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Analysis of enantiomers giving partially overlapped peaks by using different treatments of the chromatographic ultraviolet signals: quantification of pseudoephedrine enantiomers

J. Verdú-Andrés, R. Herráez-Hernández, P. Campíns-Falcó*

Department de Química Analítica, Universitat de València. c/Dr. Moliner 50, 46100 Burjassot, Valencia, Spain

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Abstract

Different strategies for the quantification of partially coeluting optical isomers have been investigated. The methods tested are based on the use of different features as the analytical UV signals: peak heights, perpendicular drop areas, first and second derivatives of the chromatograms, peak areas obtained by deconvolution of the overlapped peaks with data fitting optimization, and a multivariate model (principal component regression, PCR). The amphetamine-derivative drug pseudoephedrine was selected as a model compound. For chromatography, LiChrospher 100 RP_{18} and a mobile-phase consisting of methanol and a solution of carboxymethyl-β-cyclodextrin (the chiral selector) were used. The UV detector was set at 215 nm. The accuracy obtained with the tested methods at different degrees of overlapping and at different concentration ratios between enantiomers was evaluated. The results of this study demonstrated that the best option for quantification of partially overlapped UV peaks of enantiomers and to obtain the enatiomeric excess is the use of a PCR model using peak heights, perpendicular drop peak areas and deconvoluted peak areas as the original variables. The predictive ability of the proposed calibration model is of about 2–8 times better (depending on the overlapping degree) than that achieved with the other models tested.

Keywords: Enantiomer separation; Pseudoephedrine

1. Introduction

Today, chiral analysis has a well recognized importance in many areas of application, and there is an increasing need for fast and reliable methods for these types of analyses. Liquid chromatography (LC) has played an important role for which different approaches have been proposed. For direct methods a number of chiral stationary phases has been developed and are commercially available. An increasing number of publications also illustrates the possibility of using the chiral selector as an additive to the mobile phase [1–3]. As a result, a great number of problems can now be resolved.

However, separation of enantiomers by LC continues to be a difficult task, as the mechanisms of interaction are often unknown. Some theories have been proposed to explain the dispersion of band profile, according with the stochastic model of chromatography and the mobile phase dispersion [4,5]. From a practical point of view, the analysts

*Corresponding author.

E-mail address: pilar.campins@uv.es (P. Campíns-Falcó).

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need to discover which type of chiral selector is best suited for a given problem. Moreover, although it is relatively easy to find a chiral chromatographic system that enables some degree of enantioseparation for the compounds of interest, total separation is often impossible within reasonable times of analysis [6]. The literature shows numerous examples of chiral methods in which the enantiomers are not baseline resolved. Indeed, the analytical responses measured for one of the enantiomers (typically peak heights, tangent-skimming peak areas or perpendicular drop peak areas) may be affected by the presence of the other enantiomer. However, the information may be sufficient for certain purposes, for example, to obtain crude information about whether a sample is racemic. Obviously, the reliability of such methods for quantification purposes would depend on peak resolution, but possible errors due to overlapping are rarely taken into consideration. Even in methods with nearly baseline resolution, significant errors can be encountered in the quantification of enantiomers if they are present at different concentrations [7].

To enhance enantioselectivity different approaches have been proposed. For example, enantiomers containing suitable functional groups (amino, carboxyl, hydroxyl) can be derivatized to introduce a suitable tag for enantioseparation in a chiral stationary phase. In some instances the analytes can be transformed into diastereomeric derivatives by using homochiral reagents, and then separated on a conventional achiral stationary phase (indirect method) [8,9]. Another possibility is the simultaneous use of two chiral selectors (for example a mobile phase containing two chiral additives) [6].

Alternatively, enantiomers giving partially overlapped peaks could be quantified through the mathematical treatment of the chromatographic signal. Over the last decade several methods for curve resolution of highly (or even completely) overlapped chromatographic peaks have been proposed [8]. However, a major requisite in most of them is the existence of spectral differences among the coeluting species. Indeed, this is not the case for optical isomers, and consequently, such methods cannot be applied in chiral analysis. Other methods have been developed for peak resolution based on the chromatographic profile. Examples in this group are the first and second derivatives of the chromatograms, or the deconvolution of the overlapped peaks to a previously defined mathematical function [10–12]. However, the application of such methods to the resolution of optical isomers has received limited attention [13]. Another approach is the combination of UV detection and polarimetric detection, which has been successfully used for the determination of enatiomeric excess [14]. Under conditions of poor chromatographic resolution, the use of two Gaussian functions to model the bimodal response is necessary [15]. A similar approach, applied for preparative separation, and without any shape assumption, is presented in [16].

In the present work we have evaluated different strategies for quantification of enantiomers based on different treatments of the chromatograms: first and second derivative of the chromatograms, deconvolution of the overlapped peaks with data fitting optimization (using the Levenberg–Marquardt algorithm to fit the peaks to an exponential modified gaussian peak model), and a multivariate model (principal component regression, PCR). The results have been compared with those found by using peak heights and perpendicular drop areas. The amphetamine-derivative drug pseudoephedrine (Ψ-ephedrine) has been selected as a model compound, as the possibility of differentiating Ψ-ephedrine enantiomers with β-cyclodextrin chiral selectors has been previously illustrated [17]. In this case, carboxymethyl-β-cyclodextrin was used as a chiral additive to the mobile phase in combination with a conventional achiral C_{18} column. The mobile phase composition was modified in order to produce two different degrees of resolution representative of moderate and severe overlapping. Differences displayed by the tested methods for different concentration ratios between enantiomers have been evaluated.

