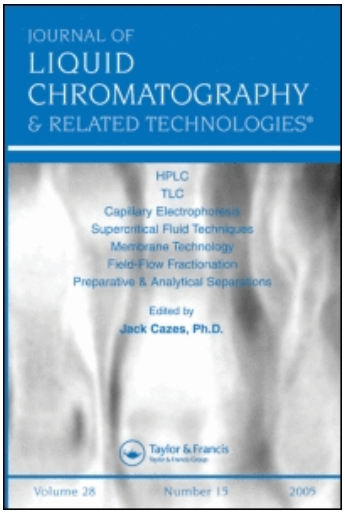

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Polarimetric Detection in Liquid Chromatography: An Approach to Correct Refractive Index Artefacts

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Polarimetric Detection in Liquid Chromatography: An Approach to Correct Refractive Index Artefacts

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Abstract: The study of factors influencing the quality of polarimetric signals from chromatographic analysis of mixtures of enantiomers shows that changes in refraction index of mobile phase, analyte concentration, and flow rate of mobile phase can give unacceptable errors that preclude polarimetric measurements to be used in enantiomeric purity determinations. Although, recent advances in polarimetric detectors (enhancing S/N ratio) avoid, in part, this effect, errors arising from quantitative measurements and calibration functions when analyzing compounds having small specific rotations or/and mixtures with small enantiomeric excess may be serious and must be corrected. In this paper, a simple equation was derived to correct deviations arising from refractive index artefacts (RIA effect), effectively useful in mixtures of enantiomers closest to racemic composition and at the extreme of the scale. Styrene glycol and citramalic acid enantiomers were used as examples of the efficacy of the approach to correct the RIA effect.

Keywords: Calibration factor, Enantiomeric excess, Liquid chromatography, Polarimetric detection, Ria effect

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INTRODUCTION

Enantiomers have identical physical and chemical properties in an isotropic environment, except that they rotate the plane of polarized light in opposite directions. The liquid chromatography analytical approaches include the use of chiral stationary phases, chiral mobile phase additives, and the use of chiroptical detectors. Currently, chiroptical detectors are based either on polarimetry (optical rotation, OR) or circular dichroism (CD).[1,2] CD detectors have intrinsic higher sensitivity than polarimetric detectors because CD is based on differences in absorbance on the left and right circularly polarized light passing through the sample cell, whilst OR is a dispersive phenomenon based on the rotation angle of the emerging linearly polarized light. However, CD chiroptical detectors are limited to samples with a cromoforic group associated to the chiral center because of the absorbance nature of the signal (this precludes the application to carbohydrates, such as sugars, without cromofores). In theses cases, polarimetric detectors give the solution to chiroptical chromatographic detection.

HPLC has become an efficient technique for enantiomeric separations, and several reviews [3–10] have summarized the progress in the field of enantiomeric separations. Polarimetric (OR) and circular dichroism (CD) have been used as chiroptical detectors in the field of enantiomeric analysis, normally associated to non-specific detectors (UV or fluorescence) in a variety of samples.[11–17]

The physical property that is unique to chiroptical detectors is their sensitivity towards the direction and magnitude of rotation of an incident linearly polarized light beam after its interaction with a chiral molecule. The magnitude of the rotation angle, α, is proportional to the difference in the refractive index values ($n_L - n_R$) (left and right values) phenomenon referred to as birefringence. The calibration factors obtained using a polarimetric detector for the R and S enantiomers are not identical in the analysis of compounds with small specific rotation and for mixtures with small enantiomeric excess, due to the refractive index-related effect.[8]

Performances of polarimetric detectors for liquid chromatography fall dramatically by pseudo-rotation signals, due to refractive index artefacts. These pseudo-rotation signals occur as the analyte passes through the detection cell and disturbs the analytical signal (optical rotation). The origin of these spurious signals is the rapid change in the refractive index of the eluent when high concentrations of analyte are incorporated in the injection.

The suppression of this artefact has been carried out by instrumental modification of the optical path by means a refractive index equalizer[19] or by correction of the artefacts producing pseudorotation.[18] In this paper, we give the fundamental, and some applications of a new approach, to detect and correct the refractive index artefact (RIA). Applications

to the determination of enantiomeric excess in several samples using achiral chromatography assess the possibilities of this simple approach.

THEORY

In HPLC polarimetric detection, the analytical signal (OR) arise when an optically active compound passes through the optical cell and the plane polarized light beam was rotated according with the Biot law.

$$\alpha = [\alpha] \cdot c \cdot l \tag{1}$$

where α is specific rotation (degrees), $[\alpha]$ is analyte specific rotation (deg), c is analyte concentration ($g \cdot mL^{-1}$) and l is optical path (dm).

