Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 995 (2003) 79–85
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

True or apparent reversal of elution order during chiral high-performance liquid chromatography monitored by a polarimetric detector under different mobile phase conditions

Christian Roussel[a,*], Nicolas Vanthuyne[a], Muriel Serradeil-Albalat[b], Jean-Claude Vallejos[a]

[a]*ENSSPICAM, Faculté Sciences St. Jérôme, UMR 6516, Université Aix-Marseille III, Avenue Escadrille Normandie-Niémen, 13397 Marseille CEDEX 20, France*

[b]*Clariant (France), Life Sciences & Electronic Chemicals Division, BP1, 60350 Trosly Breuil, France*

Received 15 October 2002; received in revised form 3 March 2003; accepted 14 March 2003

Abstract

The use of all chromatographic modes (i.e. reversed-phase, normal-phase and polar organic modes) is becoming more and more systematic in chiral HPLC method development. When this method development is monitored by polarimetric detection, which is a widespread technique, one should not forget that the observed positive or negative rotation angle for a given absolute configuration depends on the solvent. As a cautionary illustration, a new example is reported for a racemate which is separated with the same elution order in ethanol or acetonitrile mobile phase on a CHIRALCEL OD-R column whereas the signs of polarimetric detection are reversed.

Keywords: Enantiomer separation; Polarimetric detection; Elution order reversal; Mobile phase composition

1. Introduction

The determination of the elution order is a key issue in chiral HPLC analysis of enantiomers [1,2]. The configuration of the more- and the less-retained enantiomer on a CSP is of primary importance in chiral recognition mechanism studies [3], in the analysis of enriched samples (issued from chirotechnologies) or in preparative aspects [4,5]. On-line chiroptical detectors are now routinely used in many single column or column screening units [6–12]. These chiroptical detection methods are perfectly safe to trace the elution order on different CSPs using the same mobile phase. On the other hand, all chromatographic modes (i.e. reversed-phase, normal-phase and polar organic modes) are now systematically used to optimize a separation or to enlarge the application field of a given CSP [13]. This is already providing a lot of data on so-called elution order, determined by chiroptical detectors using various mobile phases. The fact that it is now so easy to determine whether the dextrorotatory or the levorotatory form is eluted first or second should not mask the fundamentals behind the use of chiroptical detection: the observed positive or negative rotation angle for a given absolute configuration depends on the solvent. Every chemist is aware of the possibility that a single enantiomer can present a

*Corresponding author. Tel.: +33-4-9128-8257; fax: +33-4-9102-7776.

E-mail address: roussel@spi-chim.u-3mrs.fr (C. Roussel).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00533-8

positive or negative rotatory power depending on the solvent in which it has been measured [14–16]. It is thus obvious that false reversal of elution order based on polarimetric response may be claimed on a given CSP when different mobile phases are compared. Francotte has already provided one example in his recent review on ''enantioselective chromatography as a powerful alternative for the preparation of drug enantiomers'' [5]. The point is that such information is diluted among a lot of very other important aspects and might escape the readers' attention. The reported example of warfarin showed that the signal of optical rotation was inverted when chiral separation was performed in *n*-hexane–2-PrOH (90:10, v/v) or *n*-heptane–ethylacetate (80:20, v/v). The latter mobile phase is not so commonly used in HPLC chiral separation and the CSP was a prototype of immobilized cellulose tris(3,5-dimethylphenylcarbamate) on silica gel. Our present study is focused on a new example we encountered using two very classical mobile phases (EtOH and CH_3CN) which are now systematically tested when preparative (batch or SMB) chromatographic separations are evaluated on commercially available polysaccharide CSPs.

2. Experimental

n-Hexane, 2-PrOH, EtOH and CH_3CN are HPLC-grade from SDS (Peypin, France); the solvents for chromatography experiments were degassed and filtered on a 0.45 μm Millipore membrane before use.

Cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phases, CHIRALCEL OD-H (250×4.6 mm), CHIRALCEL OD-R (250×4.6 mm), CHIRALCEL OD (250×10 mm) DAICEL columns and cellulose tris(4-methylbenzoate) chiral stationary phases, CHIRALCEL OJ (250×4.6 mm and 250×10 mm) DAICEL columns are available from Merck-Eurolab.

