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Chiroptical Detectors for the Study of Unusual Phenomena in Chiral Chromatography

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Abstract Differentiation of enantiomers in chromatography requires specific detectors, based on polarimetry or circular dichroism. Their use is limited to chiral HPLC and SFC. We explain the operating principles of the different chiroptical detectors available and stress the influence of working wavelength and mobile phase on the output signal. Current and relevant applications of chiroptical detectors are absolute configuration assignment, measurement of enantiomeric excesses in complex mixtures and determination of elution order. We focus on the reversals of enantiomeric elution order, an important subject for the understanding of the chiral recognition mechanisms. We review the main parameters which can induce a reversal, show the usefulness of chiroptical detectors to easily identify reversals and emphasize the significance of the isoenantioselective temperature. The aim of this chapter is to highlight the valuable information provided by chiroptical detectors to study unusual behaviour in chiral HPLC and SFC, reversals of enantiomeric elution order and exchange phenomena as dynamic chromatography and self-disproportionation on achiral columns.

Keywords Absolute configuration assignment · Dynamic chromatography · Electronic circular dichroism · Isoenantioselective temperature · Polarimetry · Reversal of enantiomeric elution order · Self-disproportionation

Contents

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Abbrevations

1-PrOH	Propan-1-ol
2-PrOH	Propan-2-ol
AcOH	Acetic acid
CD	Electronic circular dichroism
CIP	Cahn–Ingold–Prelog priority rule
CSP	Chiral stationary phase
DEA	Diethylamine
ee	Enantiomeric excess
FA	Formic acid
HPLC	High performance liquid chromatography
k	Retention factor
L	Liter(s)
LED	Light-emitting diode
min	Minute(s)
MPLC	Medium pressure liquid chromatography
nd	Not determined
nm	Nanometer(s)
ORD	Optical rotatory dispersion
RI	Refractive index
SFC	Supercritical or subcritical fluid chromatography
SMB	Simulated moving bed
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
T_{iso}	Isoenantioselective temperature
UV	Ultraviolet
α	Enantioselectivity, ratio of retention factors for two enantiomers
β	Phase ratio, ratio of the volume of the mobile phase and stationary phase in the column
μg	Microgram
μL	Microliter

Chiral Stationary Phases

Chiral-AGP	α1-Acid glycoprotein
Chiralcel OB	Cellulose tris benzoate (Daicel)
Chiralcel OD	Cellulose tris(3,5-dimethylphenyl-carbamate) coated (Daicel)
Chiralcel OJ	Cellulose tris(4-methylbenzoate) (Daicel)
Chiralpak AD	Amylose tris(3,5-dimethylphenyl-carbamate) coated (Daicel)
Chiralpak AS	Amylose tris([(*S*)alpha-phenethyl]-carbamate) coated (Daicel)
Chiralpak IA	Amylose tris(3,5-dimethylphenyl-carbamate) immobilised (Daicel)
Chiralpak IB	Cellulose tris(3,5-dimethylphenyl-carbamate) immobilised (Daicel)
Chiralpak IC	Cellulose tris(3,5-dichlorophenyl-carbamate) immobilised (Daicel)
Chiralpak ID	Amylose tris(3-chlorophenyl-carbamate) immobilised (Daicel)
Lux-Cellulose-1	Cellulose tris(3,5-dimethylphenyl-carbamate) coated (Phenomenex)
Lux-Cellulose-2	Cellulose tris(3-chloro-4-methylphenyl-carbamate) coated (Phenomenex)
Lux-Cellulose-4	Cellulose tris(4-chloro-3-methylphenyl-carbamate) coated (Phenomenex)
Regispack	Amylose tris(3,5-dimethylphenyl-carbamate) coated (Regis Technologies)
Ultron ES-OVM	Ovomucoid protein bonded
(*S*,*S*)-Whelk-O1	(3*R*,4*S*)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy) propyl]-1,2,3,4-tetrahydrophenanthrene (Regis Technologies)

1 Introduction

Chiral chromatography is a powerful tool for enantiomer differentiation, on an analytical scale to determine the ee and on a preparative scale to obtain enantiopure molecules [1]. This chirotechnology is extensively used in various fields, such as drug discovery [2], agrochemicals, fragrances and functional materials. Many chiral stationary phases, with different chiral recognition mechanisms, are now available to separate chiral compounds [3, 4]. The understanding of the chiral recognition mechanisms, by the development of a chiral recognition model or by computational chemistry [5], requires knowledge of the absolute configuration of the eluted enantiomers.

From a practical point of view the control of the elution order is convenient, on an analytical scale, for the measurement of high ee by eluting the trace component

before the major enantiomer [6]. Indeed, the accuracy of this measurement is crucial to lower the limit of detection (LOD) and to validate chiral methods: interference from the tailing of the major enantiomer should be avoided when peaks are relatively broad, particularly on protein columns [7]. On a preparative scale, for batch separations (not with SMB), the best purity will be attained for the first eluted enantiomer compared to the second because of the tailing of the first eluted enantiomer occurring with high loading. The control of elution order is so important that original methods have been developed to reverse the enantiomeric elution order by altering the type of reagent used in the derivatization of amino-acids on vancomycin based CSP [8], or by using the temperature effect for enantiomeric pairs separated by chiral HPLC under a significant enthalpy-control [9].

So fundamental and practical reasons for the monitoring of the elution order during analysis by chiral chromatography exist. Some articles have already highlighted the need of well reported elution orders [10], detailed unusual effects [11] and reversals of enantiomeric elution order [12]. In this review we emphasize the use of chiroptical detectors to detect unusual phenomena, and particularly reversals of enantiomeric elution order. Thus we restrict our study to the field of chiral chromatography in which chiroptical detectors, polarimeters and circular dichroism detectors are operational: chiral HPLC and SFC (SFC in the sense of the term defined by Guiochon [13]).

2 Principle and Applications of Chiroptical Detection

2.1 Historical Background

Bench polarimeters were first adapted for use as chromatography detectors, working at low pressure. In 1976, Hesse and Hagel reported the first chromatogram obtained with a polarimetric detector [14]: a Perkin Elmer 141 was used to follow the elution of the 2-phenyl-cyclohexanone enantiomers at 436 nm on microcrystalline cellulose triacetate. In 1980, Drake, Gould and Mason published the first use of circular dichroism for detection in liquid chromatography on microcrystalline cellulose triacetate for pavine and Tröger's base [15]. These detectors were very useful in the early years of chiral chromatography, especially in the case of partial separations [16], occurring frequently at low pressure.

Cells improvements allowed the use of chiroptical detectors in HPLC. In different reviews, Mannschreck [17] and then other groups [18, 19] described their main applications and developments and prophesied their future widespread use.

2.2 *Operating Principle of Different Chiroptical Detectors*

2.2.1 Use of a Bench Polarimeter or Circular Dichroism Spectrometer

Circular dichroism spectrometers, able to measure the differential absorption of left and right circularly polarized light between 170 and 900 nm, or bench polarimeters, able to measure the angle of rotation caused by passing polarized light through an optically active substance at discrete wavelengths, usually the emission line of sodium or mercury lamps (589, 578, 546, 436 and 365 nm), can be used as chromatography detectors with suitable cells. Cell volume can be reduced to up to 40 μL in HPLC [20].

Differentiation of enantiomers during elution is possible with such a CD spectrometer [21] or a bench polarimeter [22]. Polarimeters usually have several available wavelengths and detect enantiomers even in the absence of chromophores. For example, a partial separation, not detected with a refractometer, was reported for heptachlor and chlordane on permethylated β-cyclodextrin [23]. CD spectrometers can also monitor partial separations at a chosen wavelength [24], and moreover allow the recording of CD spectra; in this case the flow has to be stopped [25].

2.2.2 Polarimetric and Circular Dichroism Detectors Dedicated to Chromatography

The only commercially available CD detector dedicated to chromatography, namely Jasco CD-2095, has many HPLC applications described in the literature, including SFC applications according to the supplier. Figure 1 shows typical chromatograms obtained with the CD-2095, by HPLC and by SFC: the sign and the area obtained depend on the chosen wavelength and on the nature of the eluent. The sign observed for (*S*)-(**1**) is (−) at 220 nm, (+) at 254 nm and gives no response at 300 nm (Fig. 1). Consequently, the wavelength must be given for each CD chromatogram.

Polarimetric detectors dedicated to HPLC were developed in the late 1980s. Some were used in the early 1990s but are no longer available, such as the Chiramonitor from Applied Chromatography Systems [26]. Nowadays, three polarimetric detectors for chromatography are on the market, differing in their operating mode and technical features: Chiralyser from IBZ Messtechnik (Germany) with an LED source and an operating wavelength at 426 nm, ALP from PDR (USA) with a laser source at 660 nm and OR-2090 (OR-1590 is an older model) from Jasco (Japan) with an Hg–Xe lamp, using wavelengths in the range 350–900 nm. Accordingly, the sign given by a Chiralyser is the sign of the eluted enantiomer at 426 nm in the eluent, the sign given by an ALP is the sign of the eluted enantiomer at 670 nm in the eluent, but the sign given by OR-2090 results from the addition of the optical rotation contributions at all wavelengths between 350 and 900 nm. For OR-2090 the contributions do not have the same weight and the main contributions come from the emission lines of the mercury lamp (365, 405, 436, 546 and 578 nm). OR-2090 has a multiple wavelength based operating mode, while Chiralyser and ALP are mono-wavelength based.

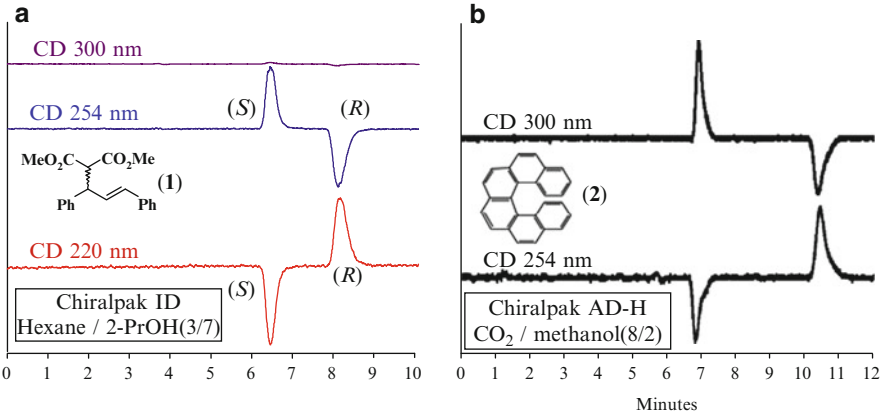

Fig. 1 CD chromatograms for (**a**) HPLC separation of (**1**) on Chiralpak ID, *n*-hexane/2-PrOH (3/7), 1 mL/min, 30°C, 4 μg injected, CD at 220, 254 and 300 nm from *bottom* to *top* and (**b**) SFC separation of [6]-helicene (**2**) on Chiralpak AD-H, CO_2/methanol (8/2), 4 mL/min, 40°C, outlet pressure = 120 bars

For compound **3**, the specific rotation is negative for the (a*R*) enantiomer on all the wavelengths, so the observed peak is negative on the polarimetric trace with OR-1590 (Fig. 2). In this case, the three commercial polarimetric detectors would have provided a negative sign. In the case of compound **4**, the ORD spectrum is more complex and less regular, with an anomalous curve with multiple Cotton effects (Fig. 2): the signal of the (*M*)-enantiomer is positive at 670 and 426 nm, but the sum of the different contributions in the range 350–900 nm gives a weak negative signal. The intensity and the sign given by a polarimetric detector depend on the operating mode of the model used. For (*M*)-**4** the three commercial polarimeters would give different signs: positive with ALP and Chiralyser and negative with Jasco detector.

2.2.3 Influence of the Mobile Phase

It is trivial to recall that the CD and above all ORD spectra depend on the solvent in which they are recorded, so that the intensity and the sign obtained with a chiroptical detector depend on the mobile phase used for chromatography. Comparisons of the sign from chiroptical detectors with data in another solvent should be carried out with caution and with a systematic check of the invariance of the sign.