Increasing concentrations of analytes will give increased OR, and the corresponding equations for linear fit are:

$$OR_+ = b_+ \cdot m \tag{2}$$

$$OR_- = b_- \cdot m \tag{3}$$

where OR_+ and OR_- is the polarimetric signal (chromatographic peak area) of each enantiomer, b_+ and b_- the corresponding slopes, and m is the mass of standard injected.

In the absence of the RIA effect, the obtained signals will give two lines passing through the origin and the same slopes but with opposite signs. However, when the RIA effect is present the lines obtained can be fitted to:

$$POL_+ = a + b'_+ \cdot m \tag{4}$$

$$POL_- = a + b'_- \cdot m \tag{5}$$

having an intercept (offset) (a) and different slope (b'_+ and b'_-).

This is because to the OR signal a parasite signal was added that is linearly related with the injected mass of analyte,[20–22] and can be expressed as:

$$RIA = a' + b''_+ \cdot m \tag{6}$$

Thus, the polarimetric detector measures a signal sum of the two signals (OR and RIA):

$$POL = OR + RIA \tag{7}$$

When applied this equality to the equations of each pair of optical isomers, we have:

$$POL_+ = [a + b'_+ \cdot m] = [b_+ \cdot m] + [a' + b''_+ \cdot m] \quad (8)$$

$$POL_- = [a + b'_- \cdot m] = [b_- \cdot m] + [a' + b''_- \cdot m] \quad (9)$$

Because rotarory power of an enantiomeric pair is the same but opposite sign, $b_+ = -b_-$, and from this, dividing (8) by (9) it can be deduced

$$a = a' \quad (10)$$

$$b_{+/-}{}'' = (b'_+ + b'_-)/2 \quad (11)$$

Where units of a′ are degrees and b″ in degrees μg^{-1}.

Equations (10 and 11)) constitute the basis of the method to correct polarimetric HPLC calibration curves from pseudo rotation due to refractive index artefacts.

By means of these equations can be calculated, from experimental known parameters, the RIA effect contribution to the polarimetric measurements. After the quantification of this contribution, its value must be subtracted to all the polarimetric measurements obtained to construct the calibration function and calculate the enantiomeric purity. The measurements were performed over samples containing a total constant concentration of analyte, thus the injected mass is also constant.

EXPERIMENTAL

Chemicals

(–)-R-styrene glycol ((–)-R-1-phenyl-1,2-ethanediol) ((–)-R-SG) 99% purity and (+)-S-styrene glycol ((+)-S-1-phenyl-1,2-ethanediol) ((+)-S-SG) 99% purity from Acros (Geel, Belgium). (–)-R-citramalic acid and (+)-S-citramalic acid were obtained from Acros (Geel, Belgium). HPLC grade acetonitrile, hexane, and methanol were purchased from Merck (Darmstadt, Germany). LC grade water was prepared by passing demineralised water to a Milli-Q filtration system (Millipore, Bedford, MA). Other chemicals were from Merck. Canadine[23] (9,10-dimethoxy,2-2,(methylendioxy)berbine) was a gift from the Department of Organic Chemistry, University of Malaga (Spain).

Instrumentation

The measurements were performed with a Merck-Hitachi (Darmstad, Germany) liquid chromatograph consisting of an L-6200 pump, an AS-4000 autosampler, an L-4250 UV-visible detector, F-1080 fluorescence detector, and a D-6000 interface. Integration was carried out with a PC computer and instrument parameters were controlled by Hitachi-Merck HM software.

A ChiralMonitor 2000 optical rotation detector (Applied Chromatography Systems Limited, Macclesfield, England) placed in series with and after the UV-visible detector, was equipped with a collimated laser diode providing up to 30 mW of light at 830 nm, and a flow cell of 0.48 dm path length, with 73 μL volume. Data acquisition and transformation were accomplished by the Pico ADC-100 (Picotechnology Ltd., Cambridge, UK) which is an analog to digital converter. Calculation of the areas (negative and positive peaks), the peaks heights, and retention times were performed with Lab-Calc LC software (Galactic, Salem, NH).

The HPLC chiral column used was Chiradex (25 cm, 4 mm internal section, 5 μm) from Merck. C_{18} and Si columns were Lichrochart from Merck.

