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Enantiomeric analysis of overlapped chromatographic profiles in the presence of interferences. Determination of ibuprofen in a pharmaceutical formulation containing homatropine

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ABSTRACT

In this work, we studied the combination of chemometric methods with chromatographic separations as a strategy applied to the analysis of enantiomers when complete enantioseparation is difficult or requires long analysis times and, in addition, the target signals have interference from the matrix. We present the determination of ibuprofen enantiomers in pharmaceutical formulations containing homatropine as interference by chiral HPLC-DAD detection in combination with partial least-squares algorithms. The method has been applied to samples containing enantiomeric ratios from 95:5 to 99.5:0.5 and coelution of interferents. The results were validated using univariate calibration and without homatropine. Relative error of the method was less than 4.0%, for both enantiomers. Limits of detection (LOD) and quantification (LOQ) for (*S*)-(+)-ibuprofen were 4.96×10^{-10} and 1.50×10^{-9} mol, respectively. LOD and LOQ for the R-(−)-ibuprofen were LOD = 1.60×10^{-11} mol and LOQ = 4.85×10^{-11} mol, respectively. Finally, the chemometric method was applied to the determination of enantiomeric purity of commercial pharmaceuticals. The ultimate goal of this research was the development of rapid, reliable, and robust methods for assessing enantiomeric purity by conventional diode array detector assisted by chemometric tools.

1. Introduction

Nowadays, the number of pure clinically available enantiomeric drugs has been growing considerably and thus, pharmacological, pharmacotechnical and toxicological knowledge of enantiomers and their degradation or metabolite products is an increasing demand [1]. However, in chiral chromatography, especially in reversed-phase conditions partial overlapping between both enantiomer profiles is often observed, causing loss in the quantitation accuracy. Besides, the possible presence of additional components in the sample, which might co-elute with the signals of interest, makes the problem even worse.

In terms of quality control and for all quantitative analysis, peak purity is a major task that can be addressed in different ways: by spectra comparison using peak purity assays offered by several chromatographic softwares when DAD detectors are used [2] or using MS detection if available. In the high-throughput analysis of metabolites, for example, overlapping peaks are ineluctable. This problem can be resolved by two dimensional data resolution methods using matrix computation combined with characteristics of spectral data [3,4]. Accurate quantitation of overlapping peaks by LC–MS is sometimes thwarted by the influence of one compound on the ionization of another, a situation that is easily diagnosed using standards of known composition [5]. Automated mass spectral deconvolution and identification system has been developed for processing GC–MS data. Most recently, Tsugawa et al. [6] proposed an open-source software data acquision for metabolite identification and quantification by mass spectral deconvolution.

The combination of suitable chemometric tools along with chromatographic-spectral data may allow the analysis of chiral sample components for which it is very troublesome to obtain an appropriate physical resolution. Kamal and co-workers have explored the role of multivariate techniques, as a mean of extracting information about enantiomeric composition of ofloxacin samples from chromatographic matrix data obtained after partial resolution of peaks in a chiral chromatographic separation fol-

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lowed by diode-array detection. Using multivariate chemometric techniques, strongly overlapped chromatographic profiles of investigated enantiomers in a sample could be successfully processed and enantiomeric purity could be accurately determined without requiring baseline enantioresolution between peaks [7]. Recently, we have demonstrated how chiral liquid chromatography combined with multivariate techniques, specifically unfolded-partial least-squares regression (U-PLS), provides a powerful analytical methodology to face unresolved profiles. Using U-PLS, strongly overlapped enantiomer profiles in a sample could be successfully processed and enantiomeric purity could be accurately determined without requiring baseline enantioresolution between peaks [8,9].