2. Experimental

2.1. Apparatus

The chromatographic system used consisted of a quaternary pump (Hewlett-Packard 1050 Series, Palo Alto, CA, USA), and an automatic sample injector (Hewlett-Packard 1050 Series) with a sample loop injector of 100 μl. For detection, a UV detector

(Hewlett-Packard 1100 Series) was used. The chromatographic signal was monitored at 215 nm. The detector was linked to a data system (Hewlett-Packard HPLC CHEMSTATION) for data acquisition and storage. The volume of sample injected was 20 μl. All the assays were carried out at ambient temperature.

All the computations were made using MATLAB for Windows, Version 5.3 (The MathWorks, Natick, MA, USA) 1999.

First and second derivative of the chromatographic data were obtained by using the algorithm proposed in [18], which is a modification of the well-known Savitzky–Golay method [19].

2.2. Reagents

All the reagents were of analytical grade. (1*S*,2*S*)-(+)-pseudoephedrine hydrochloride and (1*R*,2*R*)-(−)-pseudoephedrine were obtained from Sigma (St. Louis, MO, USA). Triethylamine (TEA) was purchased from Aldrich (Steinheim, Germany). Carboxymethyl-β-cyclodextrin was purchased from Fluka (Buchs, Switzerland), and methanol (HPLC grade) was purchased from Scharlau (Barcelona, Spain).

2.3. Pharmaceuticals

Atiramin syrup (Juste SAQF, Madrid, Spain) labelled to contain 6 mg/ml of pseudoephedrine sulphate, and Lasa syrup (Lasa, Sant Feliu de Llobregat, Barcelona, Spain) labelled to contain 6 mg/ml of pseudoephedrine chlorhydrate were analyzed. Samples were diluted appropriately in order to obtain concentrations of Ψ-ephedrine within the studied concentration interval.

2.4. Preparation of solutions

Stock standard solutions of (+)-Ψ-ephedrine and (−)-Ψ-ephedrine (1000 μg/ml) were prepared in water. Working solutions of the individual enantiomers or their mixtures were prepared by dilution of the stock solutions with water. Water was deionized and filtered using 0.45-μm nylon membranes (Teknokroma, Barcelona, Spain). All solutions were stored in the dark at 2°C.

2.5. Column and mobile phases

A LiChrospher 100 RP_{18}, 125×4 mm I.D., 5 μm column (Merck, Darmstadt, Germany) was used for separation. The mobile phase consisted of methanol and a solution of carboxymethyl-β-cyclodextrin (2.0 g/l). The solution of carboxymethyl-β-cyclodextrin was prepared by dissolving this additive in water. Then, the pH was adjusted to 5.2 by adding TEA. The percentage of methanol in the mobile phase was adjusted in order to produce different degrees of overlapping of Ψ-ephedrine enantiomers: an eluent containing 15% of methanol was used to produce moderate overlapping (resolution, $R > 0.7$) whereas a percentage of methanol of 30% was used to obtain severe overlapped compounds ($R < 0.5$). In all instances the mobile phase flow-rate was 1.0 ml/min.

All solutions were filtered through 0.45-μm nylon membranes (Teknokroma, Barcelona, Spain) and degassed with helium before use.

3. Theory

3.1. Experimental designs

In this study, a 5^2 experimental design has been used. The analytical concentrations assayed were those listed in Fig. 1. The (0,0) point was not assayed.

The latin lattice designs employed to check the validity of the calibration models consisted in a random selection of a five points subsample. Sub-

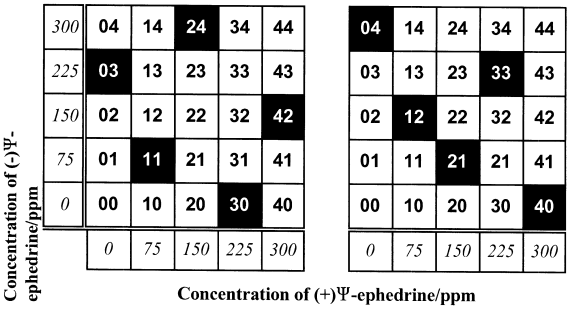

Fig. 1. Examples of latin lattice designs for the 5^2 experimental design and the corresponding experimental concentrations assayed.

samples were chosen in such a way that the row and the column of every new point selected do not repeat rows and columns of the previously selected points. Examples of these designs are also shown in Fig. 1. Under those restrictions, a great variability for the selected points was ensured [20].

3.2. Peak deconvolution

A tailed chromatographic peak can be fitted to a great variety of analytical functions, sometimes from mathematical reasons [21], and sometimes related with a theoretical chemical model [4,5]. For computational reasons, simpler functions are preferred. We have used an exponentially modified gaussian (EMG) function with four independent parameters:

$$y = \frac{A}{2\tau} e^{\left(\frac{\sigma^2}{2\tau^2} + \frac{t_R - t}{\sigma}\right)} \left[1 + erf\left(\frac{t - t_R}{\sqrt{2}\sigma} - \frac{\sigma}{\sqrt{2}\tau} \right) \right]$$

where A is the peak area, t_R is the peak center, σ is the Gaussian peak width and τ is the exponential time constant.

Two overlapped tailed chromatographic peaks can be fitted to the addition of two different EMG functions. Eight parameters must then be optimized in order to find the best fitting. Optimization is made by minimizing the summed squared error between the fitted and the experimental values. For this purpose we have used the Levenberg–Marquardt algorithm, which is based on the use of the Jacobian vector and the Hessian matrix of the function to be optimized. The reader is addressed to [22] to find the fundamentals of the method and the main algorithm.

