ANEXO 2

“Use of circular dichroism and artificial neural networks for the kinetic-spectrophotometric resolution of enantiomers.”

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Use of circular dichroism and artificial neural networks for the kinetic-spectrophotometric resolution of enantiomers

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Abstract

The circular dichroism (CD) technique was used to resolve 1-phenylethylamine enantiomers by their differential rate of reaction under non-pseudo first-order kinetic conditions with the chiral feagent (−)-citronellal. The same reaction was also monitored under pseudo first-order conditions using UV–VIS spectrophotometry and the results provided by the two techniques were compared by using principal component regression (PCR), partial least-squares regression (PLSR) and artificial neural networks (ANNs) for multivariate calibration. The best results were obtained by compressing the data matrix provided by the CD technique with principal component analysis (PCA) and using the scores of the principal components as input for the ANN. The relative standard error (R.S.E.) of prediction thus obtained was about 3% for both enantiomers.

Keywords: Circular dichroism; Artificial neural networks; Kinetic-spectrophotometric methods; Enantiomers; 1-Phenylethylamine

1. Introduction

Determination of enantiomers in samples of chiral compounds is of high interest to the pharmaceutical industry as a large number of pharmacologically active substances possess one or more chiral centers. The significance of such determinations stems from the fact that enantiomers may possess rather different pharmacological properties (e.g. one of them may be less active than the other, completely inactive or even toxic) [1].

Traditional methods for determining enantiomeric purity rely on spectrophotometric measurements of optical activity [2]. Specially prominent among them are those based on the circular dichroism (CD) technique [3], which is applied to pure chiral compounds in a manner similar to UV–VIS spectrophotometry; in addition, it has the advantage that the optical activity of a compound may be more specific than its absorption because its determination can only be interfered by another chiral compound. The problem with direct application of CD to the resolution of enantiomer mixtures is that the signals obtained are proportional to the concentration difference between the two enantiomers. This requires the use of a complementary technique (e.g. UV–VIS spectrophotometry) to determine the combined concentration of both enantiomers in order to formulate and solve an appropriate system of equations. Horválth et al. [4] used one such technical combination to assess the enantiomeric purity of phenylglycine, which requires prior purification or chromatographic separation if the sample contains any UV–VIS spectrophotometrically or CD active contaminant.

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The shortcomings of optical methods have gradually been circumvented by developing techniques to separate enantiomers. The most widely used of which is classical liquid chromatography (LC) [5] in combination with a chiral or non-chiral column and a specific (e.g. CD spectropolarimeter) or non-specific detector (e.g. a UV–VIS spectrophotometer). In many cases, chromatographic techniques involve long analysis times, use large amounts of sample and solvents, and generate waste. Another, increasingly used alternative is capillary electrophoresis (CE) combined with chiral selectors [6].

The advent of new methodologies and the inception of chemometric calibration methods have promoted a resurgence of spectroscopic techniques in recent years. Thus, Erskine et al. [7] used both UV–VIS and CD measurements in conjunction with multivariate regression to determine the enantiomeric purity of ephedrine, pseudoephedrine and mandelic acid. One alternative to this methodology is the use of chiral selectors to distinguish the CD spectra of the enantiomers [8]. Thus, Blanco et al. [9] used cyclodextrins to determine the enantiomeric purity of ketoprofen by using the CD technique and PLS regression as multivariate calibration procedure.

Kinetic methods of analysis are usually employed to resolve mixtures of highly similar species which react at a different rate with a given reagent. The analytical signal obtained is time-dependent and usually takes the form of a UV–VIS or fluorescence spectrum. When the analytes to be determined are the enantiomers of a chiral compound, these react at the same rate with a common reagent, and both their UV–VIS and their fluorescence spectra are identical. If the enantiomers react with a chiral reagent, the difference in their reaction rates can be used for their kinetic resolution. In addition, if the conventional UV–VIS detection system is replaced by one that is specific to chiral substances such as the CD detector, the kinetic-spectrophotometric discrimination is enhanced as the CD spectra for the diastereoisomers formed can be rather different. Consequently, the CD technique should provide better results than UV–VIS spectrophotometry on the basis of its increased selectivity.