Ibuprofen (2-(4-isobutylphenyl) propionic acid), the oldest of the newer non-steroidal anti-inflammatory drugs (NSAIDs), is an interesting example of a drug that is still sold as a racemic mixture. The pharmacological activity resides in the (*S*)-(+)-enantiomer, and it has been proved that the (*R*)-(−)-enantiomer causes some unwanted side effects [10]. However, ibuprofen has proven to be a challenge to purify enantiomerically. A large investment has been made in order to research and develop specific techniques for the synthesis of pure enantiomer (*S*)-(+)-ibuprofen [11]. Hence, for the production of active (*S*)-(+)-ibuprofen in an enantiomerically pure form, the study of its chiral purity control and stereoselective pharmacokinetics has become important tasks. Indeed, several cromatographic methods for the enantioseparation of ibuprofen from biological and pharmaceutical samples have been proposed [8,12–19].

Chiral reversed phase HPLC, using a permethyl-β-cyclodextrin column and isocratic mobile phase conditions were used to run standard and commercial samples. Ibuprofen peaks were partially overlapped and homatropine co-eluted with them. Ibuprofen is commonly prescribed with homatropine under combined formulations to treat muscle spasm and pain relief. Although these peaks can be separated under other chromatographic conditions, for instance, using a weaker elutropic strength mobile phase and increasing analysis time, we choose this real sample as a typical model to demonstrate the applicability of U-PLS algorithms to quantitatively obtain information under partial enantioresolution and in presence of interferences.

2. Experimental

2.1. Reagents and solutions

Racemic ibuprofen and (*S*)-(+)-ibuprofen were obtained from Sigma-Aldrich (St Louis, MO) whereas commercial pharmaceutical (*S*)-(+)-ibuprofen Cefalex® was purchased from Laboratorios Bagó (Bs. As., Argentina). Homatropine methylbromide was purchased from USP Reference Standard (Rockville, MD). HPLC-grade methanol was purchased from J. T. Baker (Edo. México, Mexico) and water was purified by means of a Milli-Q System (Simplicity, Millipore, MA). Mobile phase was a mixtures of methanol:buffer 0.1% triethylammonium acetate (TEAA) pH = 4.0 (measured in pure water). TEAA Buffer was prepared by mixing 300 μL of triethylamine (TEA, Anedra, Buenos Aires) and 700 μL of glacial acetic acid (Merck, Darmstadt, Germany) in 1 L of Milli-Q water.

Calibration solutions were prepared by dissolving stock solutions of racemic ibuprofen, (*S*)-(+)-ibuprofen and homatropine, in order to obtain duplicate solutions of three S:R ibuprofen ratios (99.5:0.5, 99:1 and 95:5) with three ratios of homatropine in each S:R ratio (0.0, 1.0 and 5.0% w/w) and three calibration levels, as reported in Table 1. The 54 calibration solutions are thus duplicates of three-component mixtures. Also, 45 test solutions were prepared, five for each S:R:H level, by dissolving the same stock solutions with final concentrations being at random in the corresponding calibration ranges (Table S1, Electronic Supplementary Materials).

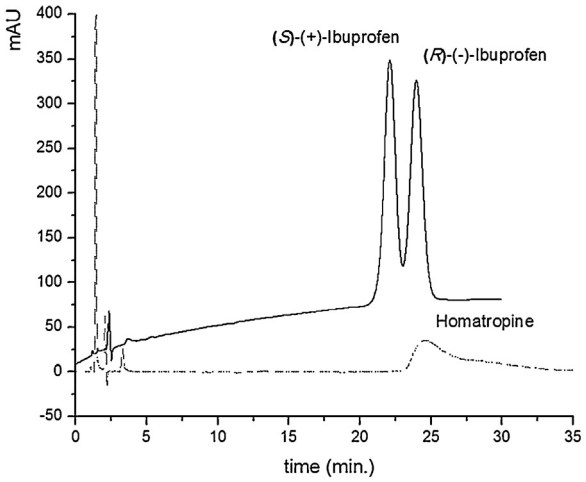

Fig. 1. Chromatographic profiles of (*R,S*)-(±)-ibuprofen (black) and homatropine (black dotted line), at the chromatographic conditions used in this work.