RESULTS AND DISCUSSION

The RIA effect gives parasite signals in two key sites of the chromatogram profile: injection peak and analyte peak. The polarimetric signal in the injection peak is a consequence of the different refractive index (RI) of the mobile phase and the solvents containing the injected sample. High concentrations of injected samples, as usual in polarimetric analysis because of lack of sensitivity, enhance this signal. When the polarimetric injection peak is very high, some overlap with the analyte peak can occur if no sufficient separation between them is attained. However, the true problem occurs at the analyte peak, especially when high concentrations are injected, due to sudden variation of the RI in the detector cell. A polarimetric signal can be obtained although no chiral substances are injected, if sufficiently high concentrations are used. Indeed, in flowing systems, solutes are transported as narrow bands characterized by high concentration gradients and any solute with a refractive index different from the solvent can generate a deflection signal proportional to the gradient.[24]

To test the applicability of this quantification strategy for routine analysis, it was applied to different data sets obtained from several enantiomeric systems having different specific rotation, concentrations, stationary phases, and eluent solvent properties.

Catechin and epicatechin are two flavonoids epimers between them; their specific rotation (in ethanol) at 20°C and at the sodium line wavelength are $[\alpha]^{20}{}_{D} = 16-18°$ (positive for catechin and negative for epicatechin). Corrected at 830 nm (argon laser line) by means of Drude Law gives $[\alpha]^{22}{}_{Ar} = 7-8°$ (ethanol), a low specific rotation. Figure 1 shows two chromatograms corresponding to the photometric (a) and polarimetric (b) detection of a mixture of catechine and epicatechin injected on a chiral Chiradex β-cyclodextrine column and a mobile phase of methanol:water (pH 2.6 with phosphoric acid), (65:35, v/v). As can be

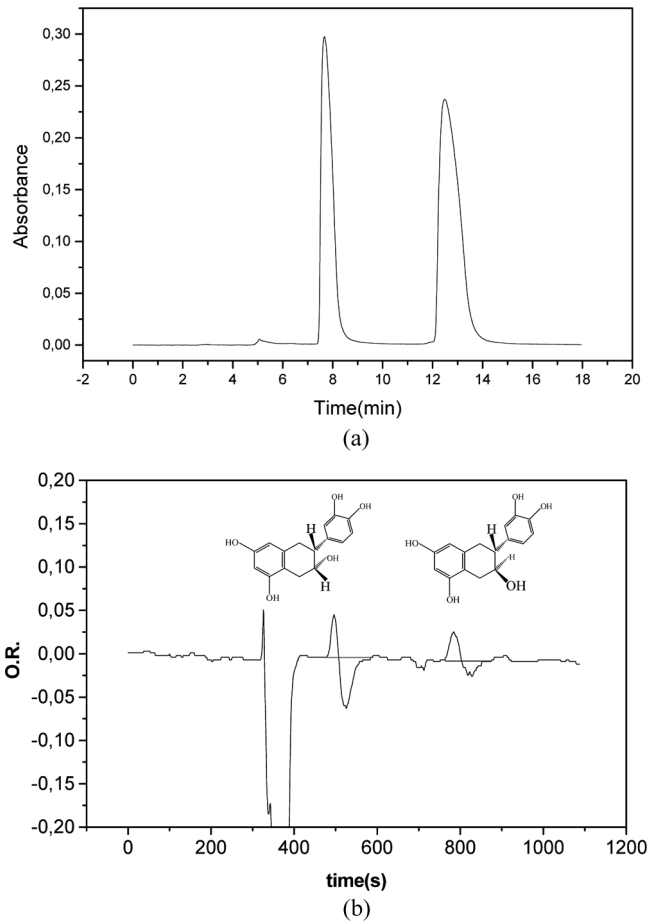

Figure 1. Photometric (225 nm) and polarimetric (830 nm) chromatograms (a and b) of a mixture of catechin and epicatechin (200 μg) on a ChiraDex-β-CD column. The mobile phase was methanol:water (pH 2.6, with phosphoric acid) at a flow rate of $0.5\,mL \cdot min^{-1}$.

seen instead, a negative peak at 520 s and a positive peak at 800 s, figure b shows two bimodal peaks at this retention times as a consequence of the RIA effect.

Total area under peaks (+ and –) were positive for catechin and negative in the case of epicatechin. This fact indicated that polarimetric signals include both effects, optical rotation and RIA effect, being similar for both substances. Several authors[1] claim that the total integrated area of the RIA peaks trends to zero (the negative part cancels the positive one), so it can be inferred that the resultant integrated area is only a contribution of the optical rotation. However, this is not always exact. In the vicinity of the racemic composition of an enantiomeric mixture the RIA effect contribution is higher than in other mixtures, even though specific rotation of the enantiomeric pair is high.