Direct comparison with specific rotation are tempting but risky. For example, the sign given by a DIP-181C polarimeter, using an Hg lamp, is (−) for the (*R*)-enantiomer of 4-hydroxy-2-cyclopentenone in *n*-hexane/2-PrOH mixtures, but the sign in chloroform at 589 nm is (+) [28]. Driffield et al. wanted to choose a molecule with a large specific rotation to avoid sensitivity problems to prove the efficiency of a new polarimetric detector. This anticoagulant compound, warfarin, is

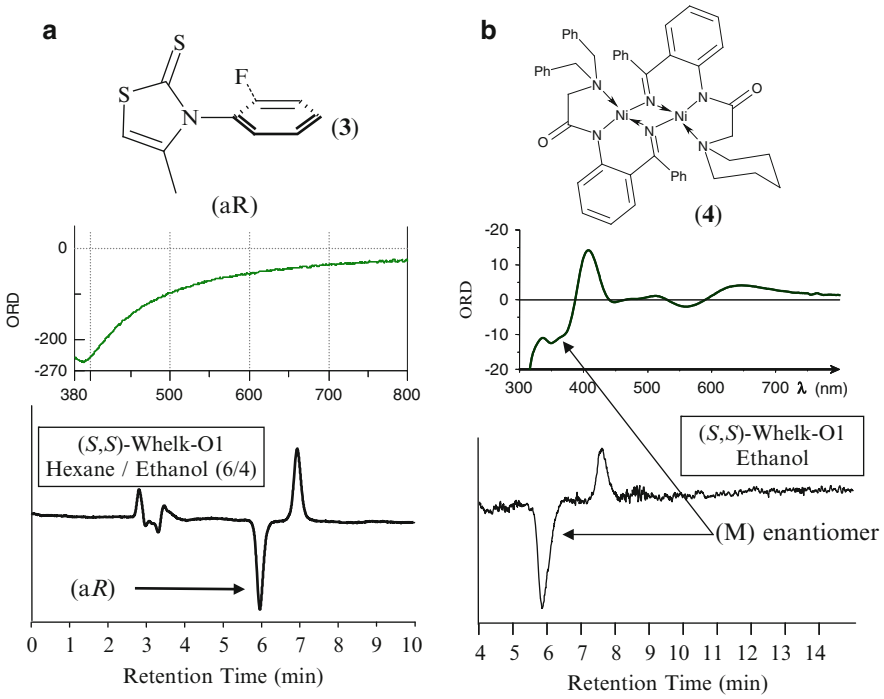

Fig. 2 ORD spectra and polarimetric traces (OR-1590) for (**a**) compound **3** on (*S*,*S*)-Whelk-O1, *n*-hexane/ethanol (6/4), 1 mL/min, 30°C and (**b**) compound **4** [27] on (*S*,*S*)-Whelk-O1, ethanol, 1 mL/min, 25°C

described with a specific rotation of −148 for the (*S*)-enantiomer in NaOH (0.5 N) [29], but the signal obtained at 635 nm is weak [30]. Indeed, the (*S*)-enantiomer has a specific rotation of +19 in 2-PrOH [29] and we have checked that (*S*)-warfarin has a low positive specific rotation at 635 nm in the eluent used (*n*-hexane/2-PrOH/AcOH 80/19.9/0.1). Moreover, the change in sign can lead to errors in the assignment of absolute configurations. Liu et al. asserted a high correlation between the magnitude and direction of the specific rotations of molecules at 675 nm and at the sodium D line [31], but also emphasized the influence of the mobile phase.

Unfortunately it is also possible to select a mobile phase in which there is no specific rotation: signals with opposite signs are observed with OR-1590 for (**5**) with 10% of ethanol, but these signals disappear with 40% of ethanol (Fig. 3b, c).

2.2.4 Case of Anomalous ORD

The type of ORD spectra, plain or anomalous, has little impact on the signal for the mono-wavelength based polarimetric detector. The specific rotation of a compound with an anomalous ORD spectrum may be zero or very low at the working wavelength, but the specific rotation of a compound with a plain curve ORD spectrum may

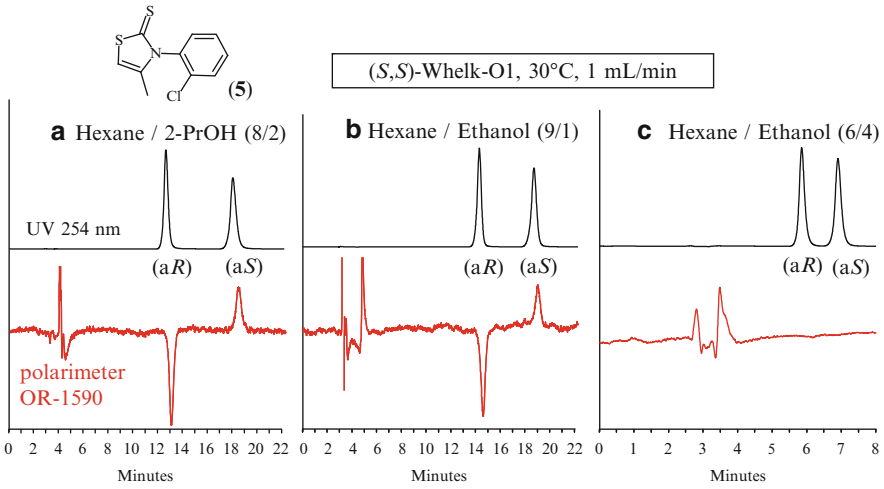

Fig. 3 UV chromatograms and polarimetric traces (OR-1590) for (**5**) on (*S*,*S*)-Whelk-O1, 30°C, 1 mL/min, with different mobile phases: (**a**) *n*-hexane/2-PrOH (8/2); (**b**) *n*-hexane/ethanol (9/1); (**c**) *n*-hexane/ethanol (6/4)

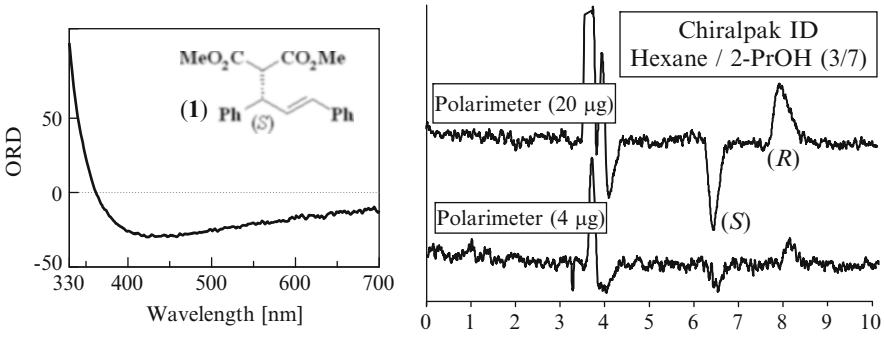

Fig. 4 ORD spectra, polarimetric traces (OR-1590) for (**1**) on Chiralpak ID, *n*-hexane/2-PrOH (3/7), 1 mL/min, 30°C, for injection of 4 and 20 µg

also be low. By using a multi-wavelength based polarimetric detector, a compound with an anomalous ORD often gives a poor signal, due to the compensation of positive and negative contributions of the specific rotation at the different wavelengths. Compound (**1**) shows an example of such behaviour: we need to increase the injected amount to 20 µg to observe a low intensity signal (Fig. 4). (*S*)-(**1**) gives a negative sign even if its specific rotation is strongly positive at wavelengths below 370 nm.

Optical filters can be used to select a part of the spectral domain and thus avoid the contributions of the low wavelengths. According to the specific rotation measured for (*S*)-warfarin (Table 1), this molecule is relevant to study both the influence of the working wavelengths and of the mobile phase.

Table 1 Specific rotation of (*S*)-warfarin reported at different wavelengths in various solvents for a concentration of 1 g/100 mL

	NaOH (0.5 N)	Ethanol	2-PrOH	Chloroform	Ethyl acetate
589 nm	−144	+8.4	+17	−19	+7.3
578 nm	−149	+9.0	+18	−20	+7.5
546 nm	−174	+9.5	+20	−24	+7.1
436 nm	−338	+5.3	+31	−52	−6.6
365 nm	nd	−51	+18	−133	−110

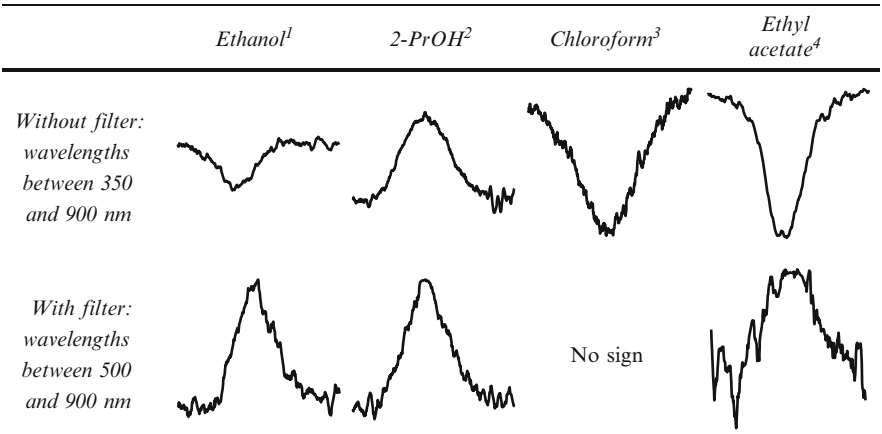

Fig. 5 Polarimetric traces (OR-1590, Chiralpak IC) obtained for (*S*)-warfarin depending on the mobile phase modifier and on the range of wavelengths used. (1) *n*-hexane/ethanol (7/3); (2) *n*-hexane/2-PrOH (1/1); (3) *n*-hexane/ethanol/chloroform (25/5/70); (4) *n*-hexane/ethyl acetate (1/1)

For instance, warfarin's specific rotations are too low in ethanol to be detected by mono-wavelength polarimetric detectors at analytical scale, but using a 350–900 nm range with multi-wavelengths polarimetric detector. Warfarin is still difficult to detect (Fig. 5), because the sign of its specific rotation changes in this range. The use of a sharp-cut filter to select the 500–900 nm range allows observation of a signal for (*S*)-warfarin by removing the highly negative contribution at low wavelengths.

Warfarin enantiomers have been separated on Chiralpak IC using different mobile phase modifiers with *n*-hexane, the (*R*)-warfarin being eluted first in each case. We could check that the sign of the (*S*)-warfarin given by the polarimetric detector changes with the chosen wavelength and modifier in accordance with the measured specific rotation. In ethanol its sign is (+) using the 500–900 nm range (with filter) but (−) using the 350–900 nm range (without filter), due to the strongly negative contribution below 400 nm. In 2-PrOH the specific rotation remains positive at all the wavelengths, so the sign given by the polarimetric detector is (+) in both cases. With chloroform and ethyl acetate as modifiers, the use of a sharp-cut filter cancels the strongest contributions to the signal between 350 and 500 nm and silences the signal. It follows that a polarimetric detector may provide a sign

either positive or negative depending on the wavelengths and the eluent used. This example also illustrates the need of a careful description of the conditions used to assign the sign of one enantiomer.

2.2.5 Artefacts

In addition to the chiroptical properties that clearly determine the response of the chiroptical detectors, other experimental parameters influence the signal. It is known that temperature affects the optical activity, while a rapid change in temperature in the laboratory may lead to a considerable drift of the baseline of the polarimetric detector (Fig. 7c). The overall performance of chiroptical detectors is good, but users should be aware that artefacts may appear for some molecules or in some conditions. In 1993, Däppen et al. [32] described the major problems encountered with polarimetric detectors. First, a signal is obtained for achiral 2-phenylethanol: this observation was explained by refractive index related effects generated by the eluted peaks. Second, distorted peak shapes in reversed phase were reported for samples with low ee or with small specific rotations, and nonlinear relationship between the area of the peaks and the ee. Such behaviour is also seen in Fig. 3a for (**5**), where the injection of a racemic mixture gives two peaks with equal areas in UV whereas on the polarimetric trace, the area of the second eluted peak is noticeably smaller than the first one. In 2008, similar observations were made and were explained by Sanchez et al. by refractive index artefacts [33].

For all the chiroptical detectors an injection peak may be present and sometimes quite intense, as a consequence of the difference in refractive index between the mobile phase and the solvent containing the injected sample.

The saturation of the detector results in a reflection of the portion of the peak beyond the saturation limit. This saturation limit depends mainly on the compound and on the wavelength, but not on the electric signal output from the detector. This saturation can be observed on only one peak (Fig. 6a) or on both enantiomers as for (**3**) (Fig. 6b). In this latter case, injection of 4 μg of *rac*-(**3**) led to a correct chromatogram, while injection of 10 μg led to the chromatogram displayed in Fig. 6b. In rare cases encountered in chiral HPLC, the CD detector provides the same sign for two enantiomers: a chromatogram with two negative peaks was reported for both enantiomers of 2-methyl-1-tetralone, separated on Chiralpak AD-RH with water/acetonitrile (6/4) and detected by CD at 230 nm [34]. We also encountered this case sometimes, as for atropisomers of (**6**) [35], which gave two positive peaks at 254 nm.