Samples of *(S)*-(+)-ibuprofen pharmaceutical tablets were treated as follows. Ten pills were crushed and dissolved in methanol and centrifuged at 4000 rpm during 20 min to yield two stock solutions of about 1500 mg mL^{-1} (considering the nominal content of active principle declared by the manufacturer). The standard addition method was applied to determine the accurate *(S)*-(+)-ibuprofen concentration. Adequate dilutions were prepared from the latter two stock solutions in (59:41% v/v) methanol:buffer TEAA 0.1% pH = 4.0, and then filtered through a 0.45 μm nylon membrane before injection.

2.2. Instrumentation

An HP 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with vacuum degasser, binary pump, autosampler, thermostated column device, and photodiode array detector (DAD) was used for acquiring chromatographic data. The chiral column was a Nucleodex-β-PM (200 × 4.0 mm, 5 μm) from Macherey-Nagel (Düren, Germany). Mobile phase composition was (59:41% v/v) (methanol:buffer TEAA 0.1%, pH = 4.0). The flow rate was set to 0.8 mL min^{-1} and column temperature to 30 °C. The injection volume was 10 μL. The output signals from the DAD detector were acquired between 190 and 290 nm every 1 nm with a frequency of 2.5 Hz. Data obtained by DAD were exported as 3D matrices from 18 to 30 min, from 190 to 290 nm every 1 nm. The final size of the matrices was 1800 × 102 data points.

2.3. Time alignment and data treatment

Chromatographic alignment, vectorization of data matrices and application of the U-PLS algorithm were made using MATLAB R2010a (The MathWorks, Inc., Natick, MA, USA). In the latter case, the MVC2 graphical interface toolbox was employed, which is available at [http://www.iquir-conicet.gov.ar/descargas/mvc2.rar]. In order to validate the results obtained from multivariate methods, classical univariate calibration without homatropine was applied. Figures of merit from univariate calibration were then compared with those from U-PLS chemometric method.

3. Result and discussion

Fig. 1 shows the chromatograms obtained from the chiral column with a mobile phase 59:41% v/v MeOH:buffer TEAA 0.1%,

Table 1
Calibration solutions and their concentrations.

Sample Calibration	Composition (S:R:H)	Concentration of S (mg L^{-1})	Concentration of R (mg L^{-1})	Concentration of H (mg L^{-1})
1	99.5:0.5:0	19.18	0.12	0.00
2	99.5:0.5:0	19.18	0.12	0.00
3	99.5:0.5:0	479.38	3.10	0.00
4	99.5:0.5:0	479.38	3.10	0.00
5	99.5:0.5:0	958.76	6.20	0.00
6	99.5:0.5:0	958.76	6.20	0.00
7	99.5:0.5:1	18.79	0.12	0.20
8	99.5:0.5:1	18.79	0.12	0.20
9	99.5:0.5:1	469.66	3.10	5.00
10	99.5:0.5:1	469.66	3.10	5.00
11	99.5:0.5:1	939.32	6.20	10.00
12	99.5:0.5:1	939.32	6.20	10.00
13	99.5:0.5:5	17.23	0.12	1.00
14	99.5:0.5:5	17.23	0.12	1.00
15	99.5:0.5:5	430.78	3.10	25.00
16	99.5:0.5:5	430.78	3.10	25.00
17	99.5:0.5:5	861.56	6.20	50.00
18	99.5:0.5:5	861.56	6.20	50.00
19	99:1:0	28.58	0.31	0.00
20	99:1:0	28.58	0.31	0.00
21	99:1:0	714.55	7.75	0.00
22	99:1:0	714.55	7.75	0.00
23	99:1:0	1429.10	15.50	0.00
24	99:1:0	1429.10	15.50	0.00
25	99:1:1	27.69	0.31	0.30
26	99:1:1	27.69	0.31	0.30
27	99:1:1	692.23	7.75	7.50
28	99:1:1	692.23	7.75	7.50
29	99:1:1	1384.46	15.50	15.00
30	99:1:1	1384.46	15.50	15.00
31	99:1:5	24.42	0.31	1.40
32	99:1:5	24.42	0.31	1.40
33	99:1:5	610.39	7.75	35.00
34	99:1:5	610.39	7.75	35.00
35	99:1:5	1220.78	15.50	70.00
36	99:1:5	1220.78	15.50	70.00
37	95:5:0	25.05	1.24	0.00
38	95:5:0	25.05	1.24	0.00
39	95:5:0	626.20	31.00	0.00
40	95:5:0	626.20	31.00	0.00
41	95:5:0	1252.40	62.00	0.00
42	95:5:0	1252.40	62.00	0.00
43	95:5:1	24.16	1.24	0.30
44	95:5:1	24.16	1.24	0.30
45	95:5:1	603.88	31.00	7.50
46	95:5:1	603.88	31.00	7.50
47	95:5:1	1207.76	62.00	15.00
48	95:5:1	1207.76	62.00	15.00
49	95:5:5	21.48	1.24	1.20
50	95:5:5	21.48	1.24	1.20
51	95:5:5	536.92	31.00	30.00
52	95:5:5	536.92	31.00	30.00
53	95:5:5	1073.84	62.00	60.00
54	95:5:5	1073.84	62.00	60.00