Canadine enantiomers have a high specific rotation, $[\alpha]_D^{20} = 317°$ in methanol solution (corrected $[\alpha]_{Ar}^{22} = 139°$). When injected, the pure enantiomers obtained signals are only positive for (+)-canadine and only negative for (–)-canadine, in this case the RIA effect is very low compared with O.R. In Figure 2 are depicted the polarimetric chromatograms obtained from three samples: pure enantiomers (A, C) and racemic mixture (B). As can be seen, when the sample composition is near a racemic mixture the RIA effect predominates over O.R. signal and a differential (bimodal) peak is obtained at the retention time of each enantiomer.

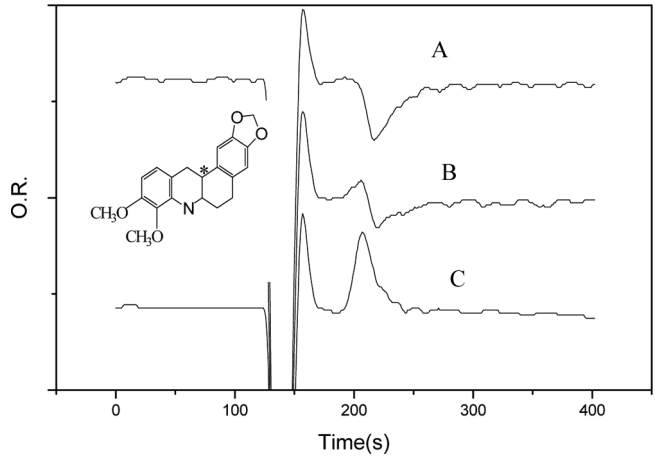

Figure 2. Polarimetric chromatograms of pure enantiomers of canadine (A, C) and a near racemic mixture (B) on a C_{-18} reverse phase column. Mobile phase methanol:water:trifluoroacetic acid (69:31:0.4) at flow rate $1\,mL \cdot min^{-1}$. Mass injected 206 μg.

Factors Influencing the RIA Effect

Although refractive index varies with detection wavelength, temperature, and concentration of the sample, the first two parameters are normally maintained constant during a chromatographic analysis. Concentration must be changed when a calibration function is sought and, as pointed out above, can generate RIA effects.

The other operational factors that affect RIA effect values are: a) refraction index (RI) of the mobile phase (specially related to the RI of the sample solvent) and b) mobile phase flow.

Refractive Index of the Mobile Phase

The polarimetric chromatographic behaviour of stirenglycol (SG) enantiomers is representative of this effect.

Figure 3a shows the chromatographic profiles corresponding to R-(–)-SG and S-(+)-SG injected (200 μg in ethanol) in a Si-25 cm-10 μm normal phase column with a mobile phase composed of hexane:ethanol:acetic acid, 80:20:0.05 v/v (A and B), and in a C_{18}-25 cm-10 μm with mobile phase water:acetonitrile, 80:20 (C and D).

In both chromatographic systems the polarimetric signal is affected by an important contribution of the RIA effect. Although pure enantiomers will give only a positive or negative signal, the obtained signals have a bimodal profile indicating a RIA effect. In the case of S-(+)-SG operating in normal phase, the RIA effect is high, with the area of negative peak higher than the positive part. In reverse phase (C and D) the mobile phase water:acetonitrile with RI near the sample solvent (ethanol), the RIA signal is lower than that of normal phase.

The RIA effect can also be affected by the presence of small quantities of pH modifiers. In Figure 3b are depicted the polarimetric chromatograms corresponding to 200 μg (in ethanol) of R-(–)-SG in a C18–25 cm-10 μm column, mobile phase water:acetonitrile 90:10 (a); with 0.1% acetic acid (b) and 0.1% trifluoroacetic acid (c). As can be seen, the RIA effect (positive part) increases in the presence of pH modifiers.

The same behaviour can be observed with an apolar mobile phase. Figure 3c shows the polarimetric chromatograms (Si-25 cm-10 μm column), hexane:ethanol 80:20 (A); 0.2% acetic acid added (B) and 0.4% acetic acid added (C), of 200 μg (in ethanol) R-(–)-SG. It can be observed that higher RIA signals are obtained increasing acetic acid content in the mobile phase. Apparently, the cause of the RIA effect on both cases is the change in RI. of the mobile phase.

