosphate buffer mixed with 0.15% TEA (v/v %) adjusted to pH 3.0 and various proportions of acetonitrile. The standards were thus injected and run with mobile phases of different composition. Practical trials showed that a single mobile phase composition could not be used to resolve the mixture. Fig. 8(a) shows the retention times obtained for the compounds TRB, GUA, SBZ and BRX as a function of acetonitrile percentage in the mobile phase. As can be seen, 30% acetonitrile provided optimum resolution with the most symmetric and well-defined peaks for TRB, GUA and SBZ, but the retention time of BRX was more than 1 h which is considered to be impractical for HPLC technique. At higher acetonitrile concentrations, BRX retention time decreased but TRB and GUA peaks became overlapped. Thus isocratic elution could not be applied. A gradient elution was applied as follows, 30% acetonitrile for 5 min to get TRB and GUA peaks with good resolution then a transition state of 3 min at which the SBZ peak comes out before applying 70% acetonitrile to get BRX at a reasonable retention time. Several gradient profiles

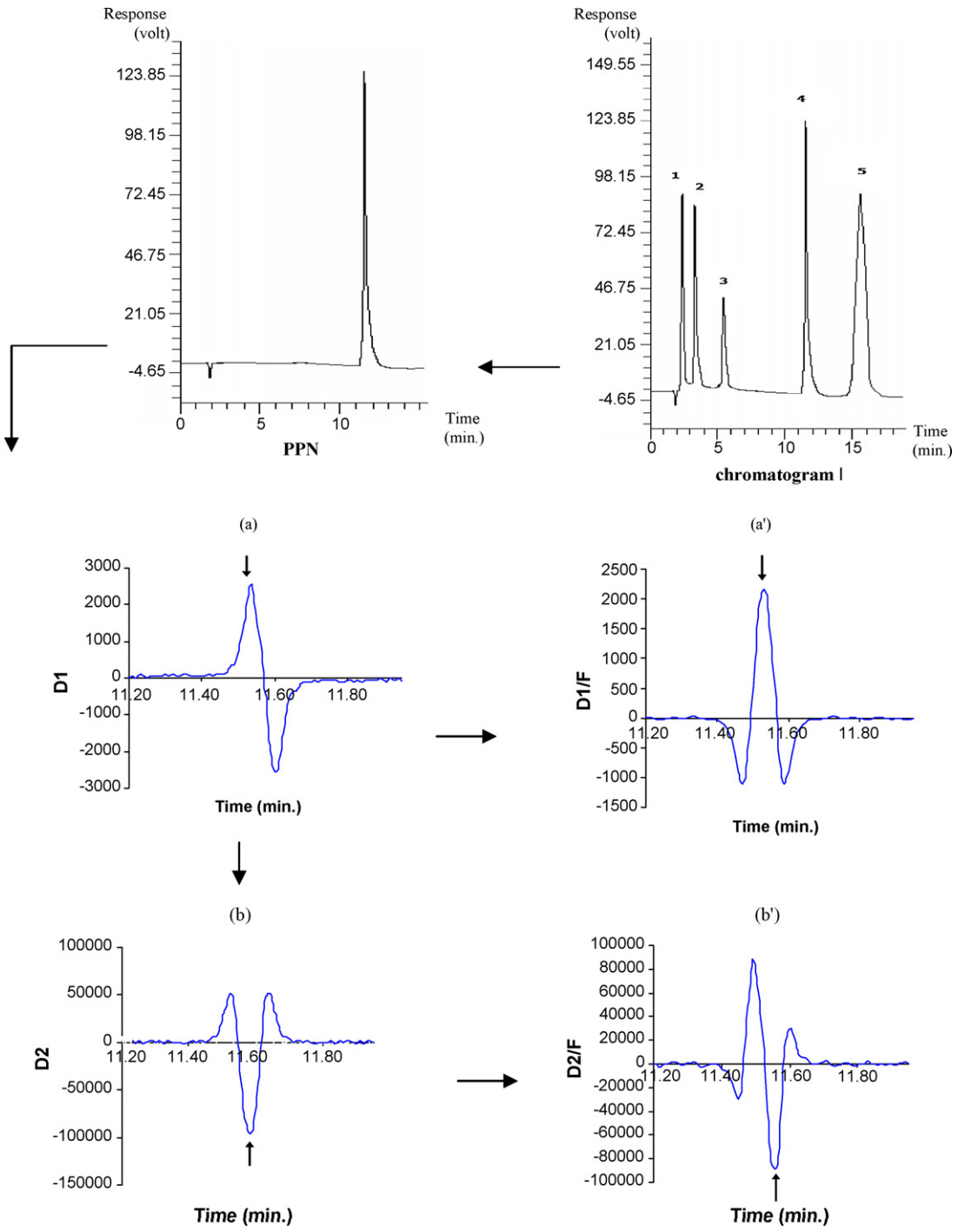

Fig. 2. First derivative (a) and second derivative (b) curves of 40 μg mL^{-1} propylparaben (PPN) and their corresponding convoluted Fourier function curves (a′) and (b′), in the ideal case I, derived from chromatogram I, which represent a synthetic mixture of the same composition as in Fig. 1.

were tested, linear, concave and convex [22]. The selectivity of the separation remained unaffected for all the tested gradients. Among all of them, a linear one was selected and it yielded the best compromise in terms of resolution, run time and noise of the base line.

4.1.3.2. Effect of pH. To facilitate the study of the pH, an isocratic system consisting of 70:30 acetonitrile: 0.02 M phosphate buffer containing 0.15% TEA was used. The influence of the pH of the buffer system was studied by using the previously mentioned isocratic system at various pH values (2.7–5.5 adjusted using orthophosphoric acid). These solutions were used as the mobile phase for the analytes. Fig. 8(b) illustrates the effect of the buffer pH on the retention times of the four compounds. The pH had nearly no effect on TRB and GUA. However, an increase in the pH results in an increase in the retention time of BRX. It was noticed that increasing the pH to 6.0 or more resulted in precipitation of the bromhexine base in the prepared solutions. Consequently, a pH 3.0 was chosen since it provided the most symmetric, well-defined BRX peak within reasonable retention time (15.0 min).

4.1.3.3. Effect of ion-pairing reagent. To facilitate the study of the ion-pairing reagent, various concentrations of TEA in the previously mentioned isocratic system were used at pH 3. Fig. 8(c) illustrates the effect of TEA concentration on the retention times