pH = 4.0 and at 30 °C. Under these conditions, the enantioseparation would be considered acceptable for quantitative purposes of the racemic ibuprofen mixture analysis, where both peaks have the same areas. However, for enantiomeric fraction determinations, real conditions consist in quantifying very low levels of the (*R*)-(−)-enantiomer, where the minor (trace) signal will be masked with the tailing edge corresponding to the (*S*)-(+)-enantiomer. The situation would be even more complex if an interference from the matrix is present. Fig. 1 also shows the superimposed profile corresponding to homatropine, a molecule that is included in some commercial tablets of ibuprofen. A co-elution between the (*R*)-(−)-enantiomer and homatropine is clearly evident. The aim of the study was to assess the quality of the enantiomeric analytical figures under this completely unacceptable condition from a chromatographic point of view. Therefore, solutions containing S:R ratios of 95:5, 99:1 and 99.5:0.5 were prepared with homatropine (at concentration levels of 0; 1 and 5%w/w). Fig. 2Ashows a three-dimensional plot of a chromatographic-spectral matrix. This plot corresponds to a mixture (S:R:H 95:5:5). (Fig. 2B has been enlarged to show the R-enantiomer profile). Clearly, a single peak without any shoulder is observed in the chromatograms, corresponding to the (*R*)-(−)-enantiomer and to the interferent.

The overlapped profiles with identical UV spectra and, furthermore, the impossibility of acquiring reproducible signals from sample to sample, makes deconvolution methods not applicable [20]. Similarly, the non-reproducible signal patterns make inapplicable some chemometric tools such as the parallel factor analysis (PARAFAC) algorithm. On the other hand, the multivariate curve resolution (MCR) algorithm can be used whenever elution profiles from different experimental runs would be shifted; however, this is not true in the event of identical spectra for both sample components [21]. U-PLS regression after a simple chromatographic