In this work, we used the CD technique for the kinetic-spectrophotometric resolution of enantiomers by their different rate of reaction with a chiral compound. As an example, the reaction of 1-phenylethylamine with the chiral reagent (−)-citronellal and the CD spectral difference between the two imine diastereoisomers formed were studied (Fig. 1). Using the UV–VIS technique, the spectra obtained for the reaction products (diastereoisomers) are too similar, so the most important source of information is the difference in reaction rate. This allows the resolution of mixtures only with similar concentrations of each enantiomers and it is inadequate when one of them is in high excess.

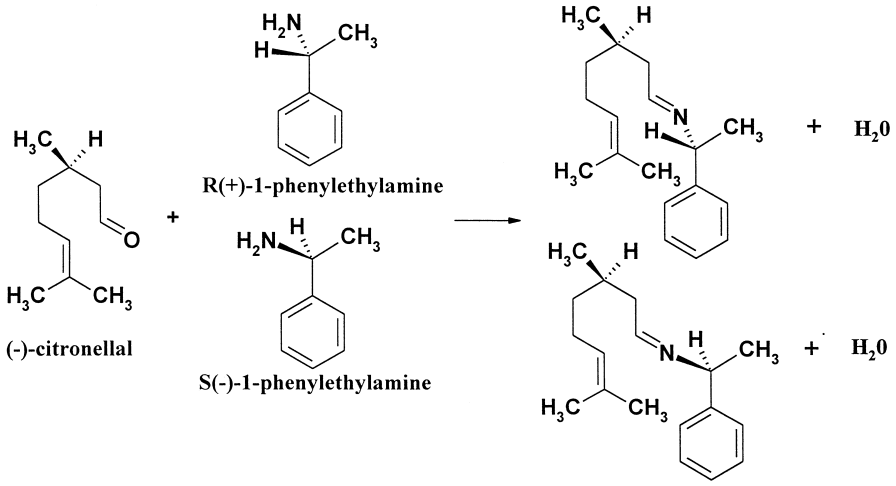

Fig. 1. Reaction between 1-phenylethylamine enantiomers and the aldehyde (−)-citronellal.

However, the use of the CD spectrum in conjunction with kinetic information allows both enantiomers to be determined. The kinetic-spectrophotometric information obtained was processed using as multivariate calibration methods: partial-least squares regression (PLS), principal component regression (PCR) and artificial neural networks (ANNs), which afford the effective resolution of chemical systems with strongly similar UV–VIS spectra and kinetics [10].

2. Experimental section

2.1. Apparatus

A Hewlett-Packard 8453 diode array spectrophotometer was used to acquire UV spectra at 2 nm intervals over the wavelength range 230–350 nm. Scans were performed at 5 s intervals for 300 s, using a thermostated cell of 1 cm path length at 25.0 ± 0.1°C.

A JASCO J-715 spectropolarimeter from Japan Spectroscopy Co. (Tokyo, Japan) equipped with a 150 W xenon lamp and a nitrogen drain, was used for CD measurements, all of which were made in rectangular quartz cuvettes of 1 cm path length. Spectra were acquired under the following conditions: scan rate 50 nm/min, bandwidth 5 nm, time constant (response) 1 s, resolution 0.2 nm, wavelength range 230–330 nm. Overall 31 scans (660 s) without a time interval were recorded. The temperature of the sample in the measuring compartment was kept at 25.0 ± 0.2°C with the aid of a PTC-351S 6-position Peltier effect cell changer.

A set of micro-pipettes PIPETMAN® P (Gilson) models P200, P1000 and BIOHIT model 720110 were used to prepare the mixtures of enantiomers. The volume ranges are: 50–200 μl for the P200, 200–1000 μl for the P1000 and 1–5 μl for the 720110 model. Before using them their performances were checked. The calibration was carried out using a gravimetric method at 22°C with distilled water in an analytical balance with readability of 0.1 mg.