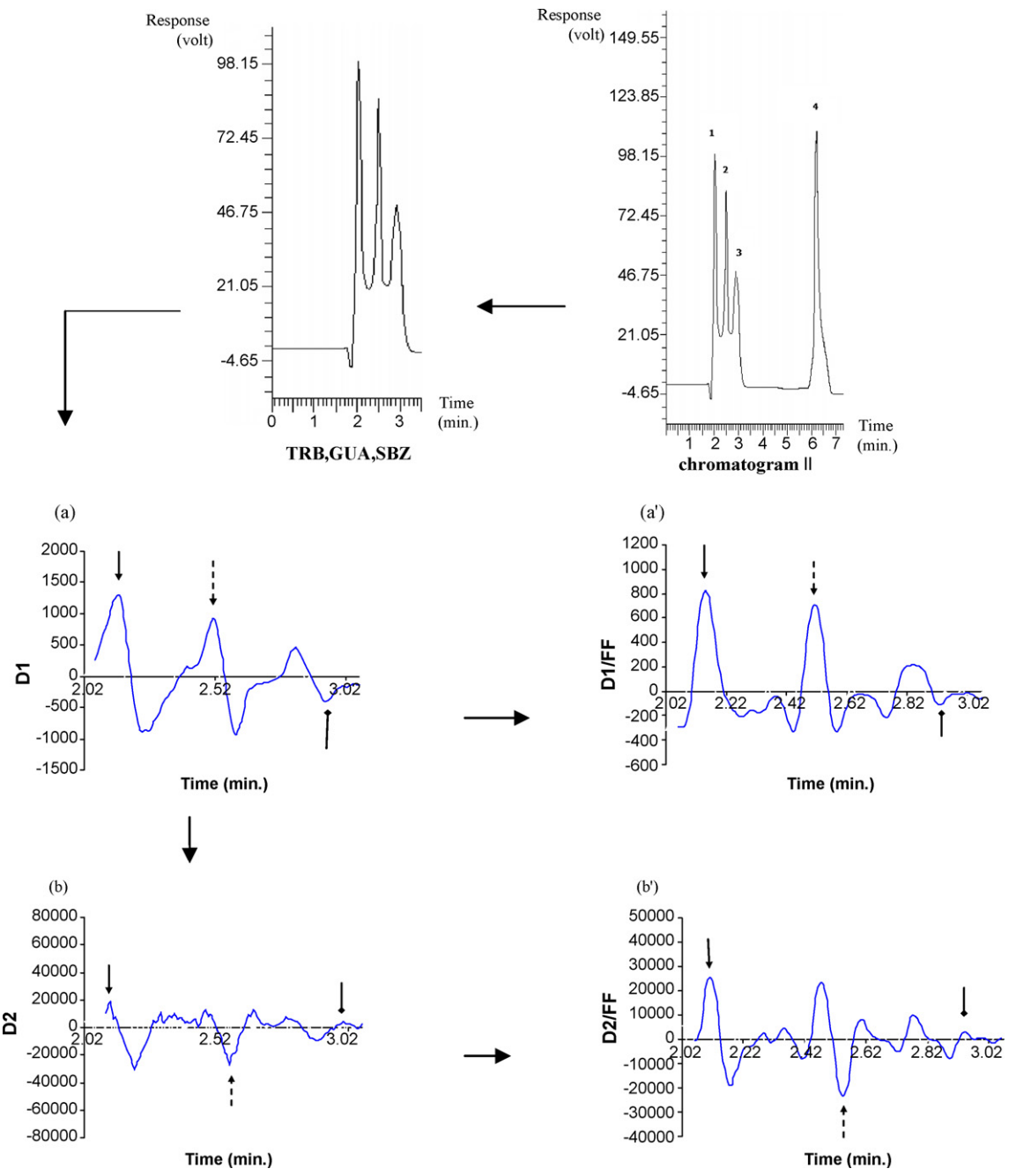

Fig. 3. First derivative (a) and second derivative (b) curves of a synthetic mixture of 50 μg mL^{-1}- terbutaline sulphate (TRB), (——▶) 50 μg mL^{-1}- guaiphenesin (GUA), (– – –▶) 20 μg mL^{-1} sodium benzoate (SBZ), (——◆) and their corresponding convoluted Fourier function curves (′) and (′, in case overlapping peaks (case II), derived from chromatogram II, which represent a synthetic mixture of (1) 50 μg mL^{-1}- terbutaline sulphate, (2) 50 μg mL^{-1} guaiphenesin, (3) 20 μg mL^{-1}- sodium benzoate, with (4) internal standard (propylparaben) 40 μg mL^{-1} in the non-ideal case II.

of the four compounds. TEA had nearly no effect on the retention time of TRB and GUA, but it had a marked effect on the retention time of BRX. It was noticed that as the concentration of TEA increases, the retention time of BRX decreases. Without using TEA, BRX retention time was more than 1 h. A concentration of 0.15% TEA in the buffer system was found optimum in increasing the sharpness and decreasing the tailing of BRX peak. This could be explained by the fact that TEA in acid medium can be used to block residual silanol groups on the silica gel backbone of bonded phase columns. This is useful for the analysis of ionized nitrogenous compounds which might interact with these silanols. In this respect, TEA was used to prevent this undesirable interaction.

4.1.3.4. Effect of buffer strength. In all cases, the aqueous phase used was phosphate buffer (0.02 M Na_2HPO_4 in water) containing TEA solution (0.15%, v/v). 0.02 M phosphate buffer strength was found optimum regarding the peak shape where lower buffer strength produced tailed peaks of BRX.

4.2. Treatment of analytical data

4.2.1. Application of derivative technique (D method) to chromatographic response data

Derivative calculations were applied to response data of the chromatographed mixtures of the previously mentioned three cases in the external and internal standard methods. As men-

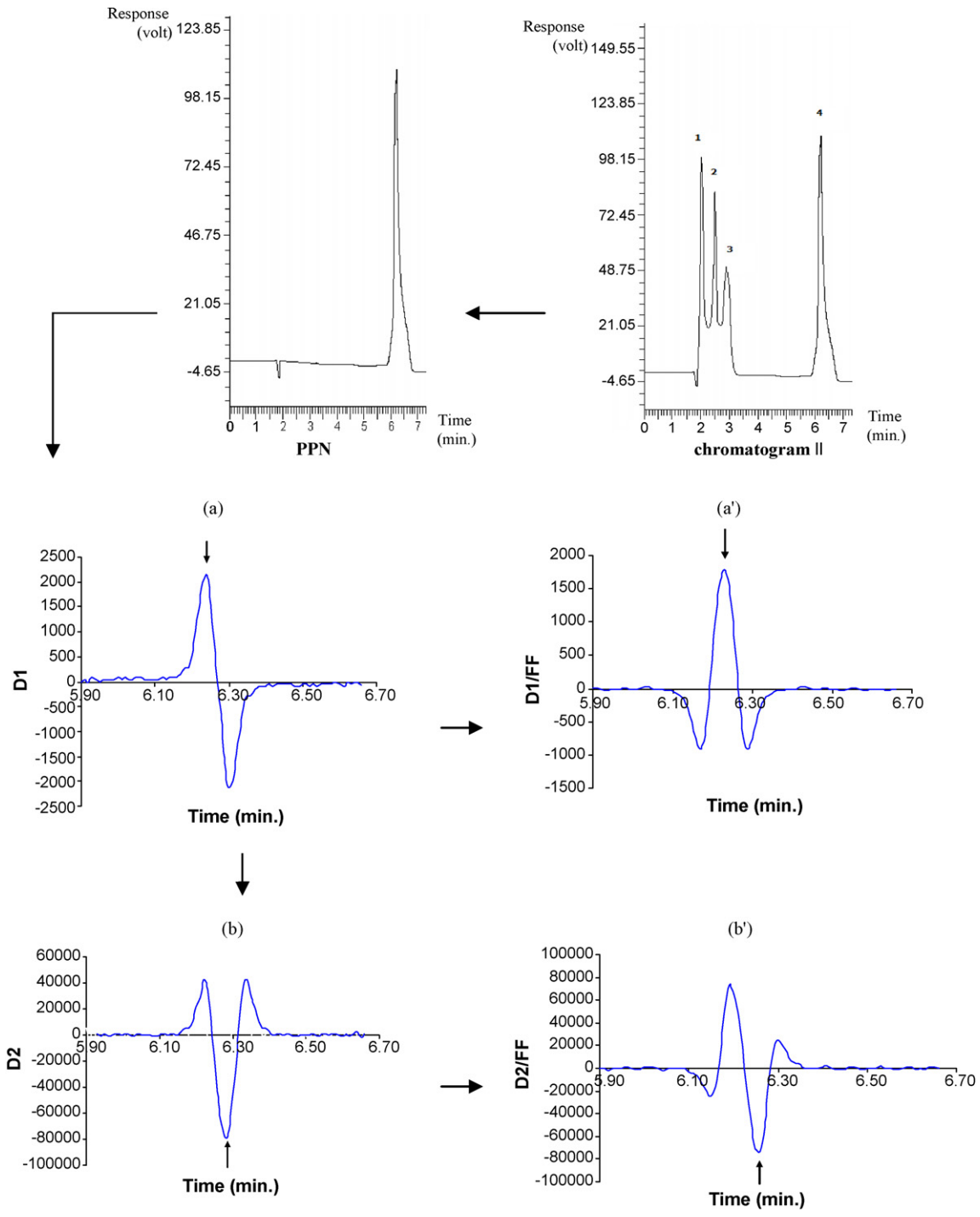

Fig. 4. First derivative (a) and second derivative (b) curves of 40 μg mL^{-1} propylparaben (PPN) and their corresponding convoluted Fourier function curves (a′) and (b′), in the non-ideal case II , derived from chromatogram II, which represent a synthetic mixture of the same composition as in Fig. 3.

tioned before in our previous work [8,9], derivative methods can be applied when direct measurement exhibits some kind of interference. Constant interferences could be eliminated by calculating the first derivative (*D*1), while second derivative (*D*2) can eliminate any linear interference. For each case, the *D*1 and *D*2 values at the selected points (Table 2) at 0.01 time interval for each of the four compounds were correlated to the concentration. As an example, Table 3 showed the parameters of GUA in cases I and II, while Table 4 showed the results of BRX in cases I and III. The points selected for case II (overlapping peaks) were based on that, maximum response was obtained for each compound at these points with nearly zero contribution of the others. Also in the case of very low concentrations (case III), the selected points were based on that, the background noise was neglected and the compound studied at each point was of maximum response.