The chiral HPLC analyses under normal-phase conditions were performed on a screening unit composed of a Merck D-7000 system manager, Merck-Lachrom L-7100 pump, Merck-Lachrom L-7360 oven which may accommodate 12 columns alimented by a Valco 12-position valve, and a Merck-Lachrom L-7400 UV detector.

The chiral HPLC analysis under reversed-phase conditions and the semi-preparative separation were performed with a Merck-Hitachi LiChrograph L-6000 pump, Merck-Hitachi L-4000 UV detector and Merck D-7000 system manager.

The on-line chiroptical detectors are a Jasco OR-1590 polarimeter and Jasco CD-1595 circular dichroism detectors. They are placed after the UV detector, so that the retention times given by the chiroptical detectors are shifted in comparison with those given by the UV detector. The OR-1590 chiral detector utilizes light of wavelengths ranging from 350 to 900 nm. As a result, the detector output is a combination of the entire wavelength range.

The optical rotatory powers were measured on a 241 MC Perkin-Elmer polarimeter with a sodium lamp and a double-jacketed cell at 25 °C.

Racemic (1-dimethoxymethyl-propyl)-[1,2,4]-triazol-4-yl-amine **1**, was obtained by addition of ethylmagnesium bromide to the hydrazone derivative obtained by reacting 1,2,4-aminotriazole with 2,2-dimethoxyethanal [17].

Absolute configuration determination for **1** enantiomers: pure enantiomers of **1** were obtained by semi-preparative chiral HPLC, they were cleaved at the $N_4–N_{exocyclic}$ bond and the corresponding free α-aminoacetals were reacted with (−)-menthyl chloroformate. The comparison of the GC retention times with those obtained from analyses of the same derivatives of the same free α-aminoacetals of known absolute configuration gave the absolute configuration of the enantiomers of **1**. The absolute configuration of the reference free α-aminoacetals was known from independent syntheses, separation, cleavage and derivatisation of an analogue of **1** prepared from a chiral aminotriazole skeleton which afforded diastereomeric compounds bearing the same (1-dimethoxymethyl-propyl) radical, and for which the absolute configuration was unambiguously determined by X-ray [17].

3. Results and discussion

(1-Dimethoxymethyl-propyl)-[1,2,4]-triazol-4-yl-amine, **1**, is a heterocyclic dimethylacetal with a single stereogenic center on carbon. The racemate was analyzed on cellulose tris(3,5-dimethylphenyl-

carbamate) coated on silica (CHIRALCEL OD or CHIRALCEL OD-R) using *n*-hexane–2-PrOH (60:40, v/v), pure EtOH and pure CH_3CN. Mono-solvent mobile phases such as EtOH or CH_3CN offer many advantages for preparative separations: improved solubility of the compounds and easy recycling.

The chromatographic data are reported in Table 1. Figs. 1–3 report the UV, polarimetric and CD HPLC traces. Fairly nice baseline separations were obtained in each of the mobile phases.

Although the first eluted peak is negative on polarimetric traces in *n*-hexane–2-PrOH and ethanol, it appears positive in CH_3CN. The three CD traces at 220 nm are positive for the first eluted enantiomer. In order to check the elution order, the first eluted enantiomer in *n*-hexane–2-PrOH eluent, the negative one which happens to have the (*S*) absolute configuration (see Experimental), was collected by semi-preparative chiral chromatography on CHIRALCEL OD (250×10 mm, flow-rate=4.5 ml/min) and injected on CHIRALCEL OD-R with EtOH or CH_3CN as eluent: it gives a negative and a positive polarimetric response, respectively. It shows that the elution order is the same ((*S*)-form first eluted) but the sign of the optical rotation is inverted. Those changes of sign are confirmed by the off-line measurements of the rotatory power in the different solvents, reported in Table 2. It is clear that a deduction of the elution order solely based on the rotation angle would have concluded in a reversal of elution order when going from ethanol to acetonitrile. Interestingly, the sign of the rotatory power is the same in CH_3CN and $CHCl_3$.