2.2. Reagents

The solvent used to prepare all solutions was a 1:1 mixture of 2-propanol and water from a Milli-Q Millipore water purification system. Stock solutions containing a 3.0×10^{-2} M concentration of either enantiomer of 1-phenylethylamine (Merck-Schuchardt, optical purity by GC > 99%) and mixtures of both were made in a 0.05 M boric acid buffer of pH 8 adjusted with 0.1 M NaOH and at constant ionic strength of 0.05 M KCl. A 0.11 M stock solution of (−)-citronellal (Fluka Chemika, optical purity by GC > 98%) was used as the common reagent. Stock solutions of each enantiomer and (−)-citronellal were prepared by weighting the appropriate volumes using an analytical balance with readability 0.1 mg.

2.3. Procedure

2 ml of the enantiomer mixture and a volume of the (−)-citronellal solution (0.75 and 0.2 ml for UV and CD, respectively) were added, with the aid of micropipettes, directly into the measuring cell. The system was kept at a constant temperature under stirring during the reaction. The experimental design for the calibration system is shown in Fig. 2 where the total amine concentration in the measuring cell lays within the range 1–5 mM. In order to keep the total concentration between the above limits and because both enantiomers are related together, a triangular design was selected. Each one of the five diagonals (from left to right) represents one concentration level. The calibration matrix was constructed from 18 samples (viz.

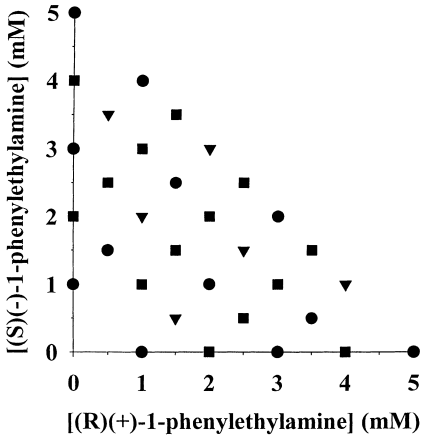

Fig. 2. Composition of the mixtures in the calibration (●), test (▼) and external prediction (■) sets.

the training and test sets for ANN computations) and an external prediction set was formed with 14 other mixtures. The reagent concentration in the cell was 0.03 and 0.01 M in UV and CD measurements, respectively. In order to include experimental variability factors, mixtures were prepared and measured on different days.

2.4. Processing of data

The UV and CD spectra for each sample were recorded at p different wavelengths (λ_1, λ_2, ..., λ_p) at k different times (t_1, t_2, ..., t_k) in order to construct 3-way data arrays that were unfolded in order to obtain a classical two-dimensional data matrix in such a way that each row contained the spectrum for a mixture recorded at different times sequentially linked together ($\lambda_1 t_1$, $\lambda_2 t_1$, ..., $\lambda_p t_1$, ..., $\lambda_i t_j$, ..., $\lambda_1 t_k$, $\lambda_2 t_k$, ..., $\lambda_p t_k$), so each column contained the absorbance measured at (λ_i, t_j) for each sample. Data matrix $\boldsymbol{X}$, which contained 3721 and 16,032 variables for UV and CD, respectively, was centred prior to processing. Data matrix $\boldsymbol{Y}$ contained the concentrations of both enantiomers.

The derivative of the data matrix was obtained independently for each time in the spectral domine by using the Savitzky–Golay algorithm with a second-order polynomial and a window size of 5 and 11 points for UV and CD, respectively.

The data matrices thus obtained were processed by using the PCR and PLS algorithms in the software Unscrambler v. 7.5 (CAMO A/S, Trondheim, Norway). PLS models were constructed by cross-validation and PCR ones by using the test set method for comparison with the ANN results. The optimum number of PLS components or PCs was determined in order to minimize the sum of the squared differences between the known and the determined concentrations:

$$\text{Press} = \sum_{i=1}^{m} (c_i - \hat{c}_i)^2 \quad (1)$$

Here m is the number of samples, c_i the known concentration and $\hat{c}_i$ the determined concentration.