4.2.2. Application of Fourier functions to derivative data (D/FF method)

For each case, the first and second derivative curves were convoluted using 8-points $\sin x_i$ polynomials at 0.01 time intervals then the optimum convoluted *D*1/FF, *D*2/FF, values selected for each of the four compounds in the external and internal standard

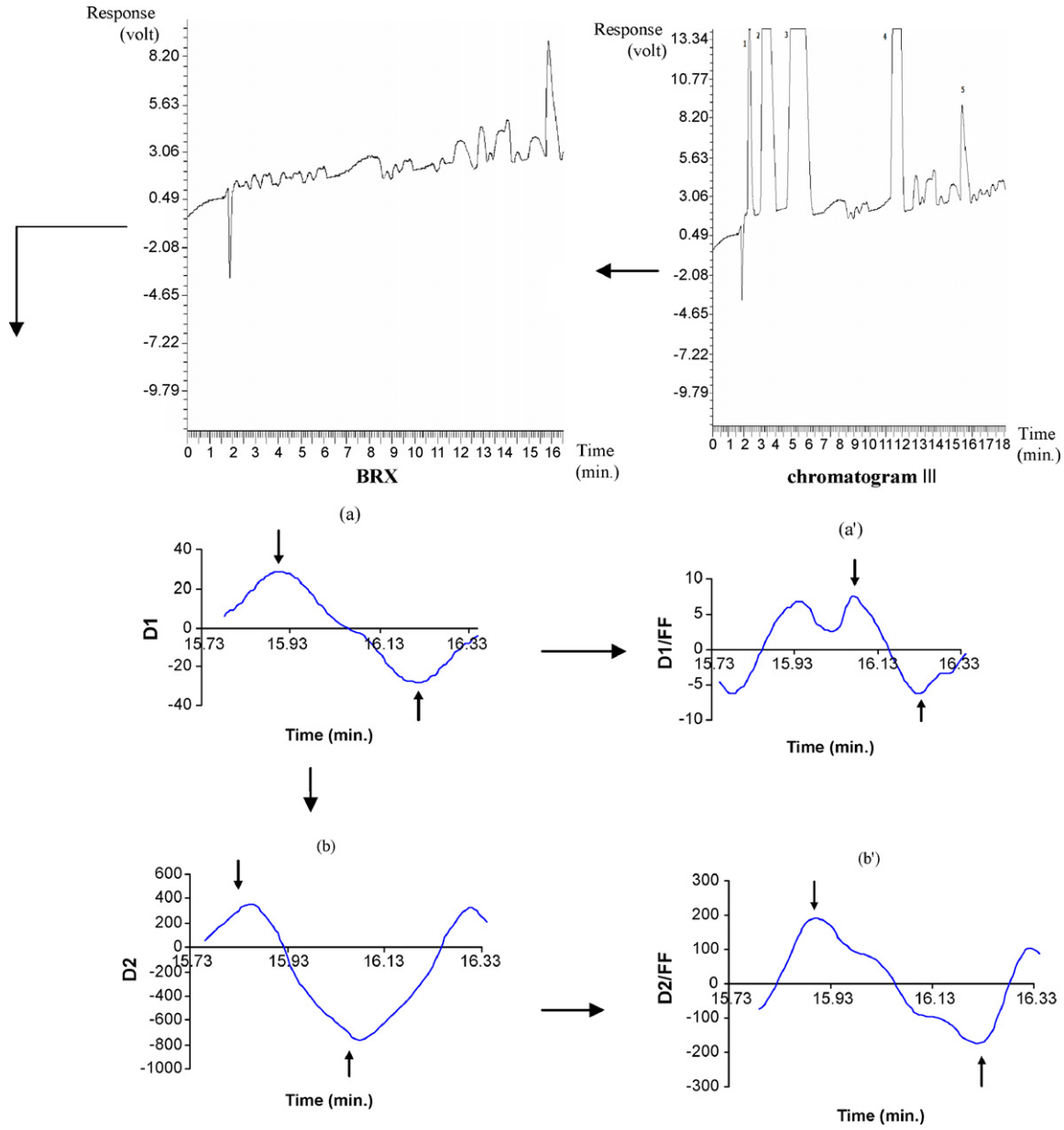

Fig. 5. First derivative (a) and second derivative (b) curves of 5 μg mL^{-1} bromhexine HCl (BRX) and their corresponding convoluted Fourier function curves (a′) and (b′), in the minor concentration case III, derived from chromatogram III, which represent a synthetic mixture of (5) bromhexine HCl 5 μg mL^{-1} with (1) terbutaline sulphate, (2) guaiphenesin, (3) sodium benzoate and (4) internal standard (propylparaben) 10 μg mL^{-1} in the non-ideal case III.

method were related to concentration. As mentioned previously in the authors' previous work on HPLC [8] and HPTLC [9] response data, convolution using Fourier functions corrects all types of interferences except for linear interference. Thus application of Fourier functions on derivative data would eventually lead to removal of all types of interference producing high degree of purity of the analytical peaks at the selected points. This would be beneficial in cases where high incidence of interferences could be found from other mixture components, as in the case of overlapping peaks (case II), at which the selected points would represent the pure compound and neglect the other interfering compounds. Also in the case of very low concentrations (case III), the selected points were based on that, the background noise was neglected and the compound studied at each point was of maximum response.

5. Methods validation

5.1. Parametric calibration graphs and statistical data

Under the previously described chromatographic conditions for each of the three cases, the graphs obtained by plotting derivative and convoluted derivative data either in the external or internal standard method versus concentration for each of the four compounds, show various degrees of linearity and were compared to those obtained using the peak area and peak height as the response signals. Using the method of least squares, regression equations, correlation coefficients (r), intercepts (a) and slopes (b) were calculated. GUA was taken as an example in cases I, II and BRX in cases I and III (Tables 3 and 4).