Artefacts can sometimes be avoided or reduced by adjusting the experimental parameters (amount injected, choice of wavelength, eluent, sample injection solvent) or the detector parameters (gain, cell size).

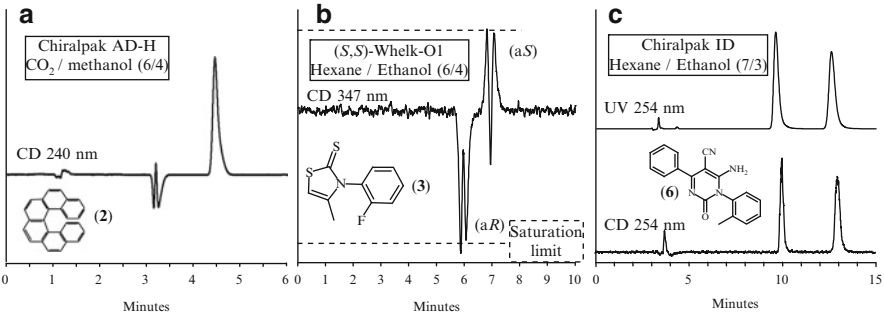

Fig. 6 CD chromatograms for (**a**) (**2**) on Chiralpak AD-H, CO_2/methanol (6/4), 3 mL/min, 40°C, outlet pressure = 120 bars, at 240 nm, (**b**) (**3**) on (*S*,*S*)-Whelk-O1, *n*-hexane/ethanol (6/4), 30°C, 1 mL/min, 10 µg injected, at 347 nm, and (**c**) (**6**) on Chiralpak ID, *n*-hexane/ethanol (7/3), 25°C, 1 mL/min, at 254 nm

2.2.6 Comparison of the Different Chiroptical Detectors

A direct comparison between the different chiroptical detectors for one or more molecules is not relevant, since each detector has a different principle or operating mode. For each detector, it is possible to find undetectable chiral molecules as well as very useful applications, so general conclusions cannot be drawn. However, generally speaking, a CD detector is more sensitive than most polarimetric detectors, but CD requires a chromophore in the chiral molecule. In the literature, discussions between polarimetric and CD detectors turn in favour of CD, considered to be more sensitive and having the advantage of the choice of the wavelength [34, 36]. Polarimetric detectors are nevertheless very helpful for molecules without chromophores [37] and have been proved to be sufficiently accurate detectors, for instance to determine an ee as high as 99.8% for mandelic acid with a detector ALP [38]. It is likely that the performance and sensitivity of chiroptical detectors will be further improved with recent advances in optics. Indeed, research to improve the sensibility of CD detectors is ongoing [39]. For polarimetric detectors, it would be interesting to be able to select the operating wavelength among several available ones displayed by the same instrument.

2.3 Assignment of Absolute Configuration

The contribution of chiral liquid chromatography to the determination of absolute configurations has already been reviewed [40]. We will recall the interest in the data provided by the chiroptical detectors in this approach and the precautions to be taken in order to not be misled.

2.3.1 From the Sign Given by a Chiroptical Detector

The correlation of the sign given by a chiroptical detector for one enantiomer with a reference value can be used to deduce its absolute configuration. This reference value may be found in the literature or predicted by a model or theoretical calculations.

In 1984, Salvadori et al. deduced the absolute configuration of a series of aryl-alkylcarbinols, binaphthol, *trans*-l,2-cyclohexanediol dibenzoate and 1-acenaphthenol benzoate by comparison of the sign of the CD detector and the sign predicted by semi-empirical methods, applying helicity rules and sector rules [21]. A correlation in a series of oxazepam analogs, the alcohol being replaced by acetate or isopropyl groups, was reported by Bertucci et al. [24], assuming that the (*S*)-enantiomers of these similar structures have a positive sign with a CD detector at 254 nm, because the isopropyl (*S*) analog has a positive CD at 260 nm. The safest way is to take as a reference the CD sign of the molecule analyzed in a given solvent at the wavelength used for analysis and verify that this sign is not changed by the eluent. This method was used to assign the absolute configuration of the naringenin enantiomers from the sign at 290 nm given by a CD detector [41].

A similar approach is possible with a polarimetric detector, being aware of the meaning of the sign obtained as outlined in the previous paragraphs. Indeed, a wrong configuration can be deduced, as in the case of Lao and Gan [42], who in 2012 studied the enantioselective degradation of warfarin in soils. They separated warfarin on a CSP with an *n*-hexane/2-PrOH/TFA (92/8/0.1) mobile phase; the sign of rotation of the resolved enantiomers was determined by an on-line ALP at 675 nm and an (*S*) configuration was assigned to the (−) sign. However, as we noted in Sect. 2.2.4, and checked, the (*S*)-enantiomer is (+) in this mobile phase. (*S*)-Warfarin is typically described with a (−) sign in the literature, in reference to the first reported specific rotation measured in NaOH (0.5 N) [29].

One of the advantages of the polarimetric detector is the ability to work with a very small amount of sample, less than 1 mg, and with mixtures. Thus, heterocyclic atropisomer absolute configurations have been determined by analysing mixtures obtained through a chemical interconversion method associated with chiroptical detection [43].

2.3.2 From On-line Circular Dichroism Spectra

An experimental CD spectrum can be recorded with CD detectors if the flow is stopped when a chiral molecule is in the cell. The first stopped-flow measurements were performed by Mannschreck in 1991 [44] and this technique is still used, particularly by Bringmann [45, 46]. With this method, the spectrum is obtained without having to collect the pure enantiomers and therefore it can be very useful for relatively unstable compounds. The use of electronic CD for the determination of absolute configuration is beyond the scope of this chapter, but has been recently reviewed by Bertucci and Tedesco [47].

2.4 Determination of Enantiomeric Excess

2.4.1 From a Partial Separation on a Chiral Stationary Phase

In the early years of chiral chromatography, CSPs were few and not very efficient, so overlapping peaks were often observed. Chiroptical detectors were then very useful to exploit these poor separations. Techniques for deconvolution of overlapping peaks have been used to determine ee from partial separations, and their accuracy was studied [48]. Nowadays, the performances of the numerous available chiral selectors allow baseline separation for all pairs of enantiomers if these are sufficiently stable. Deconvolution is therefore no longer useful in analytical chromatography to measure ee. However, the main interest remains in preparative separations [49] to collect enantiopure fractions [50]. Upon preparative separations, peak overlap is a consequence of high sample loading, and deconvolution of the chiroptical signal is a way to increase productivity [51].

2.4.2 On an Achiral Stationary Phase

On an achiral stationary phase, enantiomers are not differentiated: while the racemate gives no signal with chiroptical detection, an enantio-enriched sample provides a single peak (see self-disproportionation for exceptions), whose area is proportional to its optical purity. This method is similar to the direct use of polarimetry to measure optical purities, with the same requirements and drawbacks [52]. In the case of the Horeau effect [53], the optical purity is different from the ee due to nonlinear effects. The maximum specific rotation of the targeted compound must be known with certainty. Reference samples of known ee and a second method of detection giving the sum of the two enantiomers are required to make a calibration, i.e. to correlate the two areas obtained and ee. The first reports concerned the ee determination for permethrinic acid pentafluorobenzyl ester [54], epinephrine [55], pyrethroids [56] and amino-acids [57]. This type of dual detection as UV/CD or RI/polarimetry has limitations, especially when the chiroptical signal intensity is weak, and for the determination of high ee [58]. The method was proven to be less accurate than HPLC on CSP [59], but ees up to 99% have been determined for L-hexose during reaction monitoring using RI/polarimetry detections [60]. Sensitivity improvements of a CD detector by filter addition were used [61], but calibration and tests to validate the method are quite long compared to the development of a new method on a CSP. Articles with precise determination of ees on achiral columns have been written or sponsored by the suppliers of chiroptical detectors on particular cases. This method however does not in fact yield adequate sensitivity for pharmaceutical enantiomeric purity determinations [34].

The dual detection method has proven its interest and its efficiency in areas where the accuracy of the ee determination is not the main aim. For high-throughput analysis, the use of chromatography on achiral support with a chiroptical detector informs on the enantiopurity of the mixture components. Thus, high-throughput

screening of enantioselective catalysts [62] and investigations in organic reaction discovery by high-throughput experimentations [63] can be optimized. Obviously, care should be taken regarding the risks of overlapping peaks with other chiral components of the respective mixture such as catalysts, ligands or by-products and nonlinear responses of the chiroptical signal, owing to aggregation of some compounds. The same principle can be applied to the detection of conglomerates by chromatography on achiral support [64].

2.4.3 From Complex Mixtures

By definition, achiral molecules are not sensed by chiroptical detectors and it may be worthwhile to simplify an analysis by hiding all achiral products. Examples are described on achiral support, with the detection by polarimetric detector of sugars in human urine [18], and on CSPs, with the quantitation of the enantiomers of lorazepam in human plasma by CD detectors [65]. Chiral signatures in essential oils, i.e. polarimetric traces with peaks of the major chiral components, obtained with a polarimetric detector on a CSP, were recently proposed to study the enantiomeric diversity in essential oils [66].

2.5 Determination of Elution Order

The use of chiroptical detectors to determine enantiomeric elution order on CSPs seems quite easy by direct comparison of the observed signs, but the dependence of the signal on the experimental parameters should be kept in mind. True reversals of elution order are observed if the different analytical conditions do not affect the sign given by the chiroptical detector. Conversely, apparent reversals of elution order are observed when the observed sign is reversed with the analytical conditions for a given absolute configuration [67].

Direct comparison cannot be made for different compounds: even for analogs, CD spectra and specific rotation may change. For instance, by monitoring the enantiomeric elution order of fluoxetine, miconazole and analogs on Chiralcel OD with a CD detector at 280 nm and recording their CD spectrum, Cirilli et al. clearly explained that the apparent reversal of elution order established by on-line CD at 280 nm for the first eluted enantiomer, $(+)_{CD280}$-(*S*)-miconazole and $(-)_{CD280}$-(*S*) for analog, was only due to an inversion of the Cotton effect [68].

Using the same mobile phase, for the same compound and detector obviously, signs can be compared directly. In practice, many different mobile phases can now be screened with the widespread immobilised polysaccharide based CSPs and the sign may depend on the mobile phase modifier. The chiral HPLC separation of the enantiomers of 1-methyl-5-phenyl-5-propylbarbituric acid on Chiralpak IB monitored by a Chiralyser demonstrates that the (*R*)-enantiomer is (+) in *n*-hexane/2-PrOH (9/1) mixture but (−) in *n*-hexane/THF (9/1) [69]. So the effect of the mobile phases should always be tested [70], especially when the chiroptical signal is weak [37].

The reported examples emphasize how the deduction of the enantiomeric elution order, solely based on the sign of the chiroptical detector, could lead to mistaken conclusions. In case of doubt, the injection of a non-racemic mixture is required to confirm the enantiomeric elution order [71].

3 Reversal of Enantiomeric Elution Order

Elution order is difficult to control in chiral chromatography. Reversal of elution order can be obtained by switching the absolute configuration of the chiral selector, but it is only possible for molecular CSPs available in their two enantiomeric forms. The main objective of this section is to deal with unusual reversal of elution order, i.e. unexpected, astonishing and not easily explainable reversals.

3.1 Reversal of Enantiomeric Elution Order Due to the Chiral Stationary Phase

3.1.1 Case of Different Chiral Selector

It is obvious that different chiral selectors generate different recognition mechanisms and there is no reason why two different chiral selectors give the same elution order. However, study of the recent literature shows that the assignment of the absolute configurations from elution order by chromatography is sometimes incorrect because the authors did not compare the same CSPs, particularly in the area of asymmetric catalysis [72]. That is why we stress on this trivial point.

Reversals of elution order have been reported on molecular CSPs: for α-amino-esters from tyrosine based CSP to phenylglycine based CSP [73]; on cyclodextrins [74]; on glycopeptides: for dansyl-aminoacids, L-enantiomers are eluted first on vancomycin and teicoplanin, but second on ristocetin [75]; on polysaccharide esters: for *trans*-1,2-diphenylcyclopropane from triacetyl to tribenzoyl cellulose [22]; on polysaccharide carbamates: for naringenin from Chiralcel OD-H to Chiralpak AD-H [41]. Reversals of elution order from Chiralcel OD-H to Chiralpak AD-H, cellulose and amylose tris(3,5-dimethylphenyl)-carbamate, respectively, may occur [76], but it is not a general rule.