On the other hand the RIA effect depends mainly on the RI difference between mobile phase and sample solvent injected. Using mobile

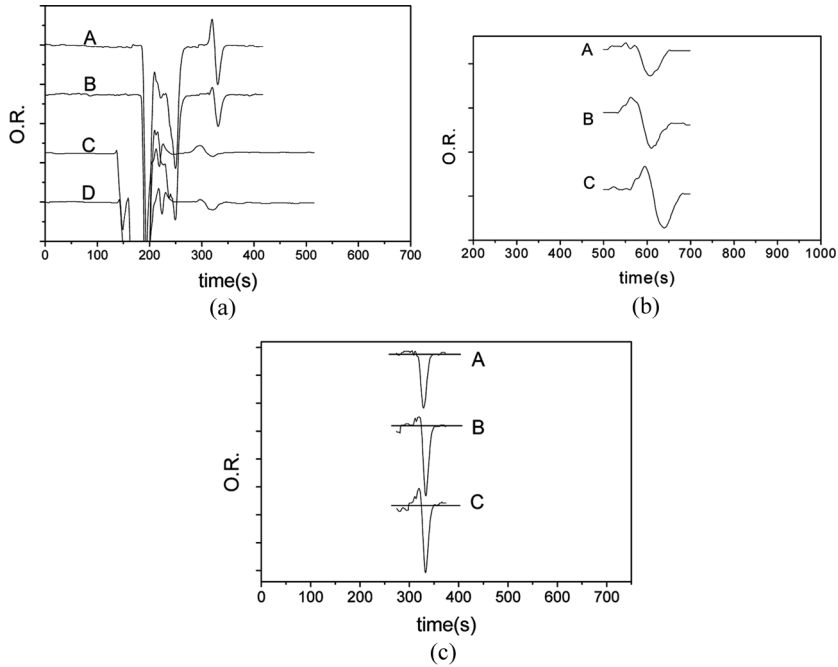

Figure 3. Chromatographic profiles corresponding to R-SG and S-SG on silica column (a), C_{-18} column (b) and on silica column with different quantities of acetic acid in the mobile phase (c). Flow rate 1 mL · min^{-1}, mass injected 200 μg.

phase solvents with RI similar to sample solvent, we can minimize the RIA effect. Unfortunately the first criteria to select mobile phase composition is directed by separative considerations, thus frequently the range of solvents to be used is restricted to only several ones. In turn, we can select a solvent for the sample with RI similar to the mobile phase, but solubility problems can arise if high concentrations of sample must be injected, as usual in polarimetry.

Figure 4 displays polarimetric chromatograms of S-(+)-SG (200 μg in ethanol) in a Si-25 cm-10 μm column. The mobile phases were: hexane: chloroform:ethanol, 70:25:5 v/v (A); 40:55:5 (B) and 37:60:3 (C). As can be seen, the RIA effect diminishes as chloroform increases in the mobile phase. This effect can be explained if we take in mind that the RI of chloroform (as halogenated solvents) has a high RI compared to hexane and ethanol (chloroform 1.4455, hexane 1.3749, and ethanol 1.3614).

This suggests that using mobile phases with RI higher than solvent sample can diminishes the RIA effect. Solvents currently used in HPLC like dichloromethane, chloroform, or 1,2-dichloroethane can accomplish this function.

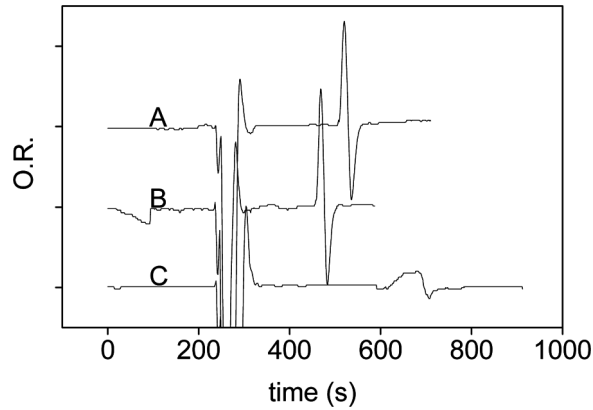

Figure 4. Polarimetric chromatograms of S-SG on a silica column and mobile phases with increasing halogenated solvent contents; hexane:chloroform:ethanol, 70:25:5, (A), 40:55:5 (B), and 37:60:3 (C). Mass injected 200 μg, flow rate $0.8\,mL \cdot min^{-1}$.

Mobile Phase Flow Effect

Mobile phase flow can affect the extension in which the RIA effect takes part in the polarimetric signal as a result of the rate in which RI changes when the analyte sample arrives to the polarimetric cell. This effect has been previously studied[18] by Däppen and col. found that small flow rates and small k′ values result in better S/N ratios.