ANNs were constructed by using the software Neural Connection v. 2.00 (SPSS, Inc., Chicago, IL), using the scores provided by the PCR models as input. The network training procedure was based on the conjugate gradient algorithm, which measures the gradient of the error surface after each sample passes through the network. The weights of the neuron inputs are altered using a compromise between the direction of the steepest gradient and the previous direction of change. So, during the training process, weights are iteratively calculated in order to minimize the Press (Eq. (1)). Overfitting is avoided by using two set of samples; thus, weights are calculated from a calibration set while the concentration of another sample set (test set) is being simultaneously predicted. Every sample in the calibration set was used in each of the four training steps. A Gaussian distribution of initial weights and a sigmoidal transfer function for the neurons of the hidden layer were used. A linear transfer function was used for the output layer.

Each input presented to the neural model has an individual range of operation. Certain inputs will have larger mean values, and greater variance, than others. So these would have larger weight values associated and a greater effect on the outputs of the model (response). In order to ensure that each input was equally used by the neural model, all inputs to the model were normalized such that they had the same mean value and range.

The training was stopped manually when the RMS error of the test set remained constant after successive iterations. Because there could be several local minima where the model could arrive, the algorithm was run from different starting values of the initial weight (seed) to find the best optimum, but the same results were obtained.

Models were tested on an external prediction set that consisted of samples belonging to neither the calibration set nor the test set.

The overall predictive capacity of the models was compared in terms of the R.S.E. defined as

$$\%\text{R.S.E.} = \sqrt{\frac{\sum_{i=1}^{m} (c_i - \hat{c}_i)^2}{\sum_{i=1}^{m} c_i^2}} \times 100 \quad (2)$$

and designated %RSEC, %RSEP and %RSEEP for the calibration, test and external test set, respectively.

3. Results and discussion

3.1. Chemical system

The reaction between a carbonyl group and a primary amine, which follows a second-order kinetics, is well-documented [11] and used in kinetic-spectrophotometric determinations [12]. In the presence of a large excess of the carbonyl compound at a constant pH, the imine formation rate can be expressed as

$$v = k_{\text{apparent}}[\text{amine}] \quad (3)$$

where $k_{\text{apparent}} = k_\nu[\text{carbonyl}]$ and the reaction follows a pseudo first-order kinetics.

The low water-solubility of the aldehyde used, (−)-citronellal, leads to the use of a 1:1 ratio of 2-propanol/water mixture as the solvent. This restricted the choice of buffer as most buffering salts are poorly soluble in alcohols. A boric acid/NaOH, 0.05 M KCl met the requirements. By altering the boric acid/NaOH concentration ratio, the reaction of each enantiomer was monitored by UV–VIS spectrophotometry at pH 8–9.5. The apparent rate constant, k_{apparent}, and as a result k_ν, were calculated from the logarithmic extrapolation method [13],

$$\log(A_\infty - A_t) = \log[A]_0 - \frac{k_{\text{apparent}}t}{2.303} \quad (4)$$

where A_∞ and A_t are the absorbances measured at the end of the reaction and after a time t, respectively; and $[A]_0$ is the initial concentration of the monitored enantiomer.

As can be seen from Fig. 3, k_ν and hence the reaction rate decreases with increasing pH, which determines the kinetic difference between the two enantiomers. Based on these results, pH 8 was chosen to resolve the mixtures and was used at different temperatures (20, 25 and 30°C) to examine the reaction. Fig. 4 shows the variation of k_ν with temperature. As expected, it increased with increasing temperature; also, from the slopes of the lines, the kinetics of the two enantiomers should be more markedly different at low temperatures. However, we decided to use 25°C as the working temperature because it was closer to the ambient temperature — a lower temperature would have involved a longer reaction time and called for