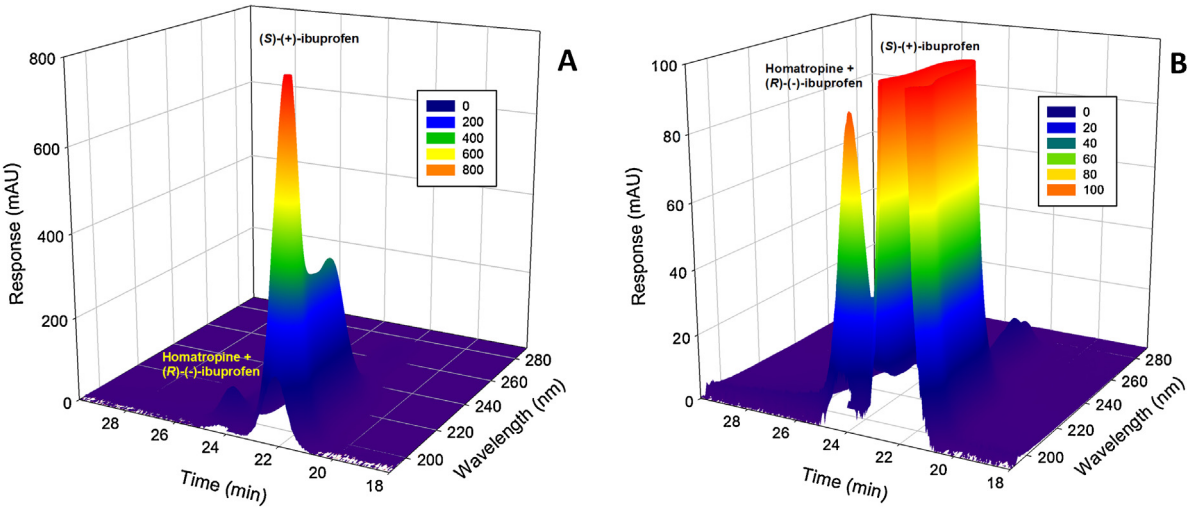

Fig. 2. Data matrix for the calibration sample (S:R:H 95:5:5). (A) Three-dimensional surface showing the absorbance as a function of retention time and wavelength for a typical sample showing the small peak due to the (*R*)-(−)-enantiomer with homatropine. (B) Vertical scale expanded for better appreciation of the small peak.

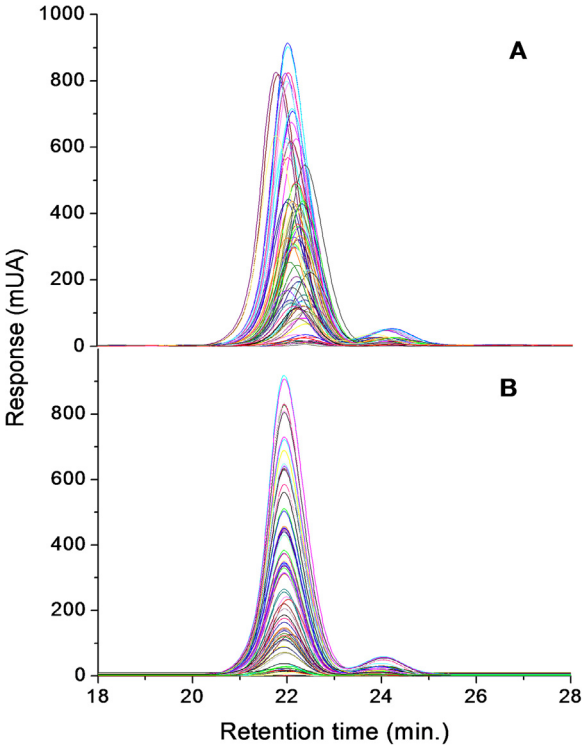

Fig. 3. (A) Raw chromatograms, plotted in a selected retention time range, at the absorption wavelength of 220 nm. They were taken from the complete data matrices employed for calibrating the U-PLS model. (B) Aligned chromatograms so that the positions of the maximum for the (*S*)-(+)-enantiomer match.

alignment procedure applied to peak positions, with the latent variables employed by this algorithm to model the changes in elution profile shapes, was tested in the present work.

Fig. 3A(left-side) shows the eluted chromatographic profiles of different samples run consecutively. The small changes in the time-scale peak positions are typical in HPLC experiments, and these shifts can be attributed to many (concurrent) events, such as temperature fluctuations, subtle changes in mobile phase compositions within the proportioning valve as well as the column history. Before the U-PLS algorithm can be applicable, the peaks have to be registered in the same time position in the data matrix, so the algorithm can recognize the signals correctly. For this reason, it is necessary to align the chromatographic profiles in the elution time direction. In some cases, alignment can be performed by seeking to maximize the correlation between a chromatogram and a reference one by using alignment algorithms [22]. However, this strategy fails when severe overlapping takes place. In this work, the matrices have been shifted by selecting one of them as a reference, and digitally moving all the others in the time direction until the maximum peak for the major component was aligned with the one in the reference matrix. The results are shown in Fig. 3B (right-side).