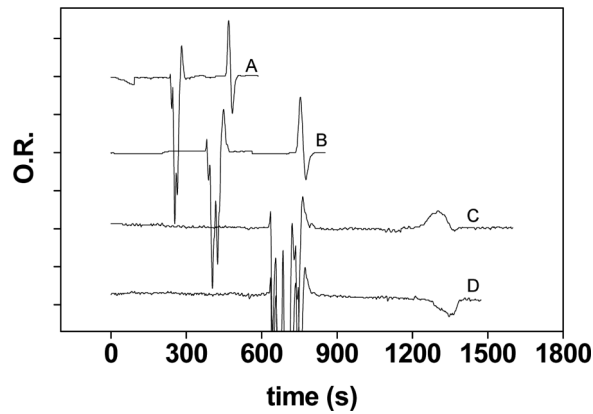

Figure 5. Polarimetric chromatograms of 200 mg of S-SG (A, B, and C) and R-SG (D) on a silica column with mobile phase hexane:chloroform:ethanol (40:55:5). At flow rate 0.8 (A), 0.5 (B), and $0.3\,mL \cdot min^{-1}$ (C and D).

Figure 5 shows polarimetric chromatograms of 200 μg (20 μL) S-(+)-SG (A,B,C) and R-(–)-SG (D) in a Si-25 cm-5 μm column, mobile phase hexane:chloroform:ethanol, 40:55:5 v/v. A) $0.8\,mL \cdot min^{-1}$, B) $0.5\,mL \cdot min^{-1}$; C, D) $0.3\,mL \cdot min^{-1}$.

Low flows produce diminution (even eliminates) of the RIA effect. However, as expected, peak broadening enhances greatly. Band broadening defined as standard deviation of a concentration profile, taking the width at 60.7% of the maximum height, is near constant[25] in the range $0.2–1.0\,mL \cdot min^{-1}$, increasing for flows $\langle 0.2$ and $\rangle 1.0\,mL \cdot min^{-1}$.

Correction of RIA Effect

In the absence of the RIA effect, obtained polarimetric signals corresponding to increasing analyte concentrations will give linear calibration curves with zero intercepts and slopes with the same absolute values but opposite sign. In accordance with the correction proposed in this article, equation[11] was applied to several enantiomeric pairs to assess this approach.

Two cases were studied to illustrate RIA effect correction. One of them is citramalic acid with specific optical rotation relatively low ($[\alpha]_D^{20} = 23.6°$ (water)), and the other stirenglycol with a moderately high specific optical rotation ($[\alpha]_D^{20} = 66°$ (chloroform)).

Table 1 contains fitted equations and correlation coefficients corresponding to SG enantiomers. By inspection of this table, it can be deduced that both enantiomers present intercepts different to zero and less different slopes. A RIA effect with resultant negative value is present in the obtained data.

To correct it, the mean value of the intercept (area values) was taken as –0.1731. By applying equation (11), the corrected value of the slope is 0.000411°/μg (area). The total correction to be applied to experimental values was calculated by the expression:

$$OR = a'' + (b'' \cdot m) \tag{12}$$

Table 1. Calibration equations from fitted data of SG enantiomers

Enantiomer	Detector	Equation	Correlation coefficient
R-(−)-SG	Absorbance (area)	$A_a = 0{,}0080 + 2{,}28 \cdot 10^{-4} \cdot m$	$r = 0{,}9982$
	Optical rotat. (area)	$P_a = -0{,}129 - 0{,}0030 \cdot m$	$r = -0{,}9980$
S-(+)-SG	Absorbance (area)	$A_a = 0{,}0053 + 2{,}39 \cdot 10^{-4} \cdot m$	$r = 0{,}9963$
	Optical rotat. (area)	$P_a = -0{,}217 + 0{,}0038 \cdot m$	$r = 0{,}9872$

All signals were plotted against m (μg).