3.1.2 Case of CSP Based on Similar Chiral Selector

This section deals with CSPs prepared from chiral selectors similar in structure but with different packing: coating, bonding or immobilization on silica for instance.

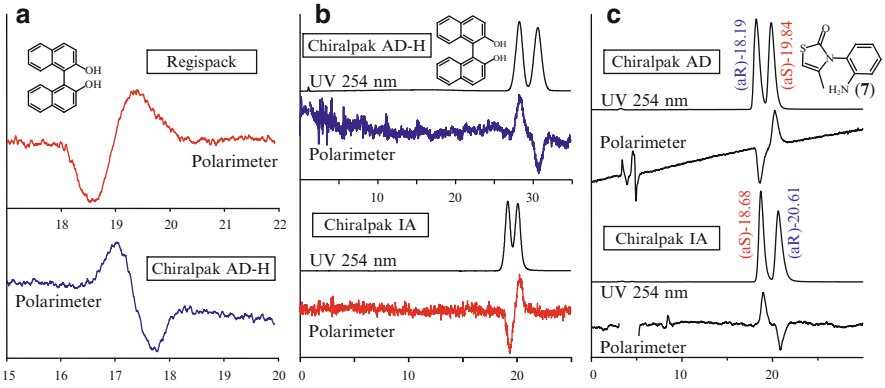

Fig. 7 Polarimetric traces (OR-1590): (**a**) for binaphthol, with *n*-hexane/2-PrOH (95/5), at 40°C, on Regispack at 2 mL/min and on Chiralpak AD-H at 3 mL/min; UV chromatograms and polarimetric traces (OR-1590); (**b**) for binaphthol on Chiralpak AD-H and on Chiralpak IA, with *n*-hexane/2-PrOH (95/5), at 1 mL/min and 25°C; (**c**) for (**7**) on Chiralpak AD and on Chiralpak IA, *n*-hexane/2-PrOH (9/1), 1 mL/min, 25°C

The quality of the silica has an impact on the performance of the column, but may also lead to a reversal of elution order. Nebivolol, a β-blocker, has opposite enantiomeric elution orders on Chiralpak AD and Chiralpak AD-RH, with 2-PrOH as mobile phase [77]. These two columns are based on the same amylose carbamate, but are coated on silica with different granulometry and hydrophobicity. According to the supplier, in reversed phase, the same chiral selector as found in the normal phase is coated on a hydrophobic high quality silica support. Influence of the silica has also been recently highlighted by a reversal of elution of the diastereomers, α- and β-thujone, on Whelk-01, depending on the characteristics of the silica used: spherical or Kromasil [66]. We have also found a reversal of elution order for binaphthol enantiomers on chiral columns coated with the same amylose carbamate chiral selector, namely Regispack and Chiralpak AD-H (Fig. 7a). The polarimetric trace shows the reversal unambiguously due to the silica characteristics, coating method of polysaccharide or packing process.

In the case of polysaccharide derivatives, the solvent used for the coating on silica plays a significant effect on the enantioselectivity of CSPs. Francotte showed that the enantiomeric elution order can be reversed depending on the solvating agent of the polymer used before its precipitation in *n*-hexane. For substituted 1-phenylethyl benzoates, elution orders were opposite using dichloromethane or nitrobenzene to dissolve cellulose tris(3-methylbenzoate) before coating [78].

The way the chiral selector is associated with silica is also an important parameter. Thus, Doyle et al. illustrated the difference between ionic and covalent CSP by a reversal of elution order on a poor separation ($\alpha = 1.03$ in both cases) of (3,5)-dinitrobenzoyl amide of 1-phenyl-2-aminopropane, on (*R*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine ionically or covalently bonded. In the ionic column, the CSP is bound to the silica through an ion pair between the carboxylate of phenylglycine and the protonated nitrogen of the amino-propyl-silica [79].

Switching from a coated chiral selector to a bonded (or immobilised) chiral selector leads to change in chiral recognition mechanisms because the chiral selector has to be modified to be bonded on silica. A spacer (alkyl or other) has to be introduced on the initial chiral selector and alters its conformation, resulting in modification of the enantioselectivity. Rigorously, it is not the same chiral selector. Examples were given on glycopeptides CSP; dansyl aminoacids have opposite enantiomeric elution orders on a chemically bonded vancomycin column or on a CSP prepared by dynamic coating of a monolithic reversed-phase HPLC column with a vancomycin-derivative as chiral selector [80]. For polysaccharide, immobilised CSPs have been introduced for cellulose and amylose (3,5-dimethylphenyl)-carbamate. In the coated version (Chiralcel OD and Chiralpak AD), the substitution degree of cellulose or amylose is 3, while in the immobilised version (Chiralpak IB and IA) the substitution degree is lower than 3, because some hydroxy groups of the glucose units are not substituted by carbamate but used for the bonding to silica. So these chiral selectors are different, as proven by the reversal of elution order obtained for atropisomer (**7**) [81] and binaphthol between Chiralpak AD and Chiralpak IA. Using the same mobile phase, polarimetric detection shows clearly the different elution orders (Fig. 7b, c). Opposite elution orders have also been reported between Chiralpak IB and Chiralcel OD-H for ten barbiturates [69].

3.2 Reversal of Enantiomeric Elution Order Due to Experimental Parameters on the Same CSP

According to the literature, almost all chromatographic experimental parameters can induce a reversal of enantiomeric elution order. Several articles have dealt with the influence of a parameter for the separation of a particular compound on a given CSP. Recently, Chankvetadze has reported an impressive study with reversals of enantiomeric elution order for Fmoc-isoleucine on Lux-Cellulose-1, depending on the content of the polar organic modifier of the mobile phase, the temperature and, for the first time, the acidic modifier content in the mobile phase [82]. We present in this section the parameters that have led to the observation of reversals of enantiomeric elution order in literature.

3.2.1 Influence of Mobile Phase Modifier

The influence of the mobile phase modifier has been shown on protein based CSP when acetonitrile replaces an alcohol. Indeed, a change from 1-PrOH to acetonitrile leads to reversal of elution order for enantiomers of clevedipine and of its hydrolysed analog on a Chiral-AGP column [83]. In the same way, on Ultron ES-OVM, a change in organic modifier from ethanol to acetonitrile causes a reversal of elution order for the *cis*-(2*S*,4*S*) and *cis*-(2*R*,4*R*) enantiomers of the

ketal tosylate intermediate of azalanstat [84] and for propanolol propyl ester enantiomers [85]. For propanolol *n*-pentyl ester, the same enantiomeric elution order is observed using ethanol, 2-PrOH and acetonitrile, but use of methanol results in the opposite [85].

One example is also described on a molecular CSP, based on a dinitrobenzoyl-tyrosine derivative, the elution order of different dinitrobenzoyl amino-esters is reversed from the mixture *n*-hexane/ethanol (85/15) to *n*-hexane/chloroform (35/65) [73].

All other reported reversals of enantiomeric elution order with the mobile phase modifier concern polysaccharide based CSPs. On Chiralcel OB, 19 different alcohols in *n*-hexane were tested for the separation of methyl 2-phenoxypropanoate enantiomers, which gave the opposite elution order according to the authors; but this case may be doubtful because the elution order was monitored by the sign from a polarimetric detector without checking that the sign was not reversed by the change of mobile phase [86]. On another cellulose ester, Chiralcel OJ, pyriproxyfen enantiomers show both elution orders depending on the 14 alcohols used as modifier in *n*-hexane [87, 88]. The absence of alcohol in the mobile phase may also lead to elution order reversal, such as for *trans*-chlordane separated on Chiralcel OD in pure *n*-hexane or with 1% of 2-PrOH in *n*-hexane [37]. When the mobile phase modifier is changed from ethanol to 2-PrOH, by HPLC, the enantiomeric elution order is reversed for binaphthol on Chiralcel OD [89], for 3-(4-fluoro)phenyl-4-benzyl-2-morpholinone on Chiralpak AS [90], and on Chiralpak AD, for omeprazole (from 2-PrOH or 1-PrOH to methanol or acetonitrile too in this case) [71, 91], nebivolol (from 2-PrOH or butan-1-ol to methanol, ethanol or 1-PrOH too) [77], morpholine derivatives [90], *N*-[2-hydroxy-2-pyridin-3-yl-ethyl]-2-(4-nitrophenyl)acetamide and its amino analog [76] and linezolid [92]. In the case of "pineno" fused terpyridyl ligands [93], ethanol, 2-PrOH, 1-PrOH and *tert*-butanol give the elution order opposite to that given by 1-butanol, 1-pentanol or 1-hexanol. On home-made amylose tris-(3,5-dimethylphenyl)-carbamate, the alcoholic modifier changes the elution order for diniconzole, uniconazole, paclobutrazol and their analogs [70]. In normal phase HPLC, methanol can be used in a mixture with ethanol, so on Chiralpak AD the enantiomeric elution order for ibuprofen and mandelic acid can be reversed when the polar alcohol modifier in isohexane, 2-PrOH, is replaced by a mixture methanol/ethanol (2/1) [94]. By SFC, the reversal is obtained from 2-PrOH to methanol for omeprazole (from 2-PrOH to ethanol too) [71, 95], naproxen, ibuprofen and ketoprofen [96]. The reversals of elution order observed on Chiralpak AD can usually be reproduced on Chiralpak IA, its immobilised version, as for omeprazole [71]. The number of solvents usable on Chiralpak IA is much larger and increases the probability of observing reversals, switching from 2-PrOH to THF in *n*-hexane, or from 100% ethanol to 100% ethyl acetate for example [97].

Such reversals of enantiomeric elution order are illustrated in Fig. 12 for compound (**1**): on Chiralpak AD-H, by HPLC, with 50% of alcoholic modifier in *n*-hexane, (*S*)-(**1**) is eluted first with ethanol and second with 2-PrOH, but also 1-PrOH, butan-1-ol, butan-2-ol, *tert*-butanol, 3-methyl-1-butanol, 4-methyl-2-pentanol, while, by SFC, with 25% of co-solvent (*S*)-(**1**) is eluted first with

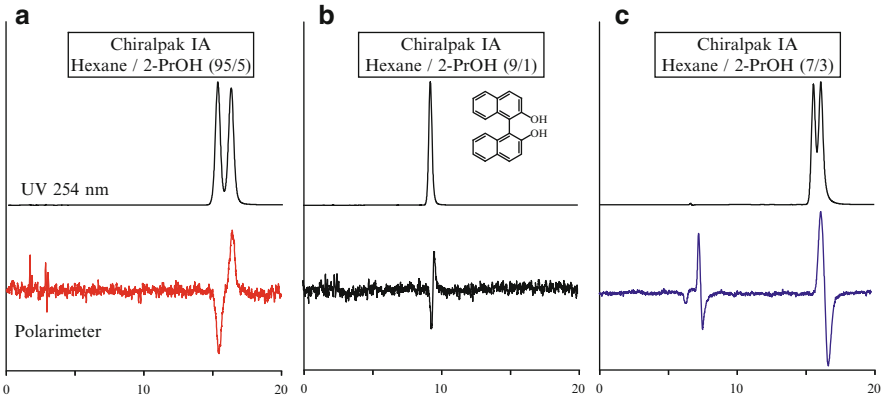

Fig. 8 UV and polarimetric traces (OR-1590) for binaphthol, on Chiralpak IA, at 35°C: (**a**) *n*-hexane/2-PrOH (95/5), at 2 mL/min; (**b**) *n*-hexane/2-PrOH (9/1), at 2 mL/min; (**c**) *n*-hexane/2-PrOH (7/3), at 0.5 mL/min

methanol and ethanol and second with 2-PrOH. A similar effect is observed for binaphthol, by SFC at 40°C with 20% of alcohol: (*R*)-binaphthol is eluted first with methanol and 2-PrOH and second with ethanol (Fig. 13). The steric bulk of the alcohols has often been cited to explain these reversals [87], but from all these examples it appears that there is no clear rule for predicting the influence of alcohols. A majority of described reversals occur on chiralpak AD but we cannot know whether this observation is due to a characteristic of this CSP or to its widespread use in laboratories.