3.3. Cross-validation and prediction errors [23]

Cross-validation is usually employed to identify the optimal complexity of multivariate methods. However, it can be also used to obtain an approximate assessment of the prediction errors that are to be expected when using the developed model. The basis for the leave-one-out cross-validation method are as follows: for the n data points of the calibration data set, n calibration models are built. For each model, one different point is left out, and the model without this point is used to predict it. Finally, n predicted values are obtained. The root mean squared prediction error based on cross-validation, rmsecv, is defined as:

$$\text{rmsecv} = \sqrt{\frac{1}{n}\sum_{i=1}^{n} [\hat{x}_i^* - x_i]^2}$$

where $\hat{x}_i^*$ represents the predicted value of the ith point by the model without using the ith point, and x_i represents the corresponding real value. The lower the rmsecv value, the better predictive ability can be expected for the model. Since the different models are rather similar to that built with the whole data set, the result is normally an optimistic assessment of the real prediction error that the model will provide.

A more realistic value for prediction ability is obtained by using one data set to build the calibration model, and a totally different data set for prediction purposes. The root mean squared prediction error, rmsep, is defined as:

$$\text{rmsep} = \sqrt{\frac{1}{m}\sum_{i=1}^{m} [\hat{x}_i - x_i]^2}$$

where $\hat{x}_i$ represents the predicted value for the ith external point and x_i represents the corresponding real value. In this case all points are predicted using the same model. Again, the lower the rmsep value, the better the predictive ability that can be expected.

3.4. Principal component regression

PCR is normally used in analytical chemistry for collinear data [23]. Calibration entails two steps. First, the column-centred $\boldsymbol{X}$ data matrix is decomposed by using the singular value decomposition: $\boldsymbol{X} = \boldsymbol{T}, \boldsymbol{P}'$, where $\boldsymbol{T}$ is the score matrix and the columns of matrix $\boldsymbol{P}$ are the loading vectors. Each score is a linear combination of the original variables ($\boldsymbol{T} = \boldsymbol{X}, \boldsymbol{P}$). In the second step, $\boldsymbol{c}$ is regressed on $\boldsymbol{T}$ using the model: $\boldsymbol{c} = \boldsymbol{T}, \boldsymbol{b} + \boldsymbol{e}$, where $\boldsymbol{b}$ are the model parameters and $\boldsymbol{e}$ are the measurement and model errors associated with $\boldsymbol{c}$. In this study, original variables for each chromatogram were peak height, perpendicular-drop area and the deconvoluted area. The use of PCR was necessary to deal with the collinearity of these data.

3.5. Measurement of peak resolution

Two parameters have been employed to measure

peak resolution: (a) The resolution factors, R, were calculated as [24]:

$$R = 2 \cdot \frac{t_2 - t_1}{w_2 + w_1}$$

where t_1 and t_2 are the retention times of the two peaks and w_1 and w_2 are the peak widths, measured at the base of the peak. This is the R definition given by the US Pharmacopeia, and it is less susceptible to errors arising from peak asymmetry than that given by the European Pharmacopeia, in which the width at half-height is used. (b) The peak separation indexes, S, were defined as [24]:

$$R' = \frac{S_1 - S}{S_1} \cdot 100$$

where S_1 is the peak height of the first-eluting enantiomer and S is the height of the valley between the first-eluting isomer and the last-eluting isomer. R' ranges from 0 (totally overlapped peaks) to 100 (totally resolved peaks).

4. Results and discussion

The chromatographic conditions were adjusted in order to produce different degrees of overlapping of Ψ-ephedrine enantiomers. An eluent containing 15% of methanol was used to obtain fairly well separated enantiomers (resolution, $R > 0.7$) whereas a percentage of methanol of 30% was used to obtain severe overlapped compounds ($R < 0.5$). (+)-Ψ-Ephedrine was the first-eluting enantiomer. For the first set of experiments, duplicate measurements were made, whereas for the second set of experiments single runs were performed. All the analysis were run in random order, to avoid possible time shifts to be incorrectly attributed to calibration features. Typical chromatograms obtained for a racemic mixture of the analytes under the chromatographic conditions assayed are shown in Fig. 2. In this figure are also depicted the chromatographic profiles obtained by application of the proposed deconvolution method.

4.1. Slightly overlapped peaks ($0.7 < R < 1.0$; $62 < S < 77$)

In the present instance Ψ-ephedrine enantiomers were fairly well separated (Fig. 2a), with resolution

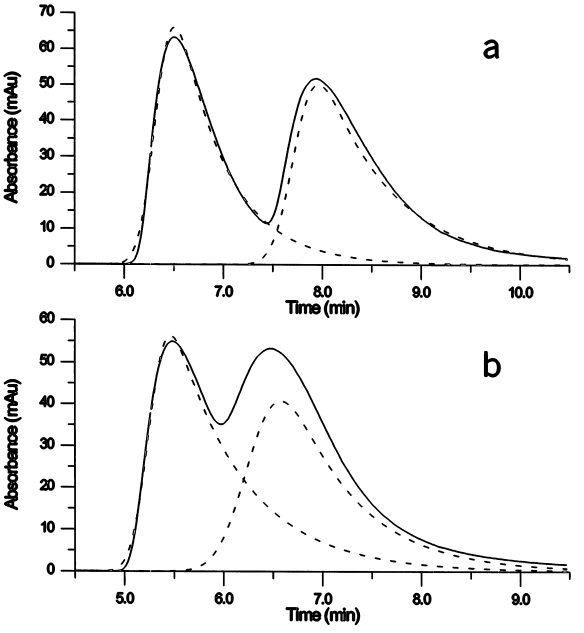

Fig. 2. Chromatograms obtained for a racemic mixture of Ψ-ephedrine (a) eluted with 15% methanol and (b) eluted with 30% methanol (in dashed line, the deconvoluted chromatograms obtained by the deconvolution method).The first eluting peak corresponds to (+)-Ψ-ephedrine. Concentration of each enantiomer, 75 ppm. For other experimental details, see text.

values (R) ranging from $R = 0.97$ for racemic samples containing 75 ppm (each isomer) to 0.69 for racemic samples containing 300 ppm of each of the isomers and separation indexes (S) ranging from $S = 77$ for racemic samples containing 75 ppm (each isomer) to $S = 62$ for racemic samples containing 300 ppm of each of the isomers. Fig. 2a also shows that the proposed deconvolution method performs rather well, and the peak profiles obtained for the deconvoluted peaks are very similar to those observed in the original chromatograms. The correlation coefficients of the regression lines for measured signal vs. predicted signal were always >0.99 for pure enantiomers, and >0.995 for enantiomeric mixtures. Good fits were also observed for samples containing different concentration ratios of the enantiomers, which in our case ranged from 4:1 to 1:4.