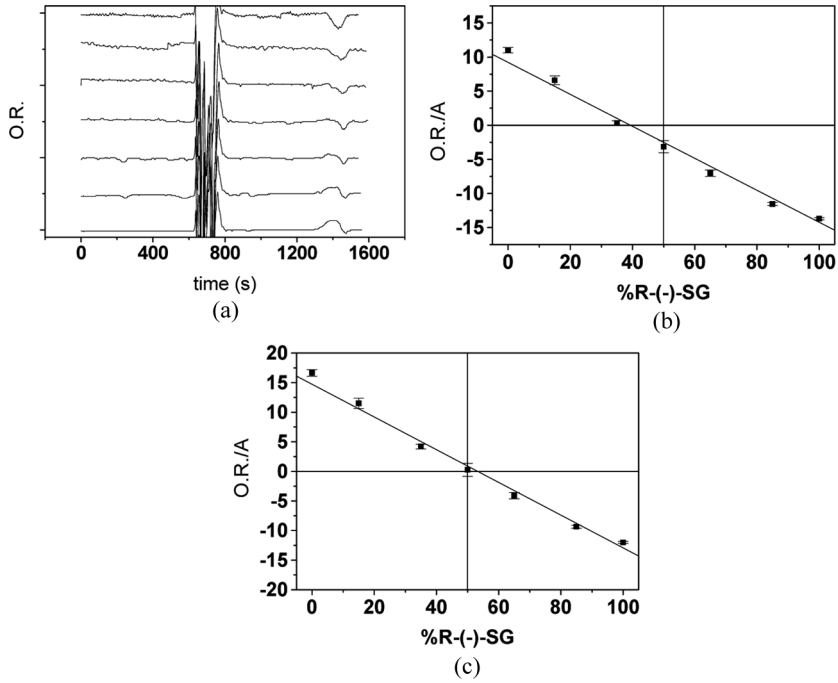

Figure 6. Polarimetric chromatograms of enantiomeric mixtures (total mass injected 200 μg, range 0–100%) (a); calibration graph OR/A (area) against% R-SG, uncorrected (b), and corrected (c). Conditions as in previous figure.

being a″ the mean value of the intercepts + and −, b″ corrected slope, and m injected mass in μg (200 μg). The resultant value to correct is −0.0909° that must be subtracted from the experimental values.

In Figure 6 can be observed the polarimetric chromatograms obtained over 200 μg (total mass injected) of enantiomeric mixtures covering the range 0−100% (a), and the calibration graph (OR/A (area) against% R-(–)-SG), (b) uncorrected values, and (c) corrected values.

As can be seen, corrected values give a calibration in which the fitted line pass through the origin at an enantiomeric percentage of 53.5 ± 1.5% instead of 40.1±1.5% for uncorrected values.

The main analytical application of this approach to correct pseudorotation or RIA effect is the determination of enantiomeric composition (enantiomeric purity) of mixtures without the need of physical (chromatographic) separation. A recovery assay of a synthetic mixture prepared from pure enantiomers and a composition of 64.7% R-(–)SG (mass injected 200 μg) was performed. All chromatographic measurements were repeated three times. Figure 7 shows the obtained chromatograms

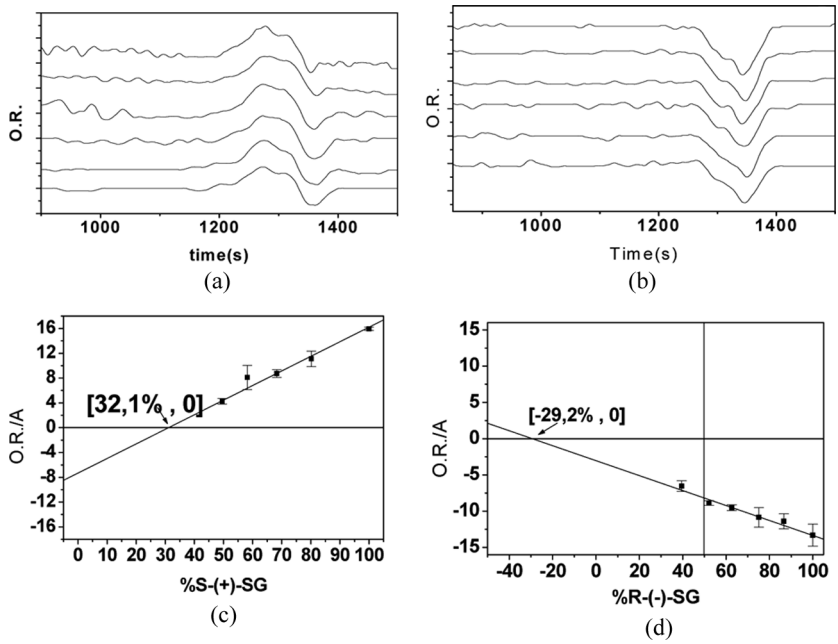

Figure 7. Recovery assay over a synthetic sample with a content of 64.7% R-(−)-SG. (a) polarimetric chromatograms corresponding to mixtures covering the range 50–100% of the pure enantiomer S-(+)-SG; (b) mixtures in the range 40–100% of the pure enantiomer R-(−)-SG; (c) uncorrected calibration curve (OR/A against% S-(+)-SG; (d) corrected calibration curve.

covering the range 49.6–100% S-(+)-SG (A) and 39.5−100% R-(–)-SG (b) of the pure enantiomers. Calibration graphs for the determination of enantiomeric composition are plotted in Figure 7 (c, d).

From the zero-crossing value of both calibration graphs, 32.1% S(+)-SG and −29.2% R-(–)-SG, we can calculate the enantiomeric proportion of the mixture assayed.