The elution order may be affected by the proportion of organic modifier in the mobile phase. Some cases are reported in the literature: for amino acids dependent on the acetonitrile content of the eluent on copper(II)-D-penicillamine chiral stationary phase [98], for metoprolol analog with the 1-PrOH ratio on Chiralcel OD [99], for methyl *N*-Boc α-methyl (4-bromo)-phenylalaninate on Chiralpak AD from 20% ethanol in *n*-hexane to 1% ethanol [100] and on Chiralpak IA dependent on the 2-PrOH content for naproxen [101] and for binaphthol with 3-methyl-1-butanol [102] or with 2-PrOH (Fig. 8). In the latter case, the reversal of elution order is monitored by a polarimetric detector. (*R*)-Binaphthol, which gives a positive sign in the three mobile phases used, is eluted second with 5% of 2-PrOH and first with 30%. This type of reversal is not often observed because decreasing the ratio of organic modifier leads to very long retention times.

3.2.2 Influence of Mobile Phase Additives

Additives are sometimes added in the mobile phase to elute basic or acidic compounds. Acids, bases or both are mixed with the eluent in amounts ranging from 0.01 to 1 vol.%. Use of acid additives may affect the elution order; thus it is

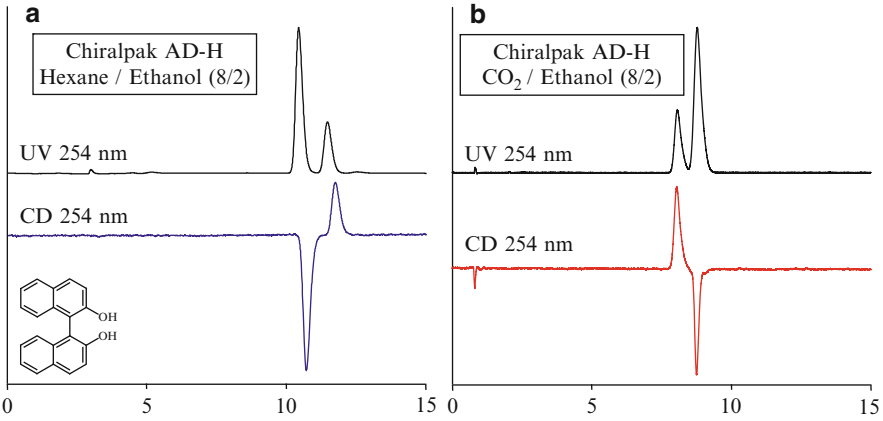

Fig. 9 UV and CD traces (CD-1595) at 254 nm for (*R*)-enriched solution of binaphthol, with 20% of ethanol, at 40°C, on Chiralpak AD-H: (**a**) by HPLC, *n*-hexane/ethanol (8/2), at 1 mL/min; (**b**) by SFC, CO_2/ethanol (8/2), 4 mL/min, outlet pressure = 120 bars

reversed for a metoprolol analog on Chiralcel OD when acetic acid (25 mM) is present [103]. In a few reported cases the nature and the ratio of these additives induce a reversal of enantiomeric elution order. On Lux-Cellulose-2 and Lux-Cellulose-4, (*R*)-ropivacaine is eluted before (*S*)-ropivacaine when using acetonitrile with 0.1% DEA and 0.1% formic acid (FA) as mobile phase, but the opposite elution order is obtained when formic acid is replaced by trifluoroacetic acid [104]. The formic acid ratio may also be an important parameter: on Lux-Cellulose-1, D-Fmoc-isoleucine is the first enantiomer eluted in *n*-hexane/2-PrOH (85/15) with 0.1% FA but the second with 0.5% FA [82]; on Lux-Cellulose-2 and Lux-Cellulose-4, with acetonitrile and 0.1% DEA as eluent, (*R*)-amlodipine eluted first with FA ratio lower than 0.02% and 0.035% respectively, but second at higher FA concentrations [105, 106], although this behaviour is not noted for other dihydropyridines tested. The influence of water contained in the eluent has also been pointed out on Chiralcel OD for a metoprolol analog: going from 0.2 to 1.6 g/L of water in *n*-hexane/2-PrOH (75/25) with DEA (10 mM) results in a reversal of enantiomeric elution order [99, 103].

3.2.3 Reversal Between SFC and HPLC

The apolar solvent may also affect the elution order, as illustrated by the reversal observed for binaphthol between SFC and HPLC techniques (Fig. 9): other experimental parameters being equal (temperature, percentage of alcohol), (*R*)-binaphthol is eluted first by HPLC with *n*-hexane and second by SFC with carbon dioxide.

3.2.4 Influence of the Injected Amount

On microcrystalline cellulose triacetate, two atropisomers, 3-(2-propylphenyl)-4-methyI-4-thiazolin-2-one and its thiazolinethione analog, show reversal of elution order when the amount of sample injected is increased from 2 to 16 mg [107], so that the enantiomer separation is lost for a given quantity. This is the only example described from analytical scale to preparative scale. Nowadays, this behaviour will be difficult to detect because the loading capacity studies are usually done gradually and stopped when the separation is lost. Compounds with such isotherms [108] are interesting for preparative purposes by sequential injections.

3.2.5 Influence of pH

On protein bonded CSPs, OVM and Chiral-AGP, the enantioselectivity of the separation of propanolol and its ester derivatives varies with the pH. It is possible to reverse the elution order by changing pH in the range 3–6.9 [109, 110]. Likewise, the enantiomeric elution order of mosapride and its main metabolite can be controled by the mobile phase pH on Chiral-AGP: pH was varied between 4.2 and 7.4 and the reversal of elution order occurred at 6.9 [111].

3.2.6 Influence of the Temperature

In chromatography on a chiral support, enantioselectivity α is related to the difference between the free energy of adsorption of both enantiomers, according to (1). The retention factors for each enantiomer, k_R and k_S, are dependent on enthalpy and entropy adsorptions on the chiral support, ΔH_R and ΔS_R for the (*R*)-enantiomer, as shown in (2).

Equation 1. Relationship between thermodynamic parameters for the adsorption process of enantiomers onto CSP and chromatographic parameters, β is the column phase ratio.

$$\Delta\Delta G = -\mathrm{RT}\ln\alpha, \tag{1}$$

$$\ln k_R = \frac{-\Delta H_R}{\mathrm{RT}} + \frac{\Delta S_R}{R} - \ln\beta, \tag{2}$$

$$T_{\mathrm{iso}} = \frac{\Delta H_R - \Delta H_S}{\Delta S_R - \Delta S_S}. \tag{3}$$

Accordingly, a temperature exists at which $\Delta\Delta G = 0$ ($k_R = k_S$). This temperature, called isoenantioselective temperature or isoelution temperature in older articles (T_{iso}, (3)) [112], was predicted by Davankov [113] and Schurig [114], and first observed [115, 116] and then studied in gas chromatography, mainly by Schurig

[117]. The Van't Hoff plot, i.e. the line obtained by plotting the natural logarithm of the retention factors against the reciprocal of temperature (in Kelvin), can be drawn for each enantiomer, after collecting data at different temperatures (Fig. 15). The relationship between ln k and $1/T$ is linear if ΔH and ΔS are temperature independent, if enantiomer retention results from a single associative mechanism [112] and if the experimental data are very accurate. The crossing of the lines obtained for both enantiomers should give the isoenantioselective temperature. However, if the temperature range is extended, T_{iso} predicted by such extrapolation may be inaccurate [118]. Moreover, linear plots seem difficult to observe in SFC [119], because ΔH is dependent on fluid density, and thus on temperature, because the critical temperature is crossed [120] or because of variation of the column phase ratio β [121]. If the surface of the chiral support is not homogeneous, this approach does not give a correct estimate of the thermodynamic parameters [122].

For each enantiomer separation it is possible to reverse the elution order beyond T_{iso}. As the temperature range used for chiral chromatography is usually narrow, between 10°C and 50°C, T_{iso} is often unreachable, but has been seen in some cases by the observation of isoelution and/or reversal of elution order. The first report in chiral chromatography by Pirkle in 1993 [123] dealt with the separation of *N*-(3,5-dinitro-benzoyl)-α-phenylethylamine enantiomers on a proline based molecular CSP: with *n*-hexane/2-PrOH (8-2) as mobile phase, (*R*)-enantiomer was first eluted at 30°C, but was second eluted at −25°C; T_{iso} is 0°C in this case. Pirkle has also shown the influence of the eluent, since T_{iso} is 25°C in *n*-hexane/ethanol (8/2), higher than 27°C in methanol/water (7/3) and lower than −25°C in *n*-hexane/dichloromethane (1/1). In one article, extreme experimental conditions were used in SFC, the temperature ranging between −10°C and 190°C with an outlet pressure of 300 bars, in order to observe reversal of enantiomeric elution order for two original molecules on Whelk-O1, with T_{iso} of 70°C and 150°C [120]. On the same molecular CSP, di-(3,5-dinitrophenyl)-carbamate of *trans*-1,2-cyclohexanediol and *trans*-1,2-cycloheptanediol have reversed elution order in the mobile phase chloroform/methanol (99/1) between 25°C and 85°C with T_{iso} around 65°C, but (*S*,*S*) enantiomer remains the first eluted in this temperature range with 15% of acetonitrile or 25% of ethyl acetate in chloroform [108]. For sotalol enantiomers, on an immobilized cellobiohydrolase Chiral-CBH, by increasing the temperature from 5°C to 40°C the retention time of the (*S*)-enantiomer rapidly decreases while the retention of the (*R*)-enantiomer is only slightly affected, resulting in a reversal with T_{iso} around 28°C [124]. On polysaccharide based CSP, reversals of enantiomeric elution order were reported for ketoprofen and naproxen by SFC on Chiralpak AD between −15°C and 30°C [96]; for binaphthol on Chiralpak IA between 10°C and 50°C [125]; for 1-(naphthylethylamino)methyl-2-naphthol analog on Lux-Cellulose-1 with $T_{iso} = 20$°C [126]; for Fmoc-isoleucine on Lux-Cellulose-1 with $T_{iso} = 20$°C [82]. The reversal of elution order for binaphthol on Chiralpak IA with *n*-hexane/2-PrOH (9/1) is illustrated in Fig. 10: at 15°C, (*R*)-binaphthol, which gives a positive sign with polarimetric detection, is first eluted; T_{iso} is reached at 30°C; at 60°C, (*R*)-binaphthol is eluted second.

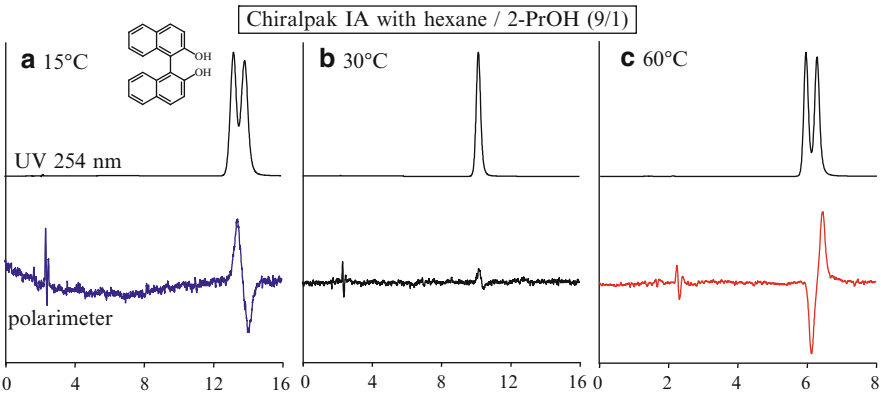

Fig. 10 UV at 254 nm and polarimetric traces (OR-1590) for binaphthol, on Chiralpak IA, *n*-hexane/2-PrOH (9/1), at 1 mL/min: (**a**) 15°C; (**b**) 30°C; (**c**) 60°C

3.2.7 Two-Dimensional Reversal of Enantiomeric Elution Order

The two main parameters prone to reverse an elution order on a given CSP are the temperature and the mobile phase. We have monitored with polarimetric detection the reversal of elution order of binaphthol enantiomers on Chiralpak IA, by varying the temperature from 15°C to 60°C and the content of 2-PrOH in the mobile phase from 5% to 30%. Polarimetric traces for this two-dimensional reversal of enantiomeric elution order are depicted in Fig. 11. For *n*-hexane/2-PrOH (8/2) mobile phase, (*R*)-binaphthol is eluted first until 40°C and second beyond 50°C. With increasing temperature, retention times decrease, the intensity of the polarimetric signal increasing at first because peaks are thinner, then decreasing because the separation is gradually lost. At T_{iso}, around 45°C, the signal is weak and above T_{iso}, the signal starts to increase again but with opposite elution order. So T_{iso} can be directly determined from the absence of polarimetric signal: 30°C with 10% of 2-PrOH, 45°C with 20% and around 52°C with 30%. Obviously, with a UV or RI detector, a single peak is obtained for both enantiomers between 30°C and 55°C; this range has been named "temperature-induced blind zone in chiral recognition, in which enantioseparation could not be obtained" by Weng and co-workers [101, 102, 125]. Obviously chiroptical detection reveals this so-called "blind zone" which does not exist anymore.