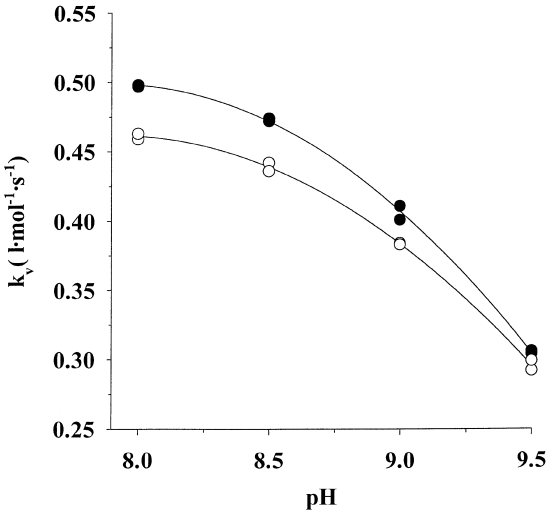

Fig. 3. Variation of k_ν for both 1-phenylethylamine enantiomers with pH over the range 8–9.5. Buffer: boric acid/NaOH/KCl; $T = 25$°C; [(−)-citronellal] $= 3 \times 10^{-2}$ M; [enantiomer] $= 1 \times 10^{-3}$ M. The kinetic constant, k_ν, was estimated from Eq. (4) and $k_{\text{apparent}} = k_\nu[(-)\text{-citronellal}]$. (●) (*S*)(−)-1-phenylethylamine. (○) (*R*)(+)-1-phenylethylamine.

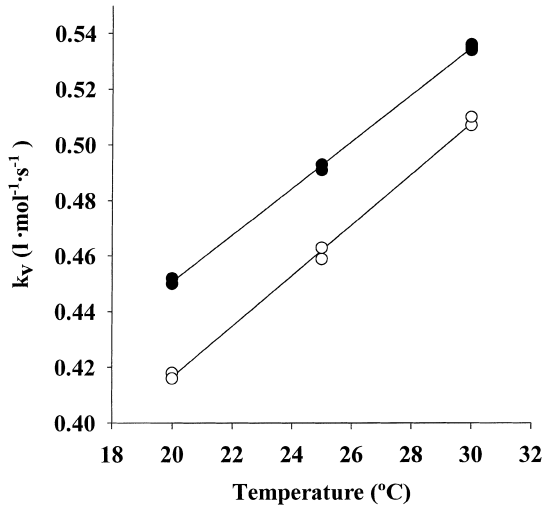

Fig. 4. Variation of k_ν for the two 1-phenylethylamine enantiomers with temperature (20, 25 and 30°C). Buffer: boric acid/NaOH/KCl at pH 8; [(−)-citronellal] $= 3 \times 10^{-2}$ M; [enantiomer] $= 1 \times 10^{-3}$ M. The kinetic constant, k_ν, was estimated from Eq. (4) and $k_{\text{apparent}} = k_\nu[(-)\text{-citronellal}]$. (●) (*S*)(−)-1-phenylethylamine: intercept $= (0.282 \pm 0.007)$ l mol^{-1} s^{-1}, slope $= (0.0084 \pm 0.0003)$ l mol^{-1} s^{-1}°C^{-1}, $r = 0.999$. (○) (*R*)(+)-1-phenylethylamine: intercept $= (0.234 \pm 0.008)$ l mol^{-1} s^{-1}, slope $= (0.0091 \pm 0.0009)$ l mol^{-1} s^{-1}°C^{-1}, $r = 0.999$.

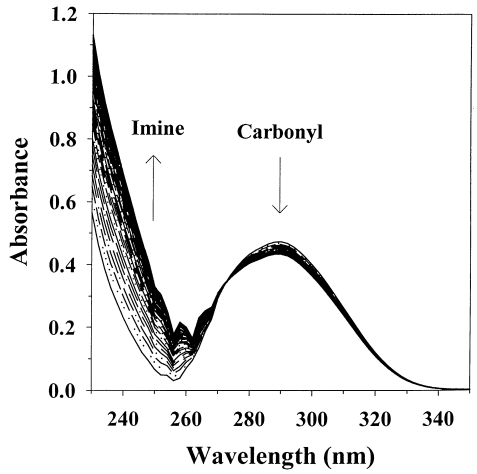

Fig. 5. Kinetic UV–VIS spectra for the reaction of pure 3×10^{-3} M (*S*)(−)-1-phenylethylamine with 3×10^{-2} M (−)-citronellal at pH 8 at 25°C. Spectra were recorded from 0 to 5 min at 5 s intervals. Arrows signal the increase in the absorption band for the imine formed and the decrease in that for the carbonyl group.