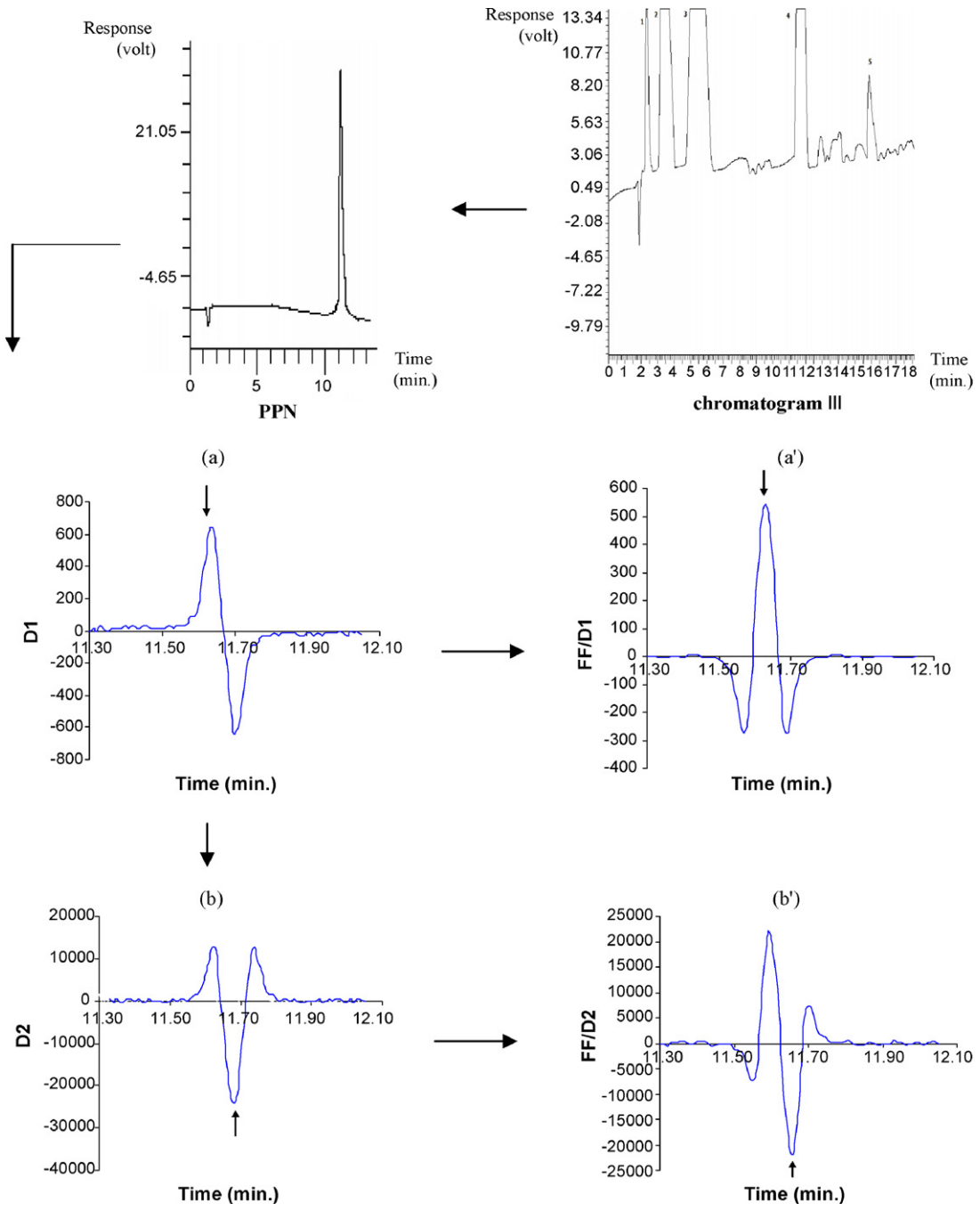

Fig. 6. First derivative (a) and second derivative (b) curves of 10 μg mL^{-1} propylparaben (PPN) and their corresponding convoluted Fourier function curves (a′) and (b′),in the non-ideal case III , derived from chromatogram III , which represent a synthetic mixture of the same composition as in Fig. 5.

5.2. Application of non-parametric regression methods

The statistical parameters concerning the parametric method have all assumed that data being examined follow the normal (Gaussian) distribution. Some support for this assumption is provided by the central limit theorem, which shows that the sampling distribution of the mean may be approximately normal. However, the theorem is not really valid for the very small data sets (often only three or four readings) frequently used in analytical work [17].

There are several further reasons of an interest in methods that do not require the assumption of normally distributed data. Some sets of data that are of interest to analytical chemists certainly have different distributions [17].

This paper introduces a statistical method for handling data that may not be normally distributed. Methods which make no assumptions about the shape of the distribution from which the data are taken are called non-parametric or distribution free methods.

There are several non-parametric methods that can be used for fitting a straight line to a set of points. Of the several methods available, perhaps the simplest is Theil's "incomplete" method which was first applied to the data in the author's previous work [8]. It was also applied to the data in our study. When the normal distribution is assumed, the arithmetic mean as the 'measure of central tendency' of a set of results is to be used. In non-parametric statistics, the median is usually used instead as in many cases it is a more realistic measure of central tendency than the arithmetic mean

Table 3
Parametric linear regression and statistical parameters for the determination of guaiphenesin (GUA) by the proposed HPLC method (ideal and non-ideal cases).

	Ideal case I					Non-ideal case II				
	r	*a*	*b*	LOD[a]	LOQ[a]	*r*	*a*	*b*	LOD[a]	LOQ[a]
(I) External standard method										
Direct measurement										
Peak area	0.9995	65,315	45,867	1.00	3.32	0.9987	87,251	44,124	5.14	17.14
Peak height	0.9994	4698	3274	1.15	3.83	0.9983	12,633	4656	5.49	18.30
Derivative technique (*D method*)										
First derivative (*D*1)	0.9996	42.65	29.93	0.92	3.08	0.9998	54.3	37.5	1.19	3.96
Second derivative (*D*2)	0.9997	1452	1019	0.91	3.04	0.9998	2119	1462	1.07	3.56
Derivative under Fourier functions (*D/FF method*)										
First derivative under Fourier functions (*D*1/FF)	0.9998	23.17	16.27	0.92	3.07	0.9998	38.11	26.3	1.14	3.80
Second derivative under Fourier functions (*D*2/FF)	0.9998	826	579.7	0.59	1.97	0.9998	1620	1118	0.95	3.15
(II) Internal standard method										
Direct measurement										
Peak area ratio	0.9996	0.027	0.014	0.86	2.86	0.9986	0.035	0.014	5.57	18.57
Peak height ratio	0.9996	0.023	0.012	1.00	3.33	0.9982	0.054	0.017	5.47	18.24
Derivative technique (*D method*)										
First derivative (*D*1) ratio	0.9997	0.022	0.012	0.75	2.50	0.9997	0.055	0.029	1.14	3.79
Second derivative (*D*2) ratio	0.9998	0.015	0.008	0.75	2.50	0.9997	0.044	0.023	1.03	3.43
Derivative under Fourier functions (*D/FF method*)										
First derivative under Fourier functions (*D*1/FF) ratio	0.9998	0.014	0.007	0.86	2.86	0.9998	0.046	0.024	1.13	3.75
Second derivative under Fourier functions (*D*2/FF) ratio	0.9999	0.012	0.007	0.43	1.43	0.9999	0.048	0.025	0.84	2.80

r: correlation coefficient, *a:* intercept, *b*: slope, LOD: limit of detection, LOQ: limit of quantitation.
[a] Concentration μg mL^{-1}.

[17]. Theil's method determines the slope of a regression line as the median of the slopes calculated from selected pairs of points: the intercept of the line is the median of the intercept values calculated from the slopes and the coordinates of the individual points [17].

For all of the previously mentioned types of linearity, and for each of the three cases, the response data were handled using Theil's method. The best-fit straight line obtained using Theil's method was compared with the least squares best-fit line calculated using the parametric regression method. Tables 5–8 represent an example illustrating that the non-parametric regression model could be considered superior over the parametric one and this was proved by calculating the percentage change in the intercept and slope. In the majority of cases, the intercept decreases and the slope increases. Taking Table 5 as an illustrating example, it can be seen that the percentage change in the intercept when applying the non-parametric relative to the parametric models was from −88.55 to −98.16 and that in most cases, the intercept decreases almost near the origin when applying the non-parametric regression model. However, the percentage change in slope was almost negligible (from +1.38 to +1.54).

As was mentioned in the authors' previous work [8], when comparing the results of the Theil's method with that of the parametric one, it was found that Theil's method had three distinct advantages over the least squares method: first, it does not assume that all the errors are in the *y*-direction; second, it does not assume that either the *x*- or *y*- direction errors are normally distributed; and third it is not affected by the presence of outlying results. Generally, an outlier value does not affect the Theil's calculation at all since it does not affect the median estimate of the slope or intercept. In the least squares calculation, however, the outlying point carries as much weight as the other points. This leads to the fact that, the least

Table 4
Parametric linear regression and statistical parameters for the determination of bromhexine HCl (BRX) by the proposed HPLC method (ideal and non-ideal cases).