3.1. U-PLS analysis

The U-PLS algorithm was successfully used, due to its intrinsic flexibility towards profile shape changes, whenever these profiles are being modelled during the calibration phase. [8,9] A latent structured unfolded partial least-squares (PLS model) is built using unfolded calibration data and analyte concentration information. The output provides regression coefficients useful for concentration predictions of new samples [23]. Figures of merit, such as sensitivity, uncertainty in predicted concentration and limit of detection (LOD) according to the latest IUPAC recommendations [24–27], can be readily estimated using known expressions.

The optimum number of factors for building the U-PLS model was obtained by leave-one-out cross validation. In this method, the model is constructed with all calibration samples except one, and the concentration for this sample is predicted and the corresponding error is calculated. Then, the procedure is repeated until all samples have been left out once. Initially a relatively large number of factors is selected and the predicted error sum of squares (PRESS) is calculated for each factor. By observing the changes of the PRESS in terms of the number of factors, we can find their optimal number. The results of (*S*)-(+)-ibuprofen, (*R*)-(−)-ibuprofen and homatropine can be seen in Fig. 4.

The accuracy and robustness of the calibration were demonstrated by predicting a set of independent test samples whose nominal concentrations are given in Table S1 (Electronic Supplementary Materials**).** The root mean square error of prediction (RMSEP), a measure of the variability of the difference between predicted and nominal values for the set of samples, provides an idea of the prediction uncertainty and bias. Larger errors for the (*S*)-(+)-enantiomer as compared to the minor peak are attributed to the overloading mass for the more concentrated solutions. The use of those data during calibration undoubtedly impaired the precision.

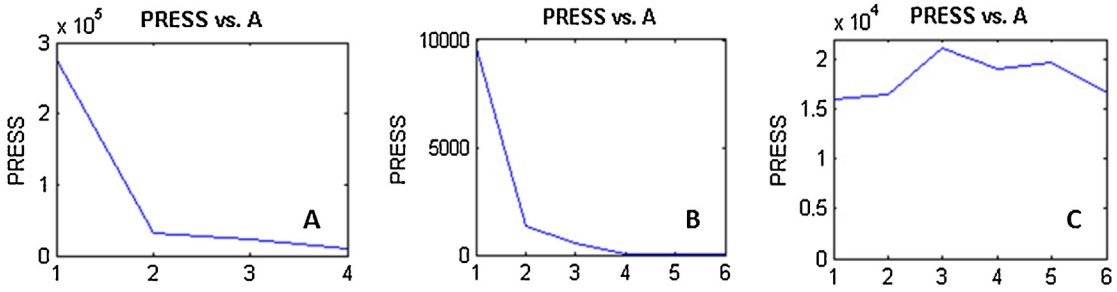

Fig. 4. Leave-one-out cross-validation analysis of (A) (*S*)-(+)-ibuprofen, (B) (*R*)-(−)-ibuprofen and (C) homatropine.

Table 2
Analytical figures of merit.

Parameters[a]	S-Ibuprofen		R-Ibuprofen	
Condition	Univariate calibration	U-PLS calibration	Univariate calibration	U-PLS calibration
Number of latent variables (*A*)	–	4	–	6
RMSEP	16.4	21	1.9	0.39
REP%	–	3.7	–	3.2
R^2	0.998	0.992	0.991	0.969
Sensitivity[b] (mAU L mg^{-1})	3.077	1.6	2.893	5.1
Analytical sensitivity (L mg^{-1})	0.5650	2.6	4.0052	23
LOD (mol)[c]	1.98×10^{-9}	4.96×10^{-10}	2.71×10^{-10}	1.60×10^{-11}
LOQ (mol)[c]	6.01×10^{-9}	1.50×10^{-9}	8.29×10^{-10}	4.85×10^{-11}
Concentration Range (mg L^{-1})	123.9–1429.1	31–1384.5	17.1–62	1–62

[a] RMSEP = root mean squared error of prediction, REP% = relative error of prediction, R^2 = squared correlation coefficient, analytical sensitivity = ratio of sensitivity to instrumental noise, LOD = limit of detection (in the U-PLS calibration, the maximum value of the LOD range), LOQ = limit of quantitation; LOQ = 10 × SD(0).
[b] In U-PLS, sensitivity is the inverse of the norm of the regression coefficients.
[c] The injection volume was 10 μL.