Three types of calibration lines were computed. In the first, calibration curves within the 75–300 ppm concentration interval, for each of the isomers, were obtained from the pure enantiomers (samples la-

belled 10, 20, 30 and 40 for (+)-Ψ-ephedrine and samples labelled 01, 02, 03 and 04 for (−)-Ψ-ephedrine in Fig. 1). In second approach, calibration curves within the 75–300 ppm concentration interval, for each of the isomers, were obtained from the racemic mixtures within the experimental design (samples labelled 11, 22, 33 and 44 in Fig. 1: these points lie in one of the diagonals of the experimental design). Finally, calibration lines from all samples assayed in the experimental design were also calculated, including the pure enantiomer samples and the racemic mixtures used in the first and second set. The results obtained by using peak height, perpendicular drop peak area and deconvoluted peak areas as the analytical signals for the three methodologies are listed in Table 1.

As expected, the results in Table 1 demonstrate that the calibration equations for each analyte and for a given analytical signal are dependent on the amount of the other enantiomer in the sample. For example, when using peak heights higher slopes of calibration were generally observed for each isomer in the presence of the other enantiomer. If perpendicular drop areas are used, the slopes of the calibration lines obtained from individual solutions of (+)-Ψ-ephedrine were higher than those obtained from mixtures of both isomers (racemic or not). In contrast, for pure (−)-Ψ-ephedrine enantiomer the slopes of calibration lines were lower than those observed for mixtures of both compounds. This means that if both isomers are present, the perpendicular drop area method overestimates first-eluting enantiomer whereas the last-eluting one is underestimated. Obviously, the amount of peak area wrongly assigned to the second-eluting peak is about the same that the area wrongly subtracted to the first-eluting peak. The opposite behavior was observed when using the deconvolution peak areas.

These findings are in agreement with those previously reported in [25,26]. In such studies, it was demonstrated that the error obtained by using the perpendicular drop area method increases when the overlapping between bands increases, and also, when the height quotient ratio between peaks decreases.

Calibration by using the first and the second derivatives of the chromatograms was also tested. However, worse correlation was observed (data not shown). This can probably be explained by the shifts in the retention times throughout the experiment and also by the high tailing peak profile, which is a typical feature of chiral separations.

Prediction abilities of a calibration model are not necessarily related with the goodness of fit parameters of the calibration lines. For this reason, we have evaluated the prediction abilities of the different approaches by calculating the previously defined rmsecv and rmsep parameters. For the all-points calibrations lines, rmsecv values have been computed by using leave-one-out cross-validation. For the pure enantiomer and racemic mixtures calibration

Table 1
Calibration results obtained for the 1st data set ($R > 0.7$). (I) data obtained from all points in the calibration design; (II) data obtained from samples 10, 20, 30 and 40, for (+)Ψ-ephedrine, in Fig. 1, and samples 01, 02, 03 and 04, for (−)-Ψ-ephedrine in Fig. 1; (III) data obtained from samples 11, 22, 33 and 44 in Fig. 1

Methodology	n	(+)-Ψ-ephedrine			(−)-Ψ-ephedrine		
		Intercept ($a \pm s_a$)	Slope ($b \pm s_b$)	Regression coefficient (r)	Intercept ($a \pm s_a$)	Slope ($b \pm s_b$)	Regression coefficient (r)
Peak height (I)	39	14±4	0.74±0.02	0.9870	18±3	0.518±0.014	0.9864
Peak height (II)	8	12±8	0.69±0.04	0.9913	13±4	0.52±0.02	0.9957
Peak height (III)	8	5±6	0.800±0.028	0.9963	15±4	0.54±0.02	0.9953
Perpendicular drop area (I)	39	−2±3	0.640_5±0.013	0.9921	5±4	0.737_5±0.020	0.986
Perpendicular drop area (II)	8	0.0±1.0	0.691±0.005	0.9999	1±3	0.687±0.013	0.9989
Perpendicular drop area (III)	8	2.6±1.9	0.599_5±0.009	0.9993	−5±3	0.818±0.014	0.9992
Deconvoluted peak area (I)	39	−6.6±1.9	0.782±0.010	0.9972	−2±3	0.719±0.014	0.9929
Deconvoluted peak area (II)	8	−1.3±0.9	0.721±0.005	0.9998	−1.1±1.2	0.737±0.006	0.9998
Deconvoluted peak area (III)	8	−11.6±2.4	0.826_5±0.012	0.9994	9±2	0.638±0.011	0.9991

Table 2
Predictive ability results for the 1st data set ($R > 0.7$)