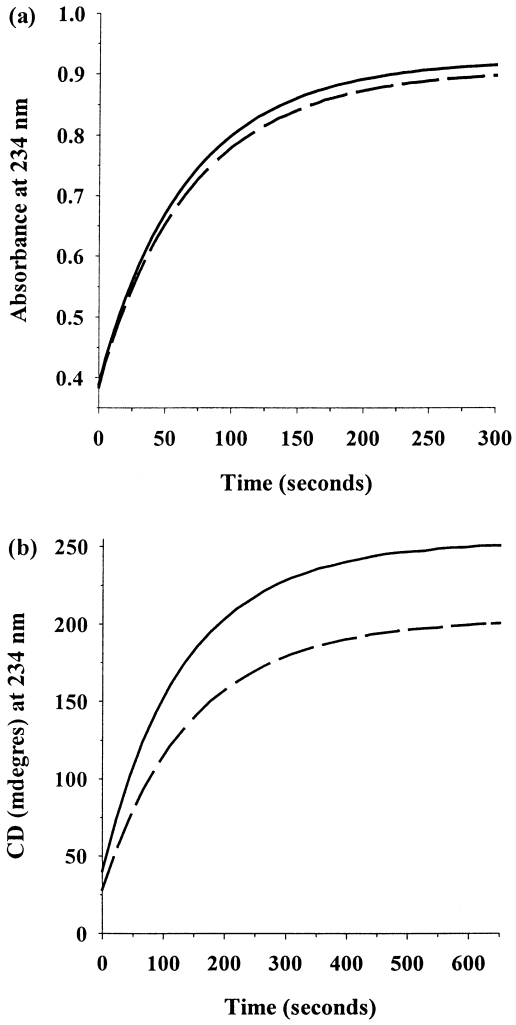

Fig. 6. Kinetic profiles obtained at 234 nm for the reaction of the pure enantiomers at a 3×10^{-3} M concentration; pH 8 and 25°C with (a) 3×10^{-2} M (−)-citronellal. UV spectra were recorded from 0 to 5 min at 5 s intervals. (b) 1×10^{-2} M (−)-citronellal. The signal sign of (*S*)-enantiomer has been changed in the plot. An overall 31 time points were recorded at 50 nm/min without time interval in CD spectra, (*S*)(−)-1-phenylethylamine. (*R*)(+)-1-phenylethylamine.

conditioning operations in order to ensure reproducibility in the kinetics.

Fig. 5 shows the kinetic-spectrophotometric spectrum for the reaction of 3×10^{-2} M (−)-citronellal with 3×10^{-3} M (*S*)(−)-1-phenylethylamine at pH 8 at 25°C. As can be seen, the carbonyl group in the aldehyde exhibits a band at 290 nm that remains unchanged throughout the reaction. The formation of the imine is signalled by the presence of a band in the wavelength range 230–270 nm. Under experimental conditions, both (*S*)(−)-1-phenylethylamine and (*R*)(+)-1-phenylethylamine exhibit a total reaction time of about 5 min (see Fig. 6 (a)).The UV spectra for the diastereoisomers formed in the reaction are too similar, so the source of analytical information allowing the two enantiomers to be discriminated will be the difference in reaction rate.

Using the CD technique the optical spectral differences between the diastereoisomers are apparent from Fig. 7. A comparison of the kinetic CD spectra for the two enantiomers reveals the presence of a band at 234 nm in both, but of different sign in the two; on the other hand, the band at 290 nm, which corresponds to the carbonyl group, changes identically in both enantiomers. Because CD spectra take longer to be recorded than do UV–VIS spectra, the concentration of (−)-citronellal was decreased in order to slow down the reaction rate. Under these conditions, the system did not follow pseudo first-order kinetics.