By SFC, a similar study was carried out by varying the temperature and the alcoholic co-solvent with an (*R*)-enriched solution of binaphthol to highlight the reversal of elution order and the isoenantioselective temperature. With 20% of co-solvent, $T_{iso} = 60$°C with methanol and 2-PrOH and $T_{iso} = 20$°C with ethanol, reversal of elution order is obtained at 40°C switching from methanol or 2-PrOH to ethanol (Fig. 14, lines a, c and e).

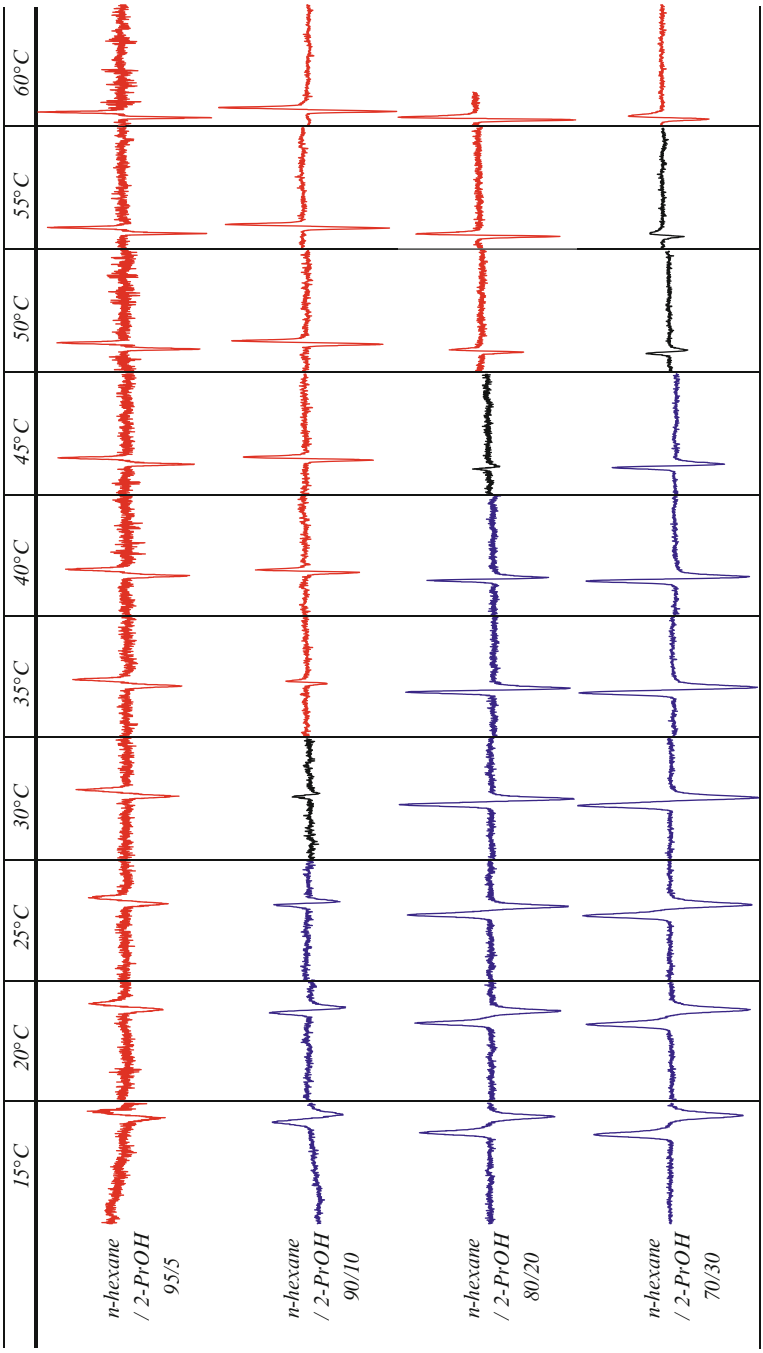

Fig. 11 Polarimetric traces (OR-1590) for binaphthol on Chiralpak IA at different temperatures and different percentages of 2-PrOH in the mobile phase; *blue*: (*R*)-enantiomer first eluted; *red*: (*S*)-enantiomer first eluted

3.3 Unusual Phenomena with Mixtures of Co-solvents

Reversed enantioselectivities ($k_{\text{second eluted enantiomer}}/k_{\text{first eluted enantiomer}}$) observed using different co-solvents or organic modifiers lead us to look at chromatographic behaviour in the case of co-solvent mixtures. Very few studies of the influence of co-solvent mixtures on the enantiorecognition have been published. This type of ternary mixtures of solvents, apart from acidic and basic additives, is sometimes described in the literature for improving the solubility of a compound in the mobile phase or to decrease retention times. Particularly, on immobilised polysaccharide CSPs, the addition of chloroform is often used to solubilize racemates in the mobile phase [127–129]. The absence of alcohol in the mobile phase can prevent the elution, so alcohol is often added, even in small amount (1–5%). In HPLC, immiscibility of methanol in *n*-hexane explains the reason why the addition of ethanol is required to obtain a unique phase.

On Chiralpak AD, reversals of elution order are observed for ibuprofen and for mandelic acid when 2-PrOH, the organic modifier in isohexane, is replaced by methanol/ethanol (2/1) [94]. The influence of methanol addition in *n*-hexane/2-PrOH mobile phase was discussed on Chiralpak AD [91]: for timoprazole, methanol addition increases the retention of the second enantiomer and, accordingly, enantioselectivity, while for omeprazole the addition of more than 4% of methanol reverses the elution order. In SFC, addition of 10% 2-PrOH in methanol co-solvent improves the enantioselectivity of omeprazole separation from 1.74 to 2.41 [71]. Again, on Chiralpak AD the reversal of elution order for nebivolol between ethanol and 2-PrOH was investigated by mixing both solvents in different proportions [77]: there is no correlation between the enantiomer retention times and the ratio of 2-PrOH in ethanol and the reversal of elution order is sudden, with 25% of 2-PrOH (*SSSR*)-nebivolol eluted first, but second with 20%. Addition of chloroform in *n*-hexane/ethanol (85/15) mobile phase for a dinitro-benzoyl amino-ester on a dinitro-benzoyl-tyrosine CSP reverses the elution order, but also decreases the retention times [73]. Thus, there are several possible behaviours when mixing several co-solvents or organic modifiers that are illustrated in several cases in the following paragraphs with mixtures of alcohols.

If we consider two different alcohols used as organic modifiers, leading to separation of enantiomers with identical elution order for a racemate on a given CSP, the expected behaviour when mixing these alcohols should be a separation with the same elution order. This is true for the chiral separation of binaphthol on Lux-cellulose-2; going from 20% ethanol to 20% 2-PrOH by addition of 5% of 2-PrOH results in a quite linear relationship between retention and ratio of 2-PrOH (Fig. 12a). However, unexpected behaviour can occur, leading to separation loss or separation with reversal of elution order. Examples are provided for binaphthol on Chiralpak AD-H by SFC (Fig. 14): at 40°C, with methanol and 2-PrOH alone, (*R*)-binaphthol is first eluted, but with an equal volume mixture of both alcohols, the separation is lost; at 50°C, a small separation exists with 100% methanol and 100% 2-PrOH, but with the mixture the opposite elution order is obtained. Improvement of enantioselectivity is possible, as for binaphthol on Chiralpak AD-H, with 20% of

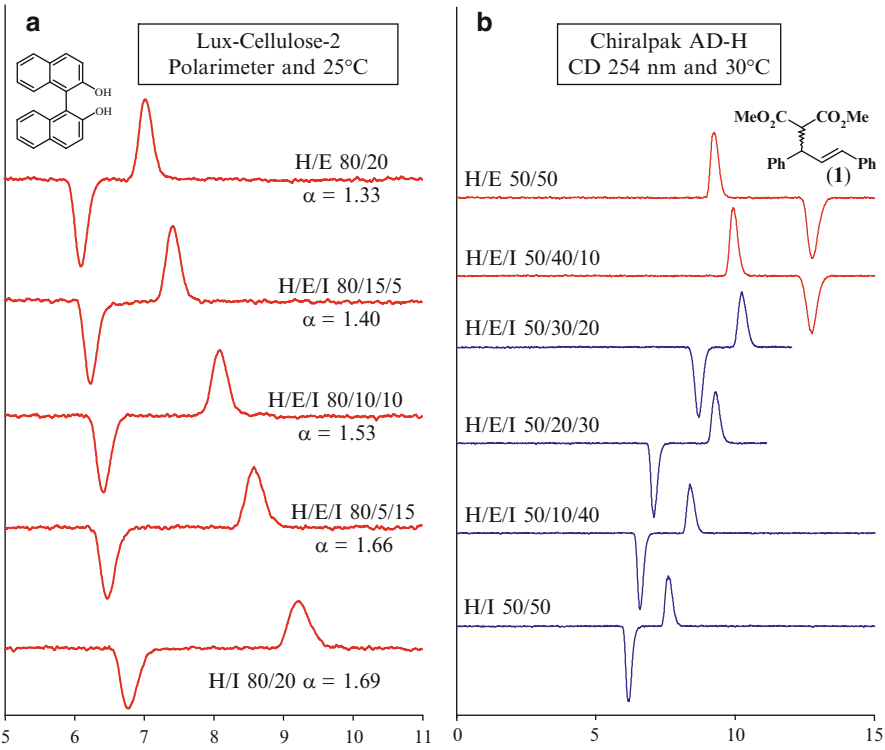

Fig. 12 Effects of mixtures of alcohols in *n*-hexane: (**a**) polarimetric traces (OR-1590) for binaphthol on Lux-Cellulose-2, 1 mL/min, at 25°C; (**b**) CD chromatograms (CD-1595) at 254 nm for (**1**), at 30°C, on Chiralpak AD-H; *H*: *n*-hexane, *E*: ethanol, *I*: 2-PrOH; *blue*: (*R*)-enantiomer first eluted; *red*: (*S*)-enantiomer first eluted

ethanol or 2-PrOH in *n*-hexane: enantioselectivity is 1.28 for both with the same elution order, but jumps to 1.70 with the mobile phase *n*-hexane/ethanol/2-PrOH (8/1/1) (Fig. 13b).

If we consider two different alcohols, giving no separation for a racemate on a given CSP, we do not expect a separation when mixing these alcohols. However, for binaphthol on Chiralpak AD-H by SFC at 60°C (Fig. 14) there is no separation with methanol and 2-PrOH, but enantiomers are separated with their mixture (1/1).