Methodology[a]	(+)-Ψ-Ephedrine			(−)-Ψ-Ephedrine		
	rmsecv (ppm)	rmsep (ppm)	rmsep (mean±SD) for 20 latin lattice designs	rmsecv (ppm)	rmsep (ppm)	rmsep (mean±S.D.) for 20 latin lattice designs
Peak height (I)	14.2	–	16±3	14.3	–	16±3
Peak height (II)	–	25.0	–	–	16.3	–
Peak height (III)	–	15.4	–	–	14.4	–
Perpendicular drop area (I)	11.1	–	12.5±1.9	14.4	–	17±3
Perpendicular drop area (II)	–	21.5	–	–	29.4	–
Perpendicular drop area (III)	–	15.2	–	–	18.2	–
Deconvoluted peak area (I)	6.7	–	8.1±1.6	8.1	–	12±3
Deconvoluted peak area (II)	–	14.1	–	–	13	–
Deconvoluted peak area (III)	–	9.1	–	–	18.2	–
PCR: 3 signals	1.7	–	2.3±0.6	3.5	–	5.2±1.9
PCR: 2 signals	3.9	–	4.8±1.4	11.7	–	13.6±1.6
1st Derivative (I)	20.0	–	21±4	40.4	–	42±13
1st Derivative (II)	–	32.4	–	–	37.9	–
1st Derivative (III)	–	20.1	–	–	40.9	–
2nd Derivative (I)	23.6	–	24±4	40.0	–	76±25
2nd Derivative (II)	–	63.6	–	–	55.4	–
2nd Derivative (III)	–	36.5	–	–	79.7	–

[a] Meaning of I, II and III as in Table 1.

lines, rmsep values have been computed for the non used points. The results of this study are summarized in Table 2.

Table 2 shows that cross-validation methodology provided the best results when using the deconvoluted areas. The rmsecv values were 6.7 and 8.1 for the first- and late-eluting enantiomers, respectively. Prediction errors when using racemic mixtures were generally lower than those achieved from the pure enantiomers. The best predictive ability for (+)-Ψ-ephedrine was achieved from the racemic mixture and using deconvoluted area as the analytical signal (rmsep = 9.1). For (−)-Ψ-ephedrine the best predictive ability was achieved from the pure enantiomer and by using as analytical signal the deconvoluted areas (rmsep = 13.0).

On the other hand, whether the use of a multivariate approach can improve the results obtained for individual signals has been studied. For this purpose, PCR with the three measured variables (peak height, perpendicular drop area and deconvoluted area) or with two of the measured variables (peak height and perpendicular drop area) have been employed. The results are also listed in Table 2.

The PCR model with three original variables and two selected principal components provided excellent results, with a cross-validation error, rmsecv, of 1.7 for the first-eluting isomer. If only peak heights and perpendicular drop areas are used, the rmsecv increases to 3.9. In other words, the inclusion of the deconvoluted peak areas substantially improved the performance of the method.

The improvements observed by using the PCR model with three original variables and two selected principal components for the late-eluting peak are even greater. For this compound the rmsecv values with three and two original variables were 3.5 and 11.7, respectively. Therefore, the deconvoluted area values are also necessary.

The reliability of the different methodologies was also tested by calculating the enantiomeric excess, e.e., defined as the difference between the proportions of the two enantiomers in the mixture divided by their total $\text{e.e.} = [(C_+ - C_-)/(C_+ + C_-)] \cdot 100$ [14]. Fig. 3 shows the added vs. computed e.e. for the four methodologies tested. The PCR model yields the best results, both in accuracy and reproducibility.