The spectra for the diastereoisomers formed from the racemic mixture exhibit a small band at 234 nm of the same sign as those obtained for

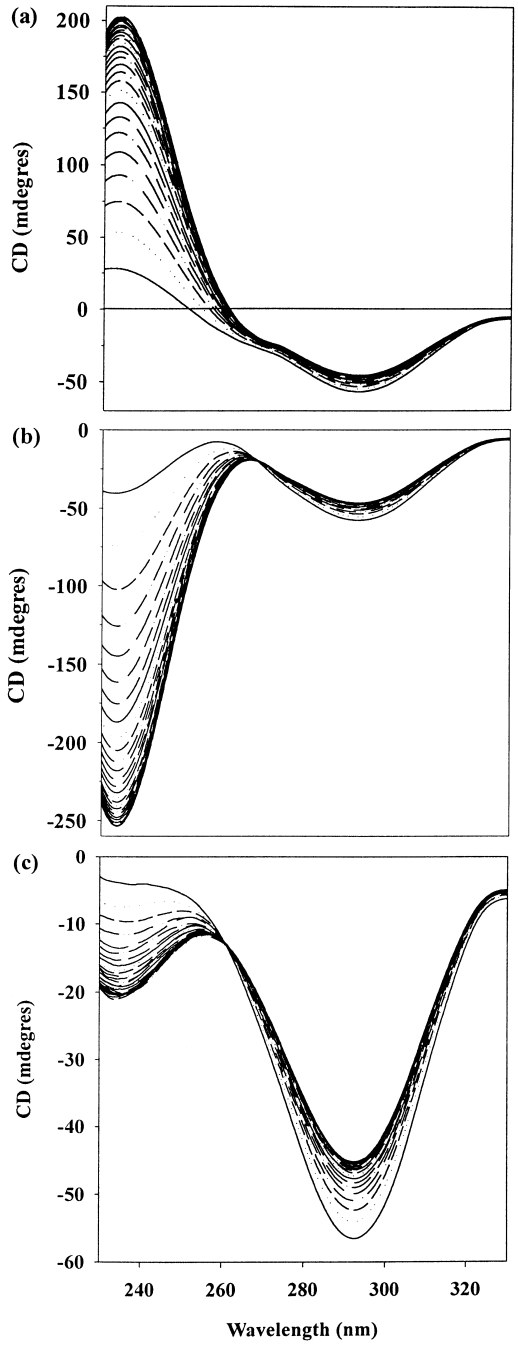

Fig. 7. Kinetic CD spectra for the reaction of the enantiomers at a 3×10^{-3} M concentration with 1×10^{-2} M (−)-citronellal at pH 8 at 25°C. An overall 31 time points were recorded at 50 nm/min without time interval. (a) (*R*)(+)-1-phenylethylamine. (b) (*S*)(−)-1-phenylethylamine. (c) Racemic mixture.

(*S*)(−)-1-phenylethylamine, as it is shown in Fig. 7(c). The difference in reaction rate between the two amines at these experimental conditions is shown in Fig. 6(b), where the signal sign of (*S*)-enantiomer has been changed in order to compare the plots. As can be seen, also at these experimental conditions (*S*)-enantiomer reacts faster than (*R*)-enantiomer. Thus, the information provided by the small difference in reaction rate between the two amines, and the optical spectral differences between the diastereoisomers formed, are used in this technique.

As can be seen, the CD technique provides spectral discrimination that is essential for resolving the enantiomers.

3.2. Determination of (S)(−)-1-phenylethylamine and (R)(+)-1-phenylethylamine

Several wavelength ranges and time intervals were used to construct calibration models and make predictions in both the absorbance and the first-derivative mode. The best results were obtained by using first-derivative spectra recorded over an interval of 200 s for UV–VIS and in 25 scans (528 s) for CD. Table 1 compares the %R.S.E. values obtained by both techniques and PLS regression. As can be seen, the CD technique provided better results, even though it operated under non-pseudo first-order kinetic conditions.