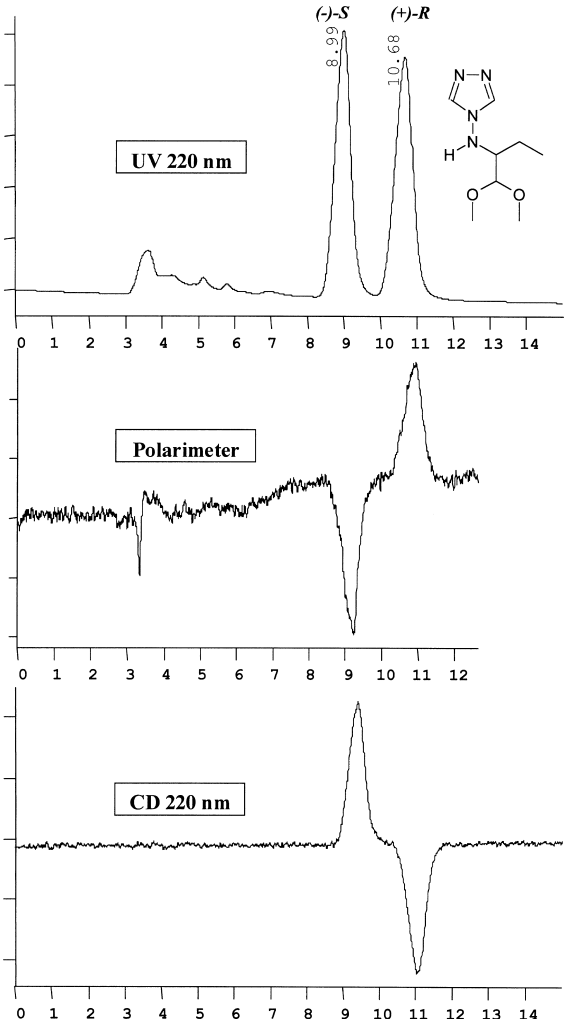

Fig. 1. UV, polarimetric and CD chromatograms of the enantiomers on CHIRALCEL OD-H in *n*-hexane–2-PrOH (60:40, v/v).

Table 1
Separations of the enantiomers on CHIRALCEL OD with different mobile phases

Mobile phase	R_{t1} (min)	k_1	R_{t2} (min)	k_2	α	R_s	Sign[a]
n-Hexane–2-PrOH 60:40[b]	8.99	2.09	10.68	2.67	1.28	1.98	(−)
Ethanol[c]	7.89	0.29	9.00	0.47	1.63	1.86	(−)
Acetonitrile[c]	9.36	0.48	12.21	0.93	1.93	1.77	(+)

R_t, retention time; k, retention factor, $k_i = (R_{ti} - R_{t0})/R_{t0}$; $\alpha = k_2/k_1$, selectivity factor; R_s, resolution.

[a] Sign of the rotation angle of the first eluted (*S*)-enantiomer.

[b] Analysis performed on CHIRALCEL OD-H (250×4.6 mm, flow-rate=1 ml/min) with both UV detection at 220 nm and on-line polarimeter.

[c] Analysis performed on CHIRALCEL OD-R (250×4.6 mm, flow-rate=0.5 ml/min) with both UV detection at 220 nm and on-line polarimeter.