According to the expression derived by Goodall:[1]

$$\mathrm{OR/A} = \mathrm{K}(100-2\cdot\%(-)),\ \text{or}\ \mathrm{OR/A} = \mathrm{K}(2\cdot\%(+)-100) \qquad (13)$$

where k is a constant, % (+) and % (–) are percentages of positive or negative enantiomer, OR optical rotation, and A absorbance. The percentage of positive enantiomer in the mixture (% +/−) can be obtained from the zero-crossing value (% enan + (0)) by using the expression:

$$\%+/- = (5000-100\cdot\%\ \mathrm{enan}+(0))/(100-\%\ \mathrm{enan}+(0) \qquad (14)$$

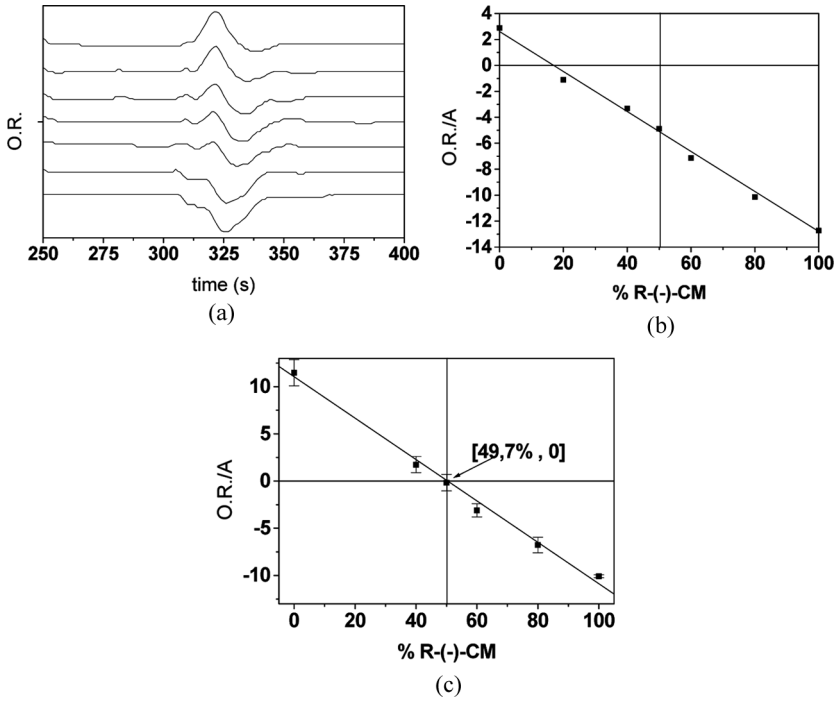

Figure 8. Calibration curve of citramalic acid enantiomers, (a) chromatograms corresponding to mixtures (from pure enantiomers) covering the range 0–100%; (b) uncorrected calibration curve; (c) corrected calibration curve.

Using corrected values, the percentage of S-(+)-SG is $32.1 \pm 5\%$ and that of R-(–)SG $61.3 \pm 3.7\%$. The average value of R-(–)-SG is 64.6 ± 3.7, very close to the theoritical value of the synthetic mixture (64.7%).

The other sample to be studied was citramalic acid (CM) enantiomers.

In this case, because specific optical rotation is low, the RIA effect affects greatly the polarimetric measurements. Figure 8 shows the polarimetric chromatograms of enantiomeric mixtures covering the range 0–100% (total mass injected 208 μg in ethanol) (a), the uncorrected (b), and corrected calibration graph (c). Chromatographic conditions were an achiral Si-25 cm-10 μm column, mobile phase hexane:ethanol 80:20 (v/v) at a flow of 1 mL/min, volume injected 20 μL.

The zero-crossing point occurs at $16.7 \pm 1.5\%$ for uncorrected data, while corrected data give the zero-crossing point at $49.7 \pm 1.4\%$, very close to the theoretical 50%. Adjusted equations for uncorrected OR/Abs = 2.578 + 0.156%(–)-CM and corrected data OR/Abs = 10.825–0.218%(–)-CM, and

correlation coefficients r = 0.9963 and r = − 0.9973, respectively, supports the quality of corrected values.

CONCLUSION

A detailed study of factors affecting spurious signals from polarimetric signals in chromatographic enantiomeric purity analysis allows us to derive an equation that correct pseudo-rotation arising from sudden changes in refraction index (RIA effect).

Correction calculus efficacy was demonstrated using synthetic samples having different specific optical rotation, e.g., styrene glycol, and citramalic acid enantiomer mixtures.

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