	Ideal caseI					Non-ideal case III				
	r	*a*	*b*	LOD[a]	LOQ[a]	*r*	*a*	*b*	LOD[a]	LOQ[a]
(I) External standard method										
Direct measurement										
Peak area	0.9997	231,158	192,187	2.74	9.13	0.994	9310	16,850	2.18	7.26
Peak height	0.9997	3640	1814	2.98	9.94	0.9947	930	1684	2.21	7.39
Derivative technique (*D method*)										
First derivative (*D*1)	0.9997	21.2	13.37	1.46	4.86	0.9993	−2.13	12.86	0.23	0.79
Second derivative (*D*2)	0.9998	281	175	1.35	4.51	0.9995	−53.3	224	0.11	0.37
Derivative under Fourier functions (*D/FF method*)										
First derivative under Fourier functions (*D*1/FF)	0.9998	4.55	2.8	0.98	3.25	0.9996	−0.80	2.96	0.10	0.34
Second derivative under Fourier functions (*D*2/FF)	0.9999	105	66	0.92	3.06	0.9996	−18.95	81.5	0.095	0.32
(II) Internal standard method										
Direct measurement										
Peak area ratio	0.9997	0.046	0.062	2.47	8.23	0.995	0.169	0.248	2.14	7.13
Peak height ratio	0.9998	0.011	0.007	2.57	8.57	0.9950	0.017	0.024	2.25	7.50
Derivative technique (*D method*)										
First derivative (*D*1) ratio	0.9998	0.004	0.005	0.66	2.00	0.9994	−0.0065	0.020	0.22	0.74
Second derivative (*D*2) ratio	0.9998	0.0012	0.0014	0.64	1.94	0.9995	−0.0019	0.007	0.17	0.57
Derivative under Fourier functions (*D/FF method*)										
First derivative under Fourier functions (*D*1/FF) ratio	0.9999	0.00136	0.00131	0.45	1.43	0.9996	−0.0011	0.005	0.15	0.5
Second derivative under Fourier functions (*D*2/FF) ratio	0.9999	0.00036	0.00075	0.24	0.81	0.9997	−0.0009	0.004	0.08	0.25

r: coefficient, *a*: intercept, *b*: slope, LOD: limit of detection, LOQ: limit of quantitation.
[a] Concentration μg mL^{-1}.

Table 5
Comparison between parametric and non-parametric regression models for the determination of guaiphenesin (GUA), by the proposed HPLC method in ideal case (I).

	$\|a\|$[a]		$\|b\|$[b]		Percentage change in $\|a\|$[c]	Percentage change in $\|b\|$[d]
	Parametric	Non-parametric	Parametric	Non-parametric		
External standard method						
Direct measurement						
Peak area	65,315.150	1263.200	45,867.40	46,499.9	−98.07	1.38
Peak height	4698.370	107.44	3274.28	3323.32	−97.71	1.50
Derivative technique (*D method*)						
First derivative (*D*1)	42.65	0.852600	29.930	30.362700	−98.00	1.45
Second derivative (*D*2)	1452.3450	29.060	1019.150	1033.790	−98.00	1.44
Derivative under Fourier functions (*D/FF method*)						
First derivative under Fourier functions (*D*1/FF)	23.1747	0.4263	16.2730	16.507300	−98.16	1.44
Second derivative under Fourier functions (*D*2/FF)	826.040	16.660	579.990	587.990	−97.98	1.38
Internal standard method						
Direct measurement						
Peak area ratio	0.027373	0.002154	0.014510	0.014720	−92.13	1.45
Peak height ratio	0.022958	0.002628	0.011972	0.012156	−88.55	1.54
Derivative technique (*D method*)						
First derivative (*D*1) ratio	0.021600	0.001500	0.011577	0.011742	−93.06	1.43
Second derivative (*D*2) ratio	0.015310	0.001105	0.008136	0.008259	−92.78	1.51
Derivative under Fourier functions (*D/FF method*)						
First derivative under Fourier functions (*D*1/FF) ratio	0.013690	0.000632	0.007427	0.007537	−95.38	1.48
Second derivative under Fourier functions (*D*2/FF) ratio	0.012270	0.001053	0.006505	0.006595	−91.42	1.38

[a] Modulus of intercept.
[b] Modulus of slope.
[c] Percentage change in $|a|$ means percentage change in $|a|$ of $P = [(|a|$ of NP $- |a|$ of $P)/|a|$ of $P] \times 100$.
[d] Percentage change in $|b|$ means percentage change in $|b|$ of $P = [(|b|$ of NP $- |b|$ of $P)/|b|$ of $P] \times 100$.

squares line passes closer to the outlier than the non-parametric line does. This can be illustrated by Figs. 9–12 which represent the *D*1/FF and *D*2/FF in the external and internal standard methods for GUA as an example.

5.3. Detection and quantitation limits

Limit of detection (LOD) according to Miller [17] is equal to $y_B + 3S_B$ where y_B is the value of the calculated intercept and S_B is the $S_{y/x}$ while limit of quantitation LOQ is equal to $y_B + 10S_B$. LOD and LOQ for each compound at each case were calculated. As an example, Tables 3 and 4 showed the results of GUA in cases I, II and BRX in cases I, III respectively. LOD and LOQ obtained after the treatment of data using the derivative and convoluted derivative in the external and internal standard method in the ideal case I were lower than those obtained before the treatment of data. For the non-ideal cases II and III, higher LOD and LOQ were obtained in the direct measurement than those in the ideal case. However,

Table 6
Comparison between parametric and non-parametric regression models for the determination of guaiphenesin (GUA), by the proposed HPLC method in non-ideal case II.

	$\|a\|$[a]		$\|b\|$[b]		Percentage change in $\|a\|$[c]	Percentage change in $\|b\|$[d]
	Parametric	Non-parametric	Parametric	Non-parametric		
External standard method						
Direct measurement						
Peak area	87,251.7	35,515.2	44,124.90	46,342.80	−59.30	5.03
Peak height	12,633.9	2024.5	4656.716	4982.71	−83.98	7.00
Derivative technique (*D method*)						
First derivative (*D*1)	54.3020	1.10870	37.470	38.0170	−97.96	1.46
Second derivative (*D*2)	2119.11	43.26	1462.56	1483.6	−97.96	1.44
Derivative under Fourier functions (*D/FF method*)						
First derivative under Fourier functions (*D*1/FF)	38.11	0.778	26.308	26.686	−97.96	1.44
Second derivative under Fourier functions (*D*2/FF)	1620.48	33.08	1118.4	1134.49	−97.96	1.44
Internal standard method						
Direct measurement						
Peak area ratio	0.034982	0.007630	0.014419	0.015305	−78.19	6.14
Peak height ratio	0.054481	0.005280	0.017075	0.018250	−90.31	6.88
Derivative technique (*D method*)						
First derivative (*D*1) ratio	0.055400	0.004300	0.028990	0.029430	−92.24	1.52
Second derivative (*D*2) ratio	0.044600	0.003470	0.023350	0.023700	−92.22	1.50
Derivative under Fourier functions (*D/FF method*)						
First derivative under Fourier functions (*D*1/FF) ratio	0.045880	0.003570	0.024012	0.024370	−92.22	1.49
Second derivative under Fourier functions (*D*2/FF) ratio	0.0480	0.0037	0.0251	0.0255	−92.29	1.59

[a] Modulus of intercept.
[b] Modulus of slope.
[c] Percentage change in $|a|$ means percentage change in $|a|$ of $P = [(|a|$ of NP $- |a|$ of $P)/|a|$ of $P] \times 100$.
[d] Percentage change in $|b|$ means percentage change in $|b|$ of $P = [(|b|$ of NP $- |b|$ of $P)/|b|$ of $P] \times 100$.

Table 7
Comparison between parametric and non-parametric regression models for the determination of bromhexine HCl (BRX), by the proposed HPLC method in ideal case I.

	$\|a\|$[a]		$\|b\|$[b]		Percentage change in $\|a\|$[c]	Percentage change in $\|b\|$[d]
	Parametric	Non-parametric	Parametric	Non-parametric		
External standard method						
Direct measurement						
Peak area	231,158.7	135,293.5	192,187	192,074.80	−41.47	−0.06
Peak height	3640.65	1666.135	1814.012	1832.67	−54.24	1.03
Derivative technique (*D method*)						
First derivative (*D*1)	21.2	9.18	13.36	13.42	−56.7	0.45
Second derivative (*D*2)	281	120.5	175	175.9	−57.1	0.50
Derivative under Fourier functions (*D/FF method*)						
First derivative under Fourier functions (*D*1/FF)	4.59	1.96	2.85	2.86	−57.3	0.35
Second derivative under Fourier functions (*D*2/FF)	105.7	44.65	66.00	66.30	−57.8	0.45
Internal standard method						
Direct measurement						
Peak area ratio	0.046070	0.040244	0.061569	0.061707	−12.65	0.22
Peak height ratio	0.011017	0.012020	0.006715	0.006696	9.10	−0.28
Derivative technique (*D method*)						
First derivative (*D*1) ratio	0.0039	0.0045	0.0052	0.0052	15.4	0.00
Second derivative (*D*2) ratio	0.0012	0.00116	0.00142	0.00142	−3.33	0.00
Derivative under Fourier functions (*D/FF method*)						
First derivative under Fourier functions (*D*1/FF) ratio	0.0014	0.0015	0.001314	0.001312	7.14	−0.15
Second derivative under Fourier functions (*D*2/FF) ratio	0.0004	0.00043	0.00075	0.00075	7.50	0.00

[a] Modulus of intercept.
[b] Modulus of slope.
[c] Percentage change in $|a|$ means percentage change in $|a|$ of $P = [(|a|$ of NP $- |a|$ of $P)/|b|$ of $P] \times 100$.
[d] Percentage change in $|b|$ means percentage change in $|b|$ of $P = [(|b|$ of NP $- |b|$ of $P)/|b|$ of $P] \times 100$.

these values became lower after the treatment of data using the derivative and convoluted derivative in the external and internal standard method.