If we consider two different alcohols used as organic modifiers, leading to separation of enantiomers with opposite elution order for a racemate, the prediction on a given CSP is tricky. If enantioselectivities are reciprocal, one can assume that the separation will be lost with the mixture, as for binaphthol by SFC at 40°C (Fig. 14): methanol and ethanol result in the separations with similar enantioselectivities and opposite elution order, and there is no separation with methanol/ethanol (1/1). This first approximation, that the retention factor for an enantiomer using a mixture of solvents is proportional to the retention factor for this enantiomer in each pure solvent and molar ratio of each solvent, is not a rule. Only three articles report

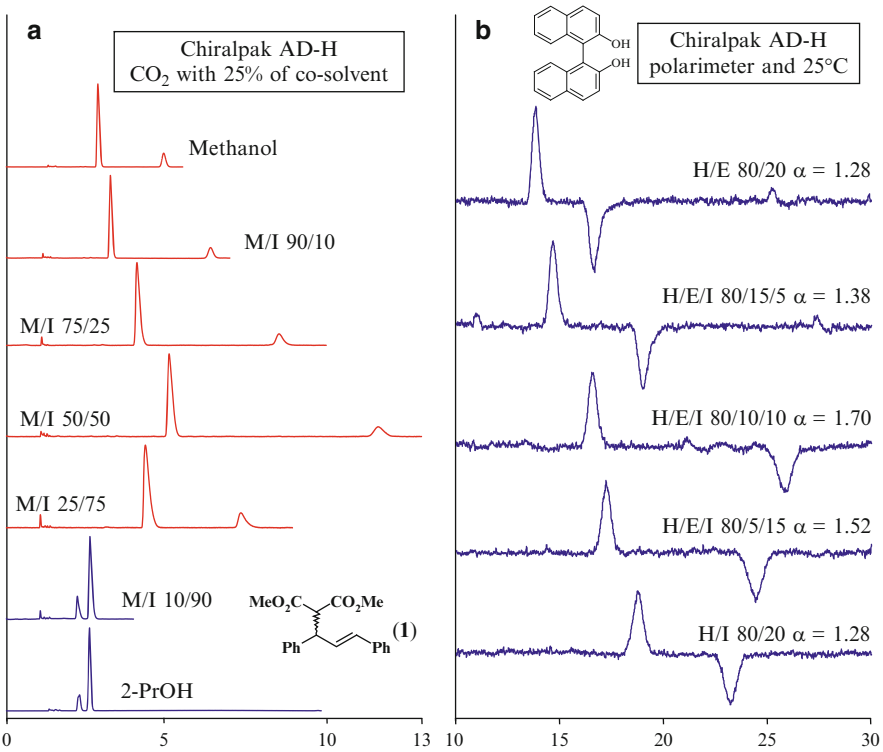

Fig. 13 Synergistic effects when mixing alcohols: (**a**) CD chromatograms (CD-1595) at 254 nm for (**1**) with mixtures of alcohol in *n*-hexane, at 30°C, on Chiralpak AD-H; (**b**) polarimetric traces (OR-1590) for binaphthol on Chiralpak AD-H, 1 mL/min, 25°C. *H*: *n*-hexane, *M*: methanol, *E*: ethanol, *I*: 2-PrOH; *blue*: (*R*)-enantiomer first eluted; *red*: (*S*)-enantiomer first eluted

the determination of the point of reversal of the elution order [73, 77, 91]. In each, the reversal is sudden, i.e. only a slight change in the mobile phase composition causes the reversal. Such behaviour is illustrated in Fig. 12b; enantioselectivities are quite similar when (*S*)-**1** is eluted first with 0–10% 2-PrOH, and (*R*)-**1** is eluted first with 20–50% 2-PrOH: the reversal is sudden between 10 and 20% 2-PrOH. In SFC, mixing methanol and 2-PrOH leads to a dramatic effect on retention for (*R*)-**1** and on enantioselectivity (Fig. 13a): retention time of (*R*)-**1** jumps from 2.3 min (2-PrOH) and 5.4 min (methanol) to 11.7 min (methanol/2-PrOH 1/1). This impressive and unusual improvement in enantioseparation of (**1**) kindles the interest in testing mixtures of modifiers in screening stategies.

The use of alcohol mixtures may induce linear, antagonist or synergistic effects on enantioselectivity. Even if these effects are not yet predictable, such unusual phenomena may be interesting for a better understanding of chiral recognition mechanisms.

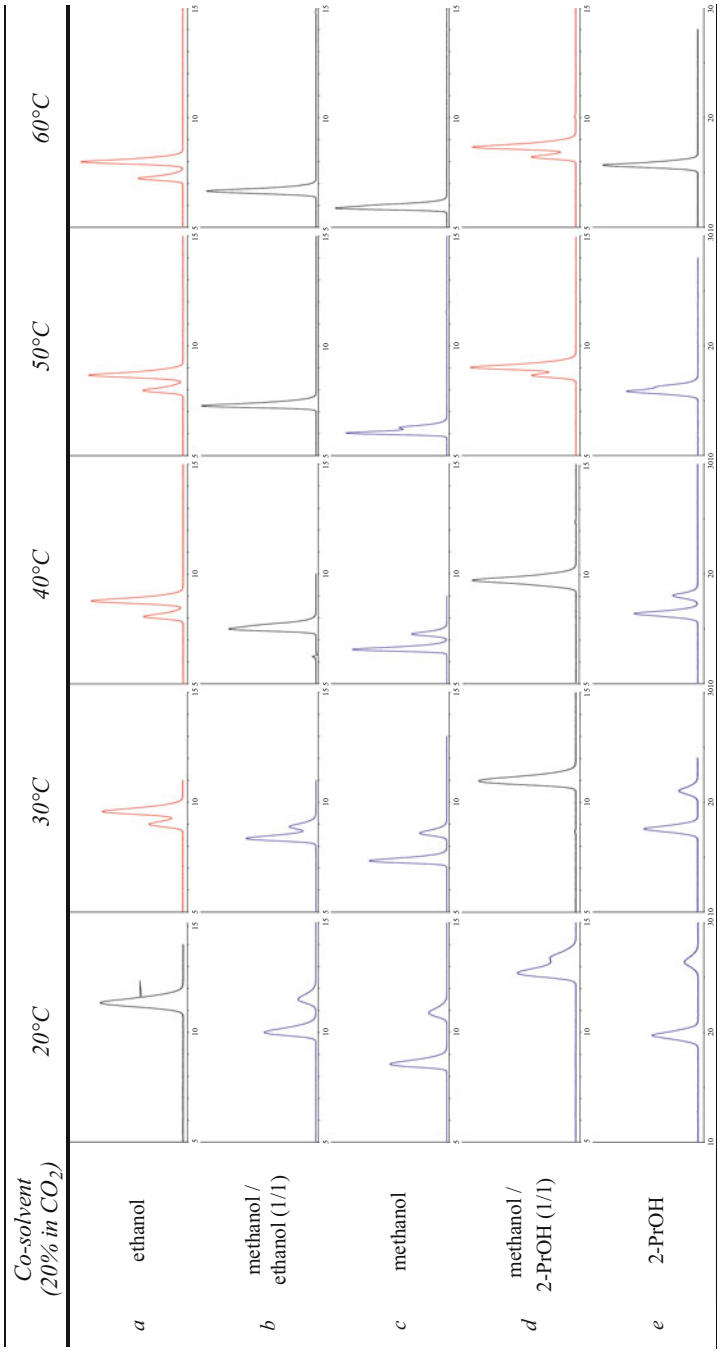

Fig. 14 UV chromatograms at 254 nm for (*R*)-enriched solution of binaphthol on Chiralpak AD-H, CO_2/co-solvent (8/2), 4 mL/min, outlet pressure = 120 bar, for different temperatures and co-solvents; *blue*: (*R*)-enantiomer first eluted; *red*: (*S*)-enantiomer first eluted

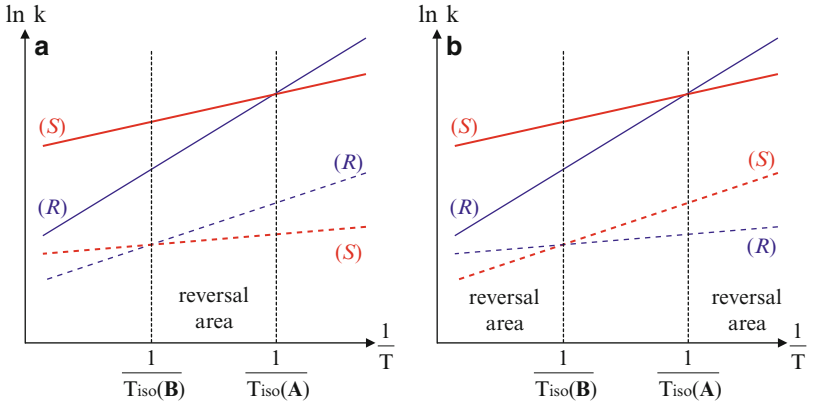

Fig. 15 Theoretical Van't Hoff plots for enantiomers (*blue* for the (*R*)-enantiomer and *red* for the (*S*)-enantiomer) separated on the same chiral selector, using two different chromatographic conditions **A** (*solid lines*) and **B** (*dashed lines*): (**a**) reversal of elution order between isoenantioselective temperatures; (**b**) identical elution order between isoenantioselective temperatures

3.4 Reversal of Enantiomeric Elution Order to Study Enantiorecognition Mechanisms

Authors who report reversals of elution order justify their observations by the complexity of the chiral recognition mechanisms, particularly on polysaccharide based CSPs, such as drastic conformational changes of the chiral selector, alteration of chiral cavities, steric effect of organic modifier and variety of binding sites. However, the observation of a reversal of enantiomeric elution order is not sufficient proof to invoke different binding sites and major modifications in the enantiorecognition mechanism or in the geometry of the diastereomeric complexes formed between enantiomers and CSP. Reversal of elution order should always be studied together with temperature dependence, and the isoenantioselective temperature should be estimated. Indeed, there are two main cases when a reversal is observed, when plotting ln k against $1/T$, assuming that ΔH and ΔS are temperature independent and that an isoenantioselective temperature exists. If we consider (*R*) and (*S*) enantiomers separated on the same chiral selector, using two different chromatographic conditions **A** and **B**, the lines ln k against $1/T$ (blue for the (*R*)-enantiomer and red for the (*S*)-enantiomer) can be drawn for each chromatographic conditions (solid lines for **A** and dashed lines for **B**). The lines for each enantiomer cross at the isoenantioselective temperature: T_{iso}(**A**) and T_{iso}(**B**) for the different chromatographic conditions (Fig. 15). **A** and **B** chromatographic conditions may differ by mobile phase (nature or content of the organic modifier, pH, water amount, ...) or by the preparation of the chiral selector (coating, immobilisation, packing, silica, ...). In Fig. 15a, elution orders are the same in the two chromatographic conditions at low and high temperature, but between $1/T_{iso}$(**A**) and $1/T_{iso}$(**B**), reversal of enantiomer elution order is observed. If the isoenantioselective temperatures are in an accessible range for

chiral HPLC or SFC (0–60°C), the reversal of elution order is experimentally reachable. We are unable, a priori, to determine whether the gap between T_{iso}(**A**) and T_{iso}(**B**) comes from a difference in chiral recognition mechanism, but we can assert that with T_{iso}(**A**) around ambient temperature it is quite easy to find conditions to reverse the elution order. This was done for binaphthol on Chiralpak AD-H and IA by changing temperature, mobile phase and column (immobilisation, supplier) in this section. The first example of multiple reversals of elution order, Fmoc-isoleucine on Lux-Cellulose-1 [82], is another example of the same behaviour with T_{iso}(**A**) = 22°C. Modifications of the chromatographic conditions lead to slight change in the solvation of the diastereomeric complexes, in conformations for the chiral selector or the solute, and so to a shift of the T_{iso}. In Fig. 15b, elution orders are different in the two chromatographic conditions at low and high temperature, but between $1/T_{iso}$(**A**) and $1/T_{iso}$(**B**) the same elution order can be observed. This is the case for compound (**1**) by HPLC on Chiralpak IA with *n*-hexane/2-PrOH (9/1); (*R*)-enantiomer is first eluted, T_{iso} is extrapolated to 160°C, and on replacing 2-PrOH by ethanol, (*S*)-enantiomer is first eluted and T_{iso} is extrapolated to 99°C, so the reversal is before both isoenantioselective temperatures. By SFC, on Chiralpak AD-H with 25% of co-solvent, the (*S*)-enantiomer is first eluted with methanol (T_{iso} around 105°C) and with methanol/2-PrOH (1/1) mixture (T_{iso} around 420°C), while the (*R*)-enantiomer is first eluted with 2-PrOH (no influence of temperature between 30°C and 55°C). In the case of compound (**1**) we can highlight two different chiral recognition mechanisms with 2-PrOH and with methanol or ethanol on Chiralpak IA and AD-H.

Estimation of the isoenantioselective temperature for a racemate on a CSP has three main points of interest. First, to optimize a chiral separation it is interesting to know whether the working temperature is above or below isoenantioselective temperature, because below T_{iso}, the higher the temperature the worse the enantioselectivity and above T_{iso}, the higher the temperature the better the enantioselectivity. Second, if T_{iso} is around ambient temperature, it will be quite easy to develop a method to reverse the elution order with accurate monitoring of the parameters. Third, the reproducibility of a chiral separation will vary from one laboratory to another if T_{iso} is around ambient temperature, particularly if the temperature is not controlled or if the CSPs are not strictly identical.

4 Chiroptical Detectors to Study Exchange Phenomena

An exchange phenomenon occurs during chromatography if analytes undergo a reversible reaction which alters their chirality (enantiomerisation, dimerisation . . .). Dynamic chiral chromatography allows the study of the interconversion of stereoisomers, while self-disproportionation is a consequence of homo- and heterodimers or oligomers formation in the mobile phase.