In order to correct intrinsic non-linearity in the kinetic system recorded by CD, the ANNs have been used as non-linear multivariate calibration method. The ANN models were constructed by using as inputs the PCs from the PCR model constructed from the same data matrix employed in the PLS models and their architecture has been optimized, especially, the number of neurons in the input and hidden layers. The calibration set was split into two subsets for this purpose, viz. a training set and a test set which consisted of 14 and 6 samples, respectively (Fig. 2). The predictive capacity of the ANN was checked on the same external prediction test used before. Table 1 gives the %R.S.E. values obtained with this model; as can be seen, ANN yielded better results than PLS. Table 2 shows the added concentrations of both enantiomers in the samples of the external prediction set and the difference (found − added) provided by the ANN model.

Table 1
%R.S.E. values (Eq. (2)) obtained in the resolution of the binary enantiomer mixture using both techniques in the first-derivative mode in conjunction with PLS1, PCR and ANN calibration methods

Technique	Range (nm)	Calibration methods	Analyte	%RSEC	%RSEP	%RSEEP
UV–VIS	234–300	PLS1[a]	(*R*)(+)-amine	6.4	–	7.2
			(*S*)(−)-amine	6.8	–	6.5
CD	231–329	PLS1[b]	(*R*)(+)-amine	4.6	–	4.0
			(*S*)(−)-amine	4.4	–	4.6
		PCR[b]	(*R*)(+)-amine	4.5	5.0	3.9
			(*S*)(−)-amine	4.7	4.1	4.4
		ANN[c]	(*R*)(+)-amine	4.1	4.0	3.4
			(*S*)(−)-amine	2.7	3.2	3.2

[a] Number of PCs used in the models = 4.
[b] Number of PCs used in the models = 2.
[c] The ANN architecture was: input nodes = 2; hidden nodes = 2; output nodes = 1.

Table 2
Results provided by the ANN, architecture (2,2,1)[a], in the resolution of mixtures of (*S*)(−)-1-phenylethylamine and (*R*)(+)-1-phenylethylamine in the external prediction set

Mixture	Added ($\times 10^{-3}$ mol l^{-1})		Found − added ($\times 10^{-3}$ mol l^{-1})	
	(*S*)(−)-amine	(*R*)(+)-amine	(*S*)(−)-amine	(*R*)(+)-amine
1a	2.11	0.00	0.09	0.07
1b	2.11	0.00	0.11	0.03
2a	4.21	0.00	−0.10	−0.02
2b	4.23	0.00	−0.08	−0.06
3a	2.63	0.53	−0.02	0.04
3b	2.65	0.53	−0.05	−0.10
3c	2.65	0.53	0.10	0.05
4a	1.05	1.05	0.04	0.04
4b	1.05	1.05	0.08	0.07
5a	3.16	1.05	−0.11	−0.09
5b	3.17	1.05	0.07	0.13
6a	1.58	1.58	0.04	0.03
6b	1.59	1.58	0.01	−0.03
7a	3.68	1.57	0.12	0.00
7b	3.69	1.57	0.05	−0.06
8a	0.00	2.10	−0.02	0.06
8b	0.00	2.10	−0.01	0.02
9a	2.11	2.10	−0.03	−0.06
9b	2.11	2.10	−0.06	−0.07
9c	2.11	2.10	0.02	0.02
10a	0.53	2.63	−0.07	−0.07
10b	0.53	2.63	−0.05	−0.08
11a	2.63	2.62	−0.03	−0.05
11b	2.63	2.62	0.12	0.18
12a	1.05	3.15	0.01	−0.02
12b	1.06	3.15	−0.02	−0.11
13a	1.57	3.67	−0.05	−0.11
13b	1.58	3.67	−0.08	−0.03
14a	0.00	4.20	−0.01	−0.01
14b	0.00	4.20	−0.15	−0.17

[a] The ANN architecture has been defined as (input nodes, hidden nodes, output nodes).

4. Conclusions

The results obtained in this work testify to the high analytical potential of the CD technique for the kinetic resolution of enantiomers in combination with multivariate calibration. This technique is an effective alternative to the kinetic-spectrophotometric determination of enantiomers provided their reaction rate with a chiral reagent is not too high and monitoring of the reaction is subject to no restrictions as regards instrumental recording.

Non-linear effects resulting from deviations from the pseudo first-order kinetics can be partially modelled by PCR and PLS; however, ANN provides much better results under these conditions.

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