5.4. Precision and accuracy

For the parametric regression method, in order to assess the precision, as percentage relative standard deviation (RSD%) and the accuracy, as mean percentage recovery, triplicate determinations were carried out on laboratory-made mixtures of different proportions with or without the internal standard for the external and internal standard methods, respectively, for the two cases I and II. For case III, the determinations were carried out on BRX different concentrations either alone or with the internal standard.

For the non-ideal cases II and III, a collective Table 9 showed bad precision and accuracy for the studied compounds in their synthetic mixtures. However, when derivative and convoluted derivative either in the external or internal standard methods were applied, the (RSD%) and mean percentage recovery became in the accepted ranges of each compound indicating good precision and accuracy.

For the non-parametric regression method, the same calculations were done as the parametric method except that the (RSD%)

Table 8
Comparison between parametric and non-parametric regression models for the determination of bromhexine HCl (BRX), by the proposed HPLC method in non-ideal case III.

	$\|a\|$[a]		$\|b\|$[b]		Percentage change in $\|a\|$[c]	Percentage change in $\|b\|$[d]
	Parametric	Non-Parametric	Parametric	Non-Parametric		
External standard method						
Direct measurement						
Peak area	9310	6390	16,850	17,210	−31.36	2.13
Peak height	930	640	1684	1725	−31.18	2.43
Derivative technique (*D method*)						
First derivative (*D*1)	2.13	2.2	12.86	12.85	3.28	−0.08
Second derivative (*D*2)	53.25	52.5	224.9	224.25	−1.40	−0.29
Derivative under Fourier functions (*D/FF method*)						
First derivative under Fourier functions (*D*1/FF)	0.8	0.775	2.96	2.95	−3.12	−0.33
Second derivative under Fourier functions (*D*2/FF)	18.95	18.5	81.5	81.25	−2.37	−0.31
Internal standard method						
Direct measurement						
Peak area ratio	0.169	0.097	0.248	0.252	−42.6	1.61
Peak height ratio	0.0167	0.0098	0.0244	0.025	−41.3	2.45
Derivative technique (*D method*)						
First derivative (*D*1) ratio	0.0065	0.006	0.0206	0.0205	−7.7	−0.49
Second derivative (*D*2) ratio	0.00192	0.0018	0.00728	0.00725	−5.25	−0.41
Derivative under Fourier functions (*D/FF method*)						
First derivative under Fourier functions (*D*1/FF) ratio	0.0011	0.0012	0.0054	0.00538	9.09	−0.37
Second derivative under Fourier functions (*D*2/FF) ratio	0.00094	0.00085	0.00372	0.0037	−9.60	−0.54

[a] Modulus of intercept.
[b] Modulus of slope.
[c] Percentage change in $|a|$ means percentage change in $|a|$ of $P = [(|a|$ of NP $- |a|$ of $P)/|a|$ of $P] \times 100$.
[d] Percentage change in $|b|$ means percentage change in $|b|$ of $P = [(|b|$ of NP $- |b|$ of $P)/|b|$ of $P] \times 100$.

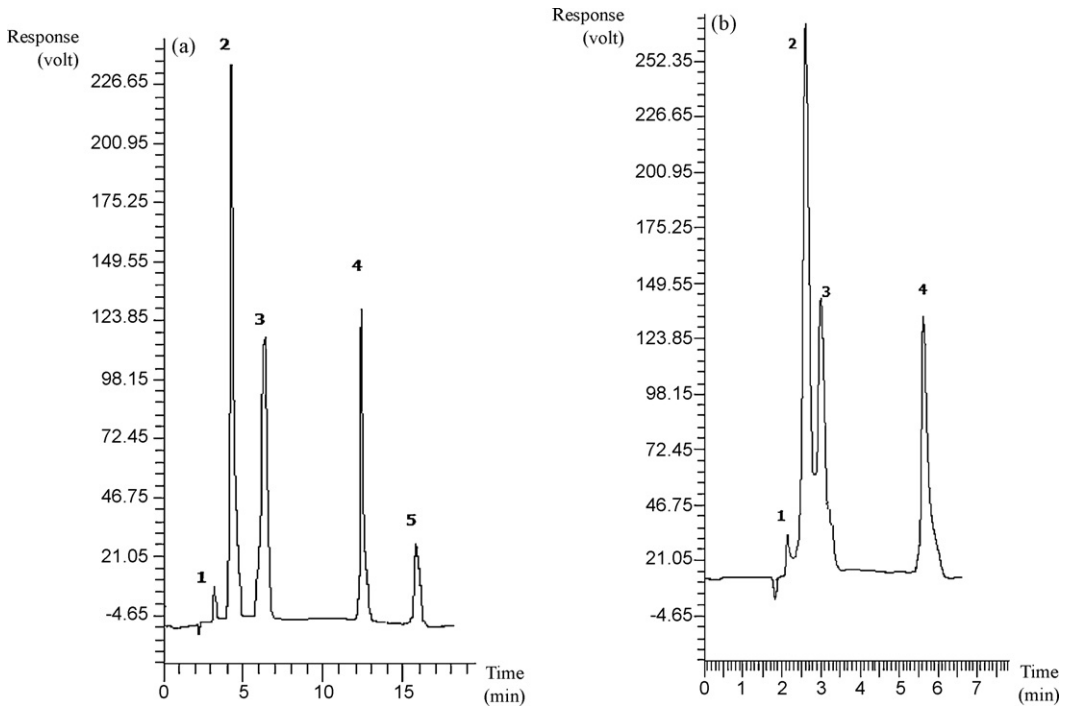

Fig. 7. Chromatograms of dosage form of (1) 5 μg mL^{-1} terbutaline sulphate, (2) 200 μg mL^{-1} guaiphenesin, (3) 50 μg mL^{-1} sodium benzoate, (4) 40 μg mL^{-1} internal standard (propylparaben) and (5) 16 μg mL^{-1} bromhexine HCl in the ideal case I (a) and in the non-ideal case II (b).