The errors were, however, acceptable for the (*S*)-(+)-enantiomer and significantly small for the (*R*)-(−)-ibuprofen.

Chemometric tools have been applied to data obtained from spectroscopic measurements in order to determine enantiomeric ratios, and relative error predictions below to 2% to more than 20% were informed for different spectroscopic data. A wide range of prediction errors were reported by Fakayode et al. [28] for enantiomeric composition of drugs using UV-absorption spectra measurements in the presence of cyclodextrins. Different chiral compound formed association complexes of different stability and, thus, their UV signals modelled in the presence of the β-cyclodextrin lead to large differences in the quality of concentration predictions. On the other hand, lower relative errors of prediction have been reported when the validation samples were prepared with solutions containing lower enantiomeric fractions, i.e., concentration conditions much less exigent for the data treatment of chemometric models [29,30].

Regarding the numbers of optimum latent variables, they are smaller in the case of the major (*S*)-(+)-enantiomer, due to the fact that the signal from the minor (*R*)-(−)-enantiomer is considerably small. The latter is greatly influenced by the signal from the major (*S*)-(+)-enantiomer and also by any residual variation of the chromatographic band from sample and to sample.

Although it was possible to determine the concentration of (*R*)-(−)-ibuprofen with almost the same relative error of prediction respect to (*S*)-(+)-enantiomer, U-PLS was unable of predicting the homatropine concentrations, even using all wavelengths. The small concentrations of this interferent, and the tailing of homatropine chromatographic profile are likely affecting the response. Fig. S1 (Electronic Supplementary Materials) shows the predicted concentrations of (*S*)-(+)-ibuprofen in commercial tablets with the addition of homatropine using U-PLS calibration. It follows that there is an excellent correlation between nominal concentration and found concentration (unit slope), even when the nominal concentrations were very high.

3.2. Validation

Guidance for impurity determinations can be taken from The International Conference on Harmonisation (ICH) [http://www.ich.org/cache/compo/276-254-1.html] [31], since no official method for the determination of enantiomeric purity of chiral compounds is stated. Table 2 shows the results of sensitivity, and limits of detection and quantification for univariate analysis measured from baseline separated peaks. The univariate figures of merit are compared with those obtained from U-PLS calculations. The statistical prediction results (RMSEP, REP%, R^2) are also shown in Table 2. The latter values are relevant, since they allow us to assess the detection capability of the developed method concerning the minor component in the studied mixtures. According to Table 2, the LOD was as low as 1.60×10^{-11} mol (when the injection volume was 10 μL). Since the mean calibration concentration of the major enantiomer is ca. 600 mg L^{-1}, the later LOD value corresponds to a ratio 6000:3.3, i.e., a composition rate 99.94:0.06.

4. Conclusion

We could obtain quantitative information of enantiomers of ibuprofen eluted from a chromatographic column as partially overlapped peaks and even under the presence of an interference, by using U-PLS data treatment. Very good figures of merit were achieved, especially for the trace peak, demonstrating that the combination of chemometric methods with chromatographic-spectroscopy data can be a powerful analytical strategy in the analysis of enantiomers or substances when complete separation is not possible.

It is also envisaged that this combination would be very powerful in the following circumstances: (1) to reduce analysis time by using stronger mobile phases to elute earlier all the interesting peaks and (2) to circumvent the usual lower enantioresolution factors achieved in reversed-phase chiral systems as compared to the

larger normal phase enantioselectivities and, therefore, to reduce analysis cost.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.05.094.

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