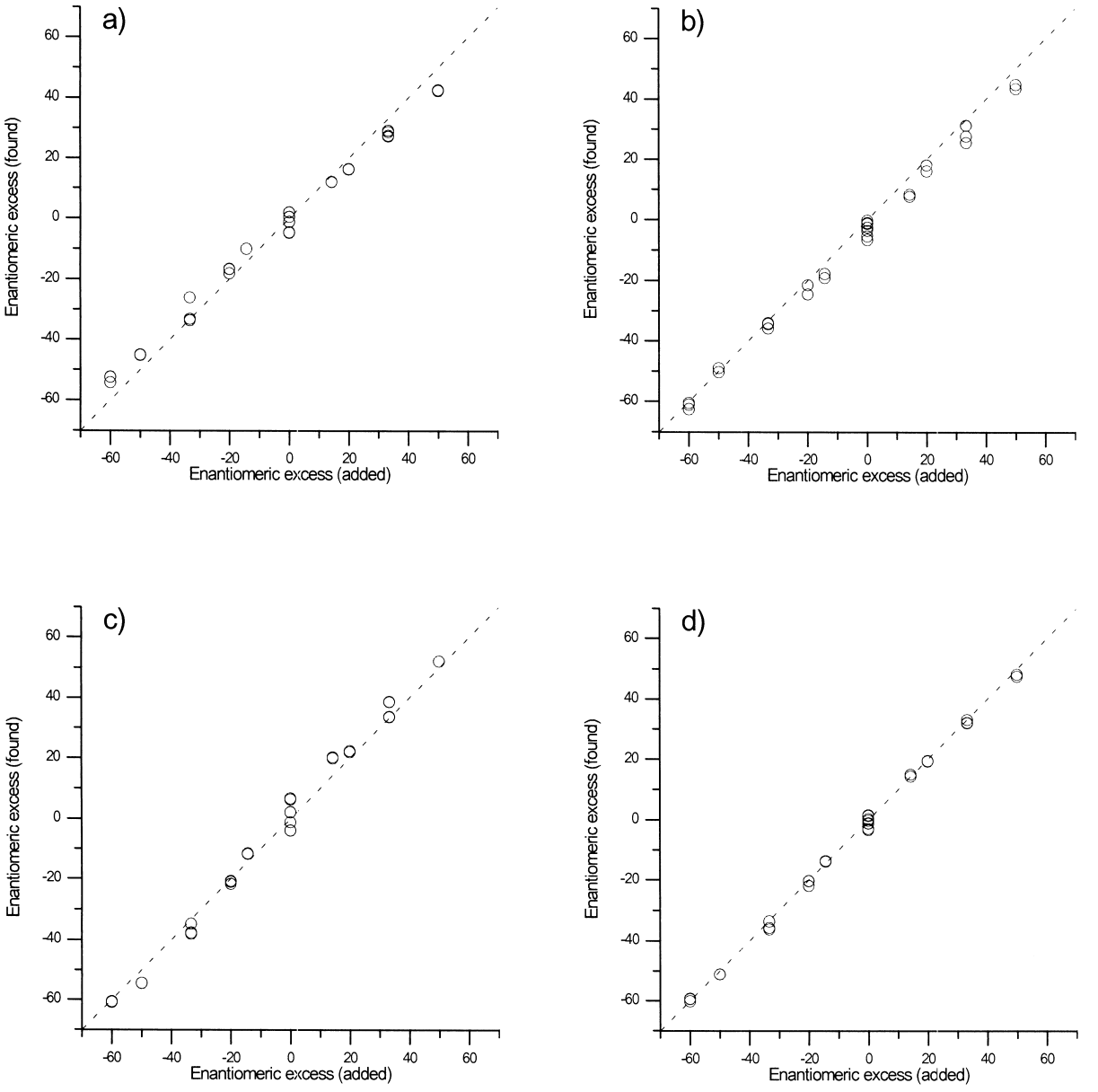

Fig. 3. Added vs. computed enatiomeric excess for the 1st data set ($R > 0.7$): (a) peak height signals; (b) perpendicular drop area signals; (c) deconvoluted area signals and (d) PCR model.

Finally, whether it is possible to use a lower number of samples to construct calibration lines with a predictive ability similar to that obtained from the complete design was tested. According to the experimental design used, twenty randomly selected five-point subsamples (by using latin lattice designs) were used. The mean and the standard deviation for the twenty rmsep values obtained are given in Table 2. Although slightly higher, mean rmsep values were fairly similar to the corresponding rmsecv values. This is in agreement with the optimistic prediction of the rmsecv values, as stated below. Therefore, five

calibration points can be considered sufficient to ensure a good calibration model, even when the PCR strategy is used.

4.2. Severely overlapped peaks ($R<0.5$; $S<35$)

In this case, Ψ-ephedrine enantiomers were poorly separated (Fig. 2b). The resolution value for the racemic mixtures containing 75 ppm of each enantiomer was $R=0.53$. At higher concentrations the R values were difficult to compute because of the high degree of overlapping, but in all instances the resolution was worse ($R<0.5$). The separation index ranged from $S=35$ for racemic samples containing 75 ppm (each isomer) to $S=14$ for racemic samples containing 300 ppm of each of the isomers. Fig. 2b also depicts the peaks obtained by applying the proposed deconvolution method. The deconvolution approach often provided poor fitting, especially for the highest concentrations assayed. This is in agreement with Vandeginste and de Galan [27], because the band position cannot be clearly established now. Although the theoretical model works well for $R>0.7$, as previously demonstrated, for this degree of overlapping, $R<0.5$, the model does not perform sufficiently well.

The calibration curves obtained for the different strategies tested are shown in Table 3. The behavior observed when using peak heights and perpendicular drop areas was similar to that previously found for the previous case (moderate overlapped peaks), but differences in the slopes of calibration were much higher. However, the slopes of the calibration lines obtained by using the deconvoluted areas showed a non-defined tendency. This is probably due to the bad performance of the deconvolution method at this severe degree of overlapping.

As regard the prediction ability (see Table 4) for the first-eluting peak and for cross-validation results, the best value was obtained by using peak heights (rmsecv=15.2). The employment of perpendicular drop areas and deconvoluted areas lead to similar results between them, but worse than the ones obtained with peak heights. Best prediction errors were achieved by using racemic mixtures and peak height measurements (rmsep=18.0). The use of perpendicular drop areas or deconvoluted areas lead to unacceptable prediction values.

For the second-eluting peak, all models resulted in comparable rmsecv values, with perpendicular drop areas giving the best result (rmsecv=27.9). For this compound, prediction errors for racemic mixtures and using peak heights and perpendicular drop areas were lower than those obtained with the pure enantiomers. Best value was obtained when using perpendicular drop areas (rmsecv=34.9). Differences among the approaches tested were lower than those observed for the first-eluting compound but, even in the best case, bad prediction results were obtained.

On the other hand, first and second derivatives only gave suitable results for the first-eluting enantiomer, as observed in Table 4. However, no improvement compared to the previous models was

Table 3
Calibration results obtained for the 2nd data set ($R<0.5$)

Methodology[a]	n	(+)-Ψ-Ephedrine			(−)-Ψ-Ephedrine		
		Intercept ($a\pm s_a$)	Slope ($b\pm s_b$)	Regression coefficient (r)	Intercept ($a\pm s_a$)	Slope ($b\pm s_b$)	Regression coefficient (r)
Peak height (I)	20	14.0±5	0.61±0.02	0.9869	30.0±8	0.43±0.04	0.9339
Peak height (II)	4	9.5±1.3	0.569±0.006	0.9999	20.0±3	0.388±0.014	0.9987
Peak height (III)	4	4.7±2.2	0.676±0.010	0.9998	9.0±4	0.567±0.018	0.9990
Perpendicular drop area (I)	20	−16.0±14	0.63±0.07	0.9131	−1.0±13	0.88±0.06	0.9578
Perpendicular drop area (II)	4	−4.0±6	0.712±0.028	0.9984	0.6±2.2	0.701±0.010	0.9998
Perpendicular drop area (III)	4	8.0±30	0.411±0.017	0.9984	−9.0±4	1.026±0.018	0.9997
Deconvoluted peak area (I)	20	−41.0±18	0.88±0.09	0.9235	−32.0±15	0.94±0.07	0.9447
Deconvoluted peak area (II)	4	4.0±5	0.743±0.023	0.9991	−3.2±0.7	0.761_5±0.004	0.99998
Deconvoluted peak area (III)	4	21.0±13	0.45±0.06	0.9809	13.0±31	0.72±0.15	0.9598

[a] Meaning of I, II and III as in Table 1.