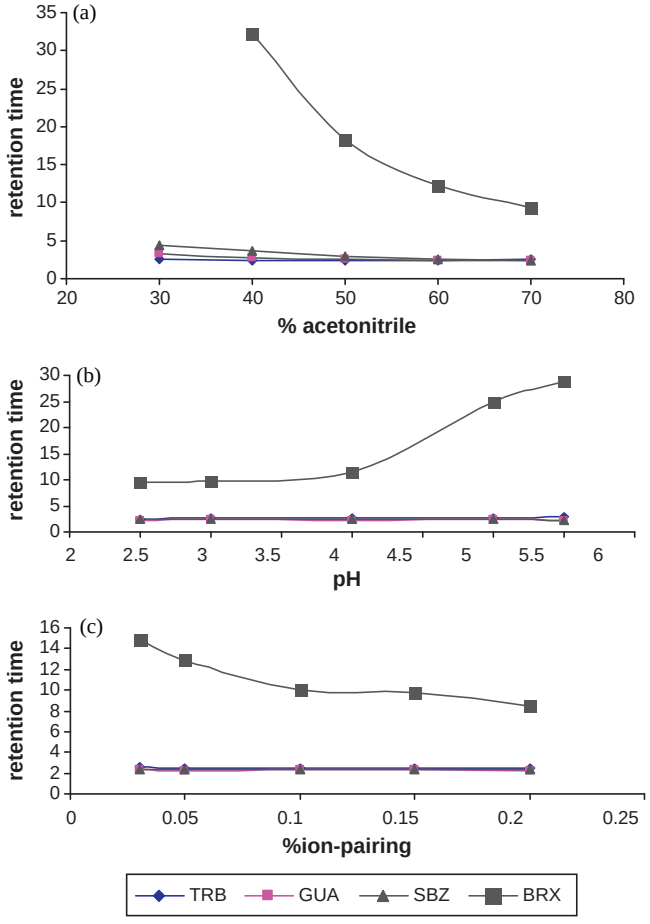

Fig. 8. Variation of the retention times of a synthetic mixture of terbutaline sulphate (TRB), guaiphenesin (GUA), sodium benzoate (SBZ) and bromhexine HCl (BRX) as a function of percentage of acetonitrile in the mobile phase (a) pH of the aqueous phase (b) and the percentage of the ion-pairing reagent in the aqueous phase (c).

and the mean percentage recovery calculations were based on the intercepts and slopes obtained by the non-parametric method. In the majority of cases, the mean percentage recovery became better and the RSD% became lower indicating better accuracy and precision. This was shown in a collective Table 9.

The internal standard method was successfully used in our study. It was useful in eliminating the proportional error that may develop during HPLC analysis as the response of the drug was divided over the response of the internal standard chosen so any common error in each of them would be cancelled. In the majority of cases, precision and accuracy results were greatly enhanced as shown in the collective Table 9. The (RSD%) and mean percentage recovery became in the accepted ranges of each indicating good precision and accuracy.

6. Analysis of pharmaceutical formulation

For the parametric method, assays of sample preparation were carried out as described under Section 3.6. The prepared solution was then chromatographed as under the method described for cases I and II (Table 1). For the non-ideal case II, Table 10 showed bad precision and accuracy in the direct measurement but the results of (RSD%) and mean percentage recovery became in the accepted ranges of each indicating good precision and accuracy after treatment of data using derivative and convoluted derivative in the external standard method. They even became better by the use of internal standard method.

For the non-parametric method, the same calculations were done as explained in Section 5.4. Table 10 showed that by using the non-parametric method, in the majority of cases, the RSD% became lower than the parametric one and the mean percentage recovery became closer to 100%, indicating that the non-parametric method was superior over the parametric one.

The effect of the internal standard method was shown in Table 10. In the majority of cases, the (RSD%) and mean percentage recovery became in the accepted ranges of each indicating good precision and accuracy.

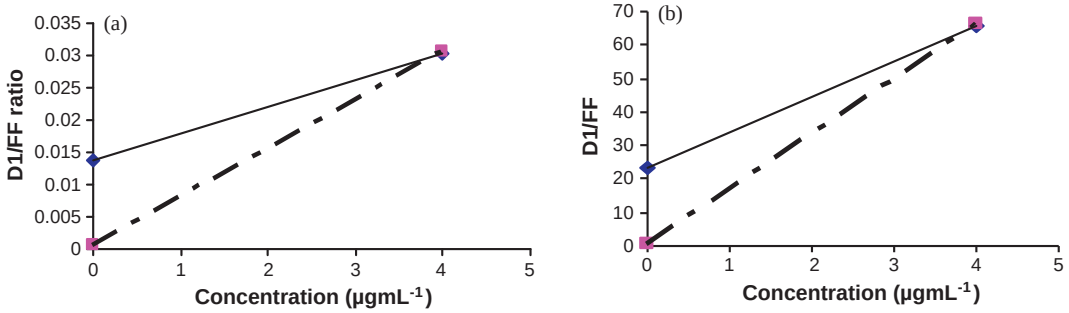

Fig. 9. Regression lines calculated by Theil's method, non-parametric(▬ · ▬), and by the least squares method, parametric (▬▬), for the determination of guaiphenesin using *D*1/FF in presence (a) and absence (b) of internal standard in ideal case.

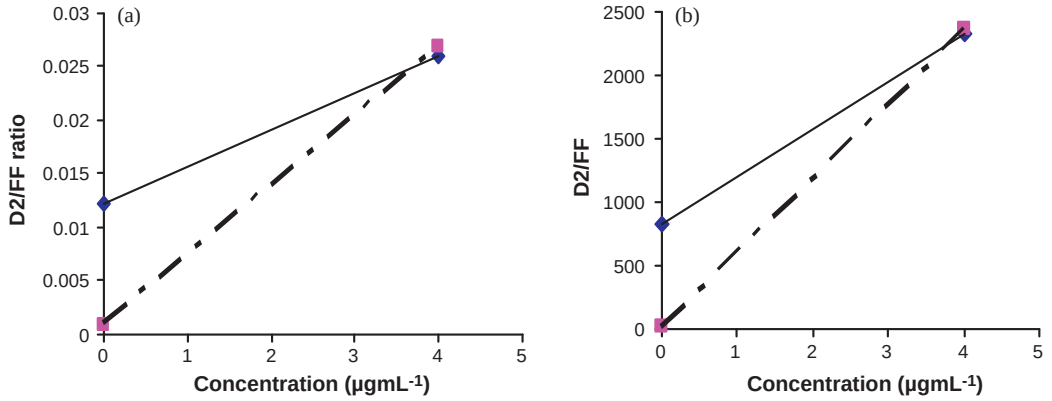

Fig. 10. Regression lines calculated by Theil's method, non-parametric (▬ · ▬), and by the least squares method, parametric (▬▬), for the determination of guaiphenesin using *D2/FF* in presence (a) and absence (b) of internal standard in ideal case.

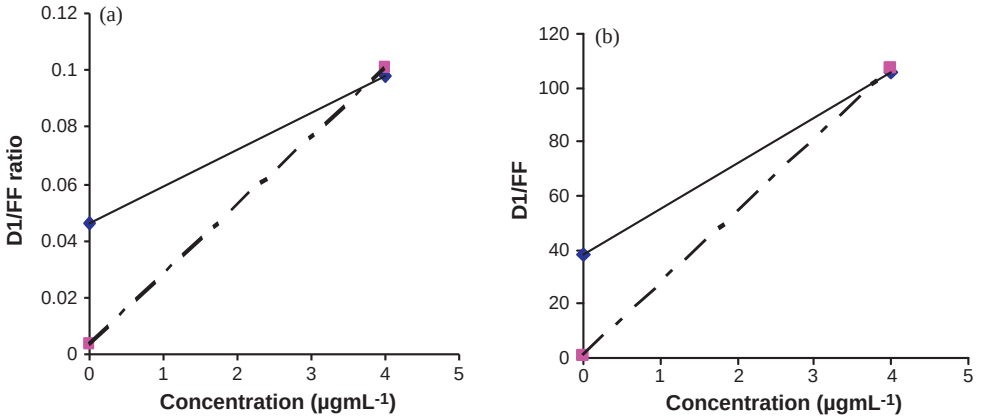

Fig. 11. Regression lines calculated by Theil's method, non-parametric (▬ · ▬), and by the least squares method, parametric (▬▬), for the determination of guaiphenesin using *D1/FF* in presence (a) and absence (b) of internal standard in non-ideal case II.

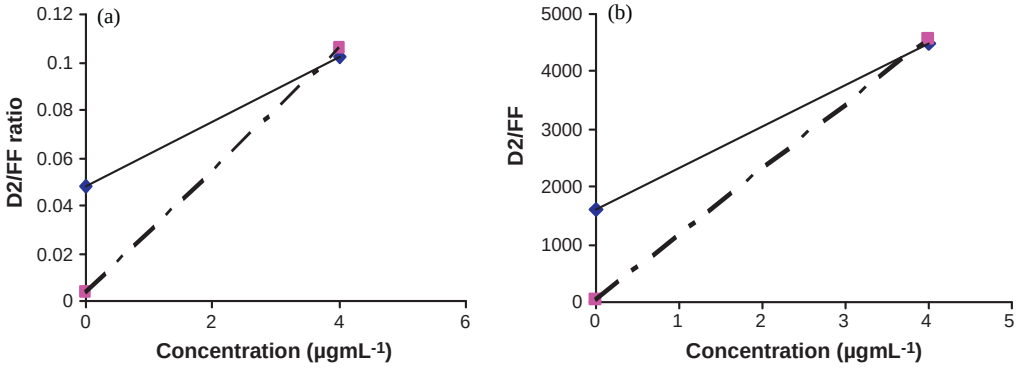

Fig. 12. Regression lines calculated by Theil's method, non-parametric (▬ · ▬), and by the least squares method, parametric (▬▬), for the determination of guaiphenesin using *D*2/FF in presence (a) and absence (b) of internal standard in non-ideal case II.