4.1 Dynamic Chiral Chromatography

Observation of the enantiomerisation of analytes during chromatography depends on the enantiomerisation rate constants. If the enantiomerisation is fast, with a barrier to enantiomerisation lower than 85 kJ/mol at 25°C, the enantiomers cannot be isolated and neither separated by chiral chromatography. If the enantiomerisation is slow, with a barrier to enantiomerisation higher than 105 kJ/mol at 25°C, the enantiomers can be baseline separated by chiral chromatography and isolated. For a barrier to enantiomerisation between 85 and 105 kJ/mol at 25°C, i.e. half-life between 43 s and 38 h at 25°C, it is possible to reveal enantiomerisation by selecting an adequate analysis temperature in order to obtain a half life on the order of a few minutes.

The typical chromatogram for racemate, subject to an interconversion process taking place inside the chiral column simultaneously with the chiral separation, shows a plateau between two peaks. Chiroptical detectors give opposite signs for the peaks and no signal for the plateau, as illustrated in Fig. 16 for the atropisomers (**8**) at 10°C. Each peak with a chiroptical sign corresponds to enantiomers which have not undergone any enantiomerisation in the column and the plateau corresponds to molecules which have undergone one or several enantiomerisations. At lower temperature the exchange would be slowed down and both peaks baseline separated. The plateau height increases and enantioselectivity decreases with temperature until coalescence of the two peaks (Fig. 16, at 35°C). The intensity of the chiroptical signal decreases with temperature because the amount of non-interconverted enantiomers decreases.

The first examples of dynamic chiral chromatography studies were performed by Schurig for gas chromatography [130] and by Mannschreck for liquid chromatography [131]. Such plateau chromatograms have been reported for different enantiomerisation mechanisms: rotation about a hindered bond for heterocyclic atropisomers [132], ring opening/ring closure for oxazepam [133] and torsion for helicenes [134]. The polarimetric [133, 134] and CD [135] detectors have been used to monitor dynamic chromatograms. Chiroptical detectors are useful to develop the analytical method to observe a suitable plateau, by varying CSP, mobile phase, temperature and flow-rate. Polarimetric signals have been deconvoluted [134] to estimate enantiomerisation rate constants. Indeed, the main interest of dynamic chromatography is to determine the barrier to enantiomerisation. Since the first estimation from the chromatogram profiles [134, 136], Trapp has developed a unified equation and software to calculate isomerisation rate constants [137–139] and the influence of the stationary phase on the enantiomerisation kinetic has been proven [140, 141]. For instance, the barrier to rotation of (**8**) was estimated at 85.3 kJ/mol in *n*-hexane/ethanol (8/2) from the UV chromatogram at 10°C.

Chiral dynamic chromatography is a versatile and now widespread tool [142–152] for studying the enantiomerisation or diastereomerisation processes in a domain of energies which was difficult or impossible to cover with dynamic NMR or classical thermal enantiomerisation kinetics. Thermal or catalyzed interchanges can thus be easily detected. In the field of dynamic stereochemistry [153], dynamic chromatography is a complementary method to dynamic NMR and classical kinetic thermal enantiomerisation, to determine the barrier to interconversion [154].

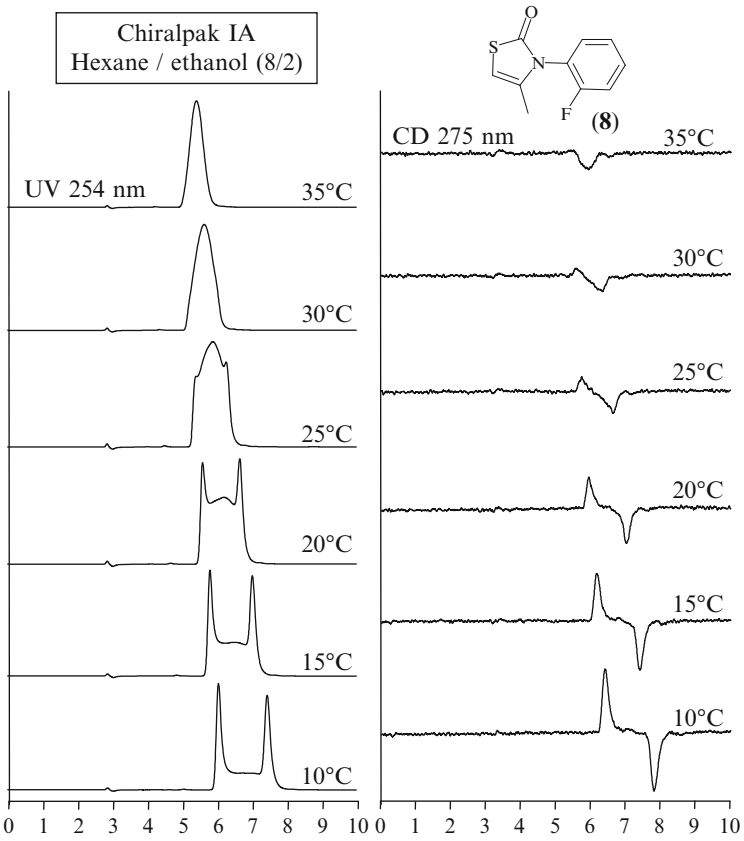

Fig. 16 UV (254 nm) and CD (275 nm) chromatograms (CD-1595) for (**8**), *n*-hexane/ethanol (8/2), 1 mL/min, on Chiralpak IA, for different temperatures

4.2 Self-Disproportionation

Enantiomers are eluted with the same retention times on achiral stationary phases. It is true when pure enantiomers are injected separately at the same concentration. Whatever the association state as homo-dimer or homo-oligomer of a pure enantiomer, the other enantiomer will produce exactly the same result. Chiroptical detection will show peaks with identical area and opposite sign. If the enantiomers in a racemic mixture behave strictly independently without the formation of associated states at a given concentration in an elution solvent, the retention of the racemate will be identical to those of the pure enantiomers. Interestingly, the injection of a racemic mixture might lead to different retention compared to the individual enantiomers. The origin of such a difference arises from the occurrence of both homo- and heterodimers (or higher order associations) when a racemate is involved. However, as expected, the composition of the mixture all along the elution peak of a racemate is strictly

composed of the racemic mixture without any enrichment whatever the type of associated states. In a racemate, a pair of enantiomeric homodimers can be formed in equal amount while heterodimers will correspond to a "meso" form. Heterodimers and homodimers are in a diastereomeric relationship and they may migrate differently on an achiral support. However, at each point of the chromatographic process the sample will be racemic due to the same migration of enantiomeric homodimers or to the occurrence of heterodimer composed of the two enantiomers. Chiroptical detection will be completely silent.

On the other hand, chromatography of a non-racemic mixture on an achiral stationary phase may lead to "self disproportionation of enantiomers" [155, 156], i.e. the enantiomeric composition of the elution peak of the mixture is not constant. One may find in the literature a handful of examples [157–170] which were recently reviewed [171, 172]. In general, a single peak is observed but the enantiomeric composition strongly varies all along the elution profile. In some extreme cases, the elution profile is composed of two distinguishable peaks without baseline separation; one shows a large increase of the ee while the other is approaching a racemic composition. Fractions with 99% ee were isolated from a mixture having an initial 40% ee on regular silica gel column [173]. In most of the reported cases the elution peak was divided into several successive fractions which were submitted to enantioselective chromatography to determine the enantiomeric excess at each point. It is thus possible to scan the actual self-disproportionation all along the peak.

On line chiroptical detection with a polarimetric or a CD detector is another way to scan the enantiomeric composition of the eluted peak. This method is perfectly suitable when HPLC columns are used but one can imagine that monitoring the output flow from MPLC is also possible. The principle is to have dual detection, e.g. UV and polarimeter or UV and CD. The use of a dual detection associating UV and polarimetry has been described in only one paper which dealt with the separation of binaphthol enantiomers through achiral chromatography [174]. We have reproduced the experiments performed on Lichrospher 100 NH_2 column eluted with chloroform. Figure 17 reports the chromatograms obtained with UV at 305 nm, CD detector at 300 nm and polarimeter for injection of 10 µL of a 2-mg/mL solution for (*R*), (*R*)-enriched and racemic binaphthol. It is clearly shown that the front of the peak is mostly composed of the pure (*R*) form while the tail is almost racemic. A signal probably due to an artefact is obtained for racemic binaphthol with chiroptical detections. The same behaviour is obtained with injection of 20 and 80 µL, but a single peak is observed for injection of 2 µL of the (*R*)-enriched sample.

The use of dual detection associating a UV or RI detector with a chiroptical detector is probably a very rapid way to spot the occurrence of self-disproportionating enantiomers during chromatography of a non-racemic mixture on achiral support. In case of disproportionation, the two signals will not have the same shape.

Modelling of chromatographic behaviour of non-racemic compounds under various conditions in achiral chromatography is generally done by simulation of elution profiles using UV detection [175–179]. Simultaneous simulation of the polarimeter or CD signals could provide a complementary useful and tutorial description of the phenomenon.

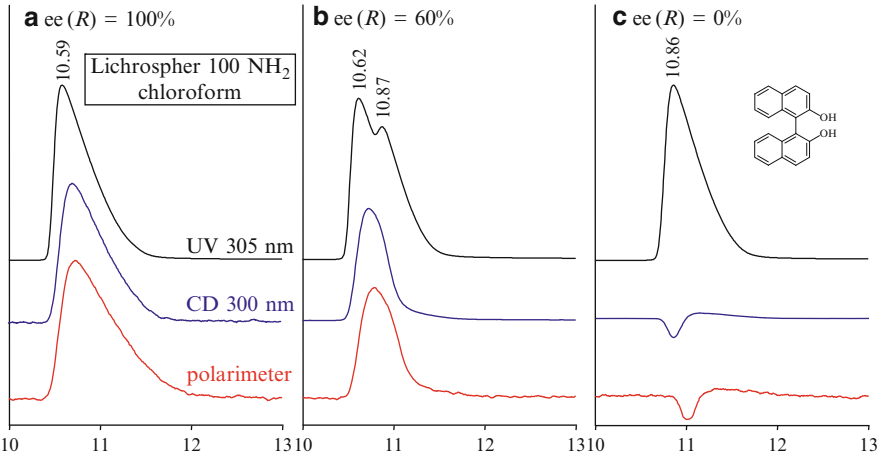

Fig. 17 UV (305 nm), CD (300 nm) and polarimetric chromatograms on Lichrospher 100 NH_2 (250 × 4 mm, 5 µm) eluted with chloroform, 1 mL/min, 25°C: (**a**) (*R*)-binaphthol; (**b**) (*R*)-enriched binaphthol; (**c**) racemic binaphthol

Chromatography of a non-racemic mixture on achiral stationary phases is a common post-reaction purification of most asymmetric catalysis media or resolution media before the determination of ee with the appropriate enantioselective chromatography on chiral stationary phases. It turns out that during the purification step particular attention shall be paid to collect the whole fraction of the non-racemic mixture in order to obtain a true evaluation of the ee. As shown above, the ratio of enantiomers may vary along the fraction containing the non-racemic mixture and thus different ees may be monitored for different zones of collection. Many more examples have probably been ignored during the chromatographic purifications of non-racemic mixtures issuing from chirotechnologies. Chiroptical detection can provide the answer.

5 Conclusion

Polarimetric and circular dichroism detectors are unique tools to differentiate enantiomers by chiral HPLC and SFC. However, the performances of these detectors depend on the chiroptical properties of the analytes: appropriate wavelength or range of wavelengths must be chosen to obtain a relevant output signal. The chiroptical sign given by the detector may serve to assign absolute configuration only after paying attention to the influence of the mobile phase on the sign. Reversals of enantiomeric elution order can thus be easily followed. These help in improving the knowledge of chiral recognition mechanisms. Many experimental parameters, such as temperature, mobile phase composition and column packing, may induce unusual and surprising

reversals. The temperature effect is thermodynamically explained and an isoenantioselective temperature exists in most cases, but is often difficult to reach in the working temperature range of chiral chromatography.

Reversals of enantiomeric elution order should not be systematically explained by different chiral recognition mechanisms involving diverse binding sites. Isoenantioselective temperature should be estimated to reveal a reversal due to a variation of this isoenantioselective temperature, caused by a slight modification in the energy of the diastereomeric complexes formed between enantiomers and the chiral selector.

Chiroptical detectors are essential to identify enantiomers in complex mixtures or during exchange phenomena where enantiomers and racemic peaks overlap while other detectors like UV or RI are inefficient in these cases. In particular, chiroptical detectors may provide very useful data for the understanding of a subject of growing interest, the self-disproportionation of enantiomers.

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