Table 4
Predictive ability results for the 2nd data set ($R < 0.5$)

Methodology[a]	(+)-Ψ-Ephedrine			(−)-Ψ-Ephedrine		
	rmsecv (ppm)	rmsep (ppm)	rmsep (mean±S.D.) for 20 latin lattice designs	rmsecv (ppm)	rmsep (ppm)	rmsep (mean±S.D.) for 20 latin lattice designs
Peak height (I)	15.2	–	17±4	35.8	–	40±15
Peak height (II)	–	28.6	–	–	59.1	–
Peak height (III)	–	18	–	–	36.8	–
Perpendicular drop area (I)	42.0	–	47±15	27.9	–	34±11
Perpendicular drop area (II)	–	57.5	–	–	47.2	–
Perpendicular drop area (III)	–	95.0	–	–	34.9	–
Deconvoluted peak area (I)	38.3	–	44±12	30.9	–	37±7
Deconvoluted peak area (II)	–	51.7	–	–	35.9	–
Deconvoluted peak area (III)	–	127.1	–	–	45.5	–
PCR: 3 signals	5.2	–	11±6	18.0	–	20±2
PCR: 2 signals	4.8	–	6.4±1.5	26.8	–	31±8
1st Derivative (I)	21.0	–	24±6	60.0	–	(8±4)·10
1st Derivative (II)	–	35.8	–	–	80.0	–
1st Derivative (III)	–	24.4	–	–	49.7	–
2nd Derivative (I)	23.0	–	27±7	82.9	–	(9±5)·10
2nd Derivative (II)	–	40.8	–	–	88.9	–
2nd Derivative (III)	–	23.4	–	–	252.0	–

[a] Meaning of I, II and III as in Table 1.

observed. In addition, unacceptable errors were observed for the late-eluting isomer. From these results, it can be concluded that derivative methods are inappropriate for the quantification of the analytes.

Application of the PCR model to the first-eluting peak with three original variables and three selected principal components yielded a cross-validation error, rmsecv, of 5.2; this value is of about one third of the value obtained when working with peak heights. However, in this case, the deconvoluted area can be excluded from the original variables, as the PCR model with peak heights and perpendicular drop areas and two selected PC performs similarly. In both cases, with the number of PCs equal to the number of original variables, the model simplifies to the original three or two variables multiple linear regression.

For the late-eluting peak, the best cross-validation error (rmsep = 18.0) was obtained for the PCR model with three original variables and one selected principal component. Interestingly, in this case deconvoluted area cannot be excluded as a PCR model with two original variables and one selected PC performs worse. These findings and results of calibration studies suggest that the deconvolution methodology performs better for the more retained enantiomer than for the first-eluting one. In previous testing we have established the performance of the deconvolution method to detect one enantiomer in the presence of the other. We observed that the proposed method provided adequate curve fitting, at least for concentration ratios ranging from 10:1 to 1:10. It is interesting to note that in the former case only one peak was observed. This is a clear advantage of the deconvolution approach, as this is the only method than can be applied when a single peak is obtained.

Fig. 4 shows the added vs. computed e.e. for this degree of overlapping for the four sets of calibrations performed. In this case, only the PCR model performs adequately in accuracy and reproducibility, which is an additional advantage of the proposed methodolgy.

Finally, another twenty different five-point random subsamples selected by latin lattice design were employed to check the validity of the obtained results. The mean and the standard deviation for the twenty rmsep values are given in Table 4. Conclusions obtained from this table are similar to those indicated for the first case.