Table 9
Summary of parametric and non-parametric evaluation of the precision and accuracy for the determination of synthetic mixtures of terbutaline sulphate (TRB), guaiphenesin (GUA), sodium benzoate (SBZ) and bromhexine HCl (BRX) in non-ideal cases.[a].

Non-ideal cases				External standard method						Internal standard method					
				Area	Height	*D*1	*D*1/FF	*D*2	*D*2/FF	Area ratio	Height ratio	*D*1 ratio	*D*1/FF ratio	*D*2 ratio	*D*2/FF ratio
II	TRB	Parametric	Mean%[b]	94.41	94.41	99.83	99.99	99.60	99.84	97.66	95.26	99.82	100.31	100.03	100.14
			RSD (%)[c]	10.81	10.54	0.82	0.83	0.65	0.40	7.64	8.91	0.60	0.35	0.47	0.17
		Non-parametric	Mean%[b]	94.25	98.07	99.80	100.07	99.81	99.86	94.41	98.19	99.99	100.31	100.02	100.11
			RSD (%)[c]	13.05	2.26	0.35	0.71	0.51	0.24	15.47	2.11	0.54	0.32	0.59	0.09
II	GUA	Parametric	Mean%[b]	91.57	92.01	99.87	100.04	99.37	99.87	95.03	93.18	99.80	100.28	99.94	100.15
			RSD (%)[c]	9.83	9.43	0.91	0.78	0.62	0.48	5.24	8.18	0.57	0.58	0.54	0.17
		Non-parametric	Mean%[b]	96.62	97.20	99.89	100.02	99.37	99.97	97.56	97.53	99.95	100.34	100.01	100.11
			RSD (%)[bc]	4.01	3.29	0.49	0.91	0.27	0.78	3.09	2.52	0.32	0.60	0.40	0.12
II	SBZ	Parametric	Mean%[b]	94.18	93.18	99.50	100.08	99.57	100.23	96.61	94.12	99.71	100.46	100.45	100.22
			RSD (%)[c]	11.61	10.65	1.11	0.69	0.37	0.59	7.60	9.33	0.74	0.43	0.36	0.21
		Non-parametric	Mean%[b]	93.13	97.15	99.67	100.06	99.69	100.16	95.84	97.32	99.42	99.33	100.66	100.26
			RSD (%)[c]	13.29	5.86	0.97	0.86	0.56	0.40	8.53	5.06	1.11	1.33	1.14	0.99
III	BRX	Parametric	Mean%[b]	94.78	95.09	99.34	99.80	100.60	100.60	95.70	96.54	100.02	100.07	100.46	100.12
			RSD (%)[c]	14.46	13.71	1.01	0.64	0.80	0.96	8.11	7.00	0.97	0.92	0.47	0.62
		Non-parametric	Mean%[b]	96.54	97.27	99.37	99.88	100.12	100.34	96.75	97.19	99.94	100.02	100.09	99.9
			RSD (%)[bc]	6.30	5.50	0.58	0.29	0.44	0.60	6.36	5.70	0.66	0.49	0.41	0.67

[a] The concentrations in μg mL^{-1} of the synthetic mixtures 's.mix' of TRB ,GUA and SBZ in the external standard method for the non-ideal case II are: (a) s.mix1(4,300,100), (b) s.mix2(10,200,50), (c) s.mix3(30,100,40), (d) s.mix4(50,50,20), (e) s.mix5(100,10,10), (f) s.mix6(200,4,4) and for the internal standard method, 40.0 μg mL^{-1} of PPN as internal standard was added to the synthetic mixtures. For non-ideal case III, different concentrations were repeated of BRX to determine the precision and accuracy in the external standard method (5,6,6.5,7,8 μg mL^{-1}), for the internal standard method, 10.0 μg mL^{-1} of PPN as internal standard was added.

[b] The mean of all recoveries of different concentration in the same method.

[c] Percentage relative standard deviation.

Table 10
Parametric and non-parametric evaluation of the precision and accuracy for the determination of terbutaline sulphate (TRB), guaiphenesin (GUA) and sodium benzoate (SBZ) in their pharmaceutical preparation in non-ideal case II in the external and internal standard method.

Nominal value (μg mL^{-1})		External standard method						Internal standard method					
		Area	Height	*D*1	*D*1/FF	*D*2	*D*2/FF	Area ratio	Height ratio	*D*1 ratio	*D*1/FF ratio	*D*2 ratio	*D*2/FF ratio
TRB 5													
Parametric	Recovery[a]	96.00	94.60	98.80	98.40	100.80	99.40	96.00	96.40	98.60	98.80	100.20	100.20
	RSD (%)[b]	2.08	2.33	0.81	0.61	0.99	1.21	3.10	2.07	1.01	0.40	0.40	1.00
Non-parametric	Recovery[a]	93.00	96.40	99.00	99.60	100.60	100.40	90.40	97.40	99.20	100.60	99.80	100.20
	RSD (%)[b]	3.87	2.07	0.81	0.40	0.80	1.08	3.32	2.46	0.83	0.40	0.34	0.40
GUA 200													
Parametric	Recovery[a]	92.84	83.34	98.75	100.17	100.76	99.67	90.84	85.00	98.95	99.25	99.96	100.00
	RSD (%)[b]	2.76	9.17	0.44	1.15	0.66	0.30	4.21	5.88	0.75	0.66	0.30	0.50
Non-parametric	Recovery[b]	94.95	97.50	99.00	100.06	100.15	99.50	95.45	96.00	99.30	99.50	100.00	100.03
	RSD (%)[b]	2.70	2.15	0.43	1.05	0.55	0.25	2.57	3.13	0.65	0.55	0.25	0.24
SBZ 50													
Parametric	Recovery[a]	94.66	96.34	100.38	100.32	99.42	99.46	94.66	96.00	100.74	99.70	100.20	99.86
	RSD (%)[b]	5.32	2.16	0.86	0.42	1.25	1.29	4.39	2.08	1.09	0.62	1.92	1.28
Non-parametric	Recovery[a]	92.20	96.60	99.80	100.20	99.60	99.80	90.40	97.20	100.60	99.56	100.38	99.90
	RSD (%)[b]	5.42	2.26	0.86	0.40	0.84	1.00	4.62	2.26	1.03	0.58	1.59	0.60

[a] Mean recovery of triplicate determinations.

[b] Percentage relative standard deviation.

7. Conclusion

The internal standard method could be successfully used in the HPLC analysis. It has a great benefit in eliminating the proportional error that may develop during HPLC analysis as the response of the drug is divided over the response of the internal standard chosen so any common error in each of them will be cancelled and the results will be enhanced.

Chemometric techniques could be successfully applied for handling complex chromatograms. This is highly needed in cases where sources of interference could dramatically affect the chromatographic response, e.g., very low concentrations or overlapping peaks.

Derivative treatment of the chromatographic response data followed by application of Fourier functions on the resulting derivative data and the use of the ratio of these data give improved quantitation of the chromatographic signals.

Non-parametric regression of the response data using Theil's method is highly advantageous over the usual least squares method. "Theil's method" could be used in cases where there are both *x*- and *y*-direction errors assuming that the errors are not normally distributed. It also has effectively circumvented the outlier problem.

As an extension to the work in the previous paper [8], not only statistical data of the different regression methods mentioned in our present study were compared but also detection, quantitation limits, precision and accuracy were also compared. This was done by the analysis of synthetic mixtures and pharmaceutical formulations. It was found that the internal standard and non-parametric methods were superior over the external standard and parametric ones, with the use of chemometrics to overcome errors caused by interferences when handling complex chromatograms.

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