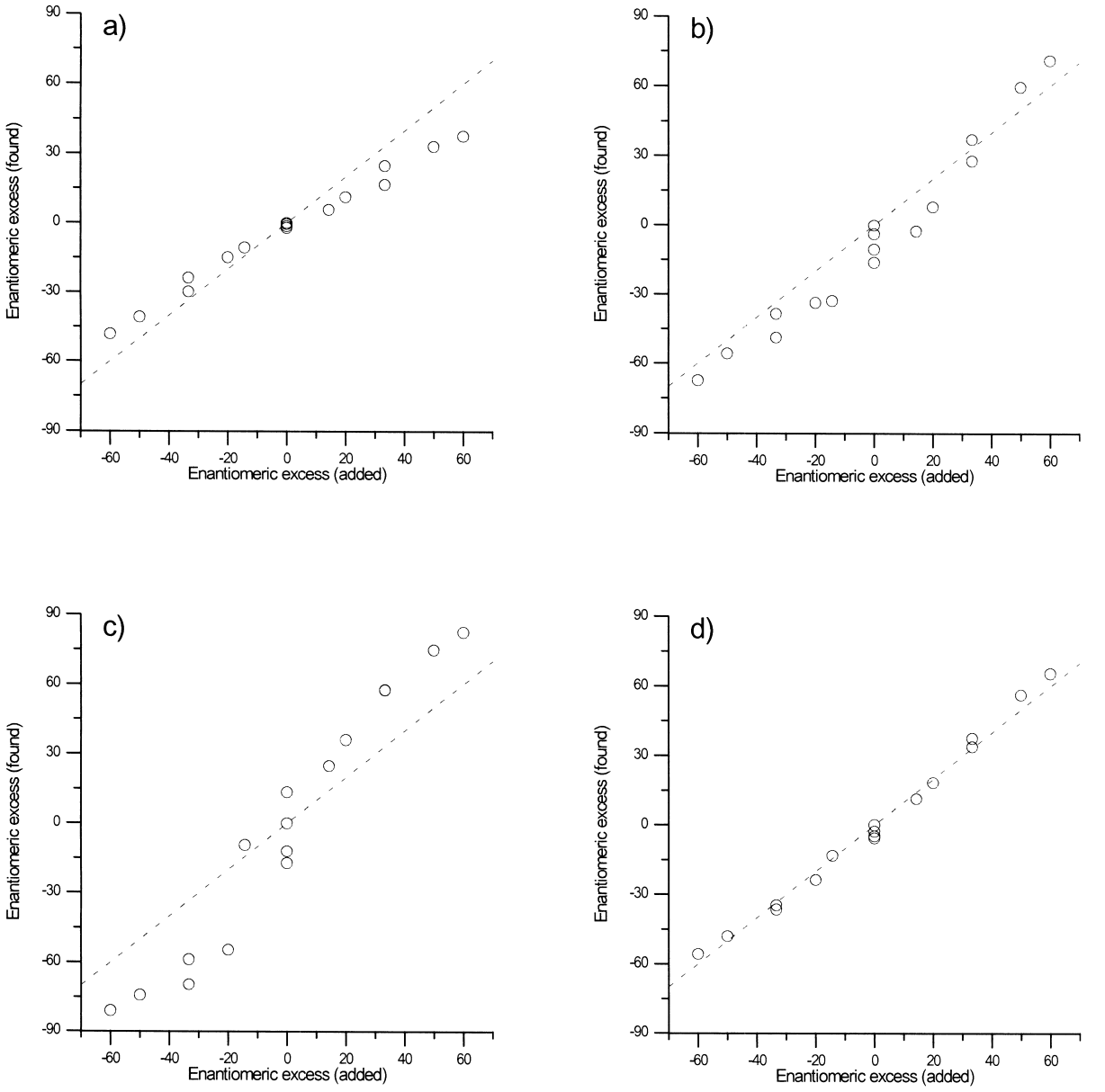

Fig. 4. Added vs. computed enatiomeric excess for the 2nd data set ($R < 0.5$): (a) peak height signals; (b) perpendicular drop area signals; (c) deconvoluted area signals and (d) PCR model.

4.2.1. Application to real samples

The reliability of the calibration methodology proposed was tested by analyzing two commercial pharmaceutical preparations containing Ψ-ephedrine. Both preparations are widely used in the treatment of nasal congestion and sneezing. The tested syrups were diluted appropriately in order to produce a concentration of the analytes within the studied concentration range, and then processed under conditions giving poor resolution (30% of methanol in the mobile phase).

In both pharmaceutical preparations only the (+)-

Table 5
Results for the pharmaceutical assayed

Product	Labelled	Found (+)-Ψ-ephedrine	*n*	RSD (%)
Atiramin	6 mg/ml (Ψ-Ephedrine)$_2$, H_2SO_4	6.30±0.07	3	1.1
Lasa	6 mg/ml Ψ-Ephedrine, HCl	6.08±0.04	3	0.7

Ψ-ephedrine enantiomer was detected. This was confirmed by using spiked samples. The concentrations determined for this enantiomer by using the best PCR model for this compound (with two original variables and two selected PCs) are given in Table 5. In both cases, good agreement between the labelled concentrations and the calculated concentrations was obtained.

5. Conclusions

The results of this study demonstrate that quantification of enantiomers giving partially overlapped peaks may be inadequate if peak heights or perpendicular drop areas are used for calibration, even when peaks are fairly well resolved ($0.7 < R < 1.0$). The employment of the first and second derivative data, which has sometimes been used for quantification of overlapped peaks, led to unacceptable results. This can be explained by the shifts in the retention times during the analytical measurements and also by the high peak asymmetries, which are a typical feature in chiral separations.

Possible alternatives such as peak deconvolution and a multivariate regression method (PCR) were tested in order to enhance the accuracy of the determinations. When the degree of overlapping is moderate, the use of deconvoluted areas improves the predictive ability for both peaks. Predictions are drastically improved if a PCR model, by using as original variables peak heights, perpendicular drop areas and peak deconvoluted areas, is employed. This strategy provided a rmsecv value, used to estimate the predictive ability of the calibration model, of about 4–8 times lower than the corresponding values obtained for the other calibration models.

For severely overlapped peaks, $R < 0.5$, the PCR strategy, with two original variables for the first peak and three original variables for the second one, was also the best option for calibration. In this situation, however, improvements in rmsecv values are only 2–3 times lower than the corresponding values obtained for the other calibration models.

The PCR strategy is also the best approach to establish the ratio between enantiomers. Moreover, for the high overlapping degree ($R < 0.5$), the enantiomeric excess can be only determined accurately by using this methodology.

On the other hand, five calibration points selected by a latin lattice design ensure a good calibration model for both isomers. In such a way, calibration involves samples containing the pure enantiomers and mixtures with different concentration ratios of the enantiomers. Calibration by using racemic samples led to worse results. Therefore, the proper selection of a calibration design in conjunction with a PCR calibration model provides substantial improvement in the quantification of partially coeluting enantiomers. With adequate software, to perform and validate the calibration model takes less time than the time needed to perform one chromatographic injection.

Since no assumptions have been made, the above conclusions could, in principle, be valid for other compounds giving similar peak shapes. Although baseline resolution is highly desirable and experimental conditions should be optimized to obtain it, the proposed approach can be an alternative in those situations where complete resolution cannot be obtain or when baseline resolution requires very long eluting times.

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