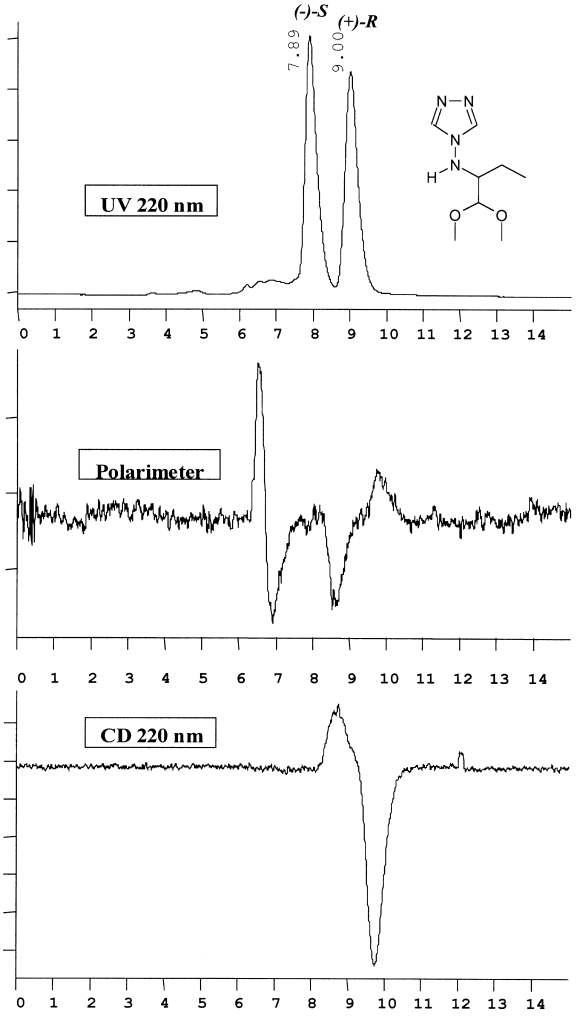

Fig. 2. UV, polarimetric and CD chromatograms of the enantiomers on CHIRALCEL OD-R in ethanol.

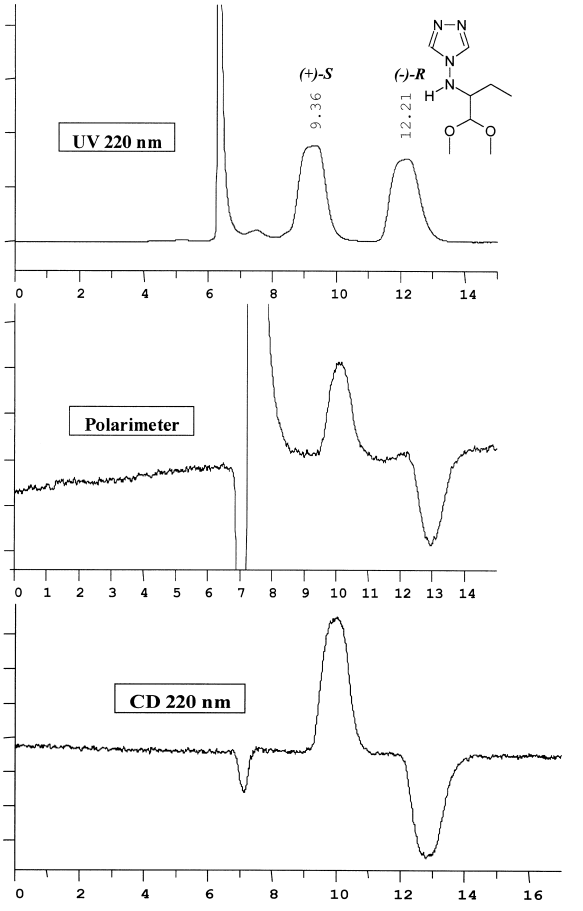

Fig. 3. UV, polarimetric and CD chromatograms of the enantiomers on CHIRALCEL OD-R in acetonitrile.

Examples and origin of the reversal of elution order using chiral HPLC have been reviewed recently by M. Okamoto [2]. Persson and Andersson in their review on ‘‘unusual effects of separation conditions on chiral separations’’ presented a paragraph on solvent effects [1]. In fact the literature dealing with reversal of elution order due to mobile phase composition can be divided into two categories.

The first one consists in established inversion of elution order using analytes enriched in one enantiomeric form. Samples are prepared starting from racemic mixtures and addition of a pure enantiomer of known configuration. This method has been used on several occasions in our group and others for the determination of elution order when several CSPs are evaluated. This is the method we recommend. In recent literature, Wang et al. [18,19] have described a series of inversions of elution order on CHIRALPAK

Table 2
Rotatory powers in different solvents of the (*S*) enantiomer, obtained after semi-preparative chromatography in *n*-hexane–2-PrOH (60:40, v/v)

Solvent	$[\alpha]_D^{25\,°C}$	*c* (g/100 ml)
n-Hexane–2-PrOH 60:40	−4.55	0.605
Ethanol	−1.14	0.550
Acetonitrile	+4.72	0.500
Chloroform	+18.37	0.615

AD upon changing the mobile phase modifier from 2-propanol to ethanol, Armstrong et al. [20] reported a true inversion of elution order for *N*-benzyl-α-methylbenzylamine analyzed on CHIROBIOTIC V using reversed and normal modes, respectively, and Karlsson et al. reported inversion of elution order on an AGP column on changing the organic modifier [21]. Elution orders were determined by spiking a pure enantiomer in the solution of the corresponding racemic compound. In the older literature, well-established inversions of elution order have been reported using enriched samples [22–24].

The second category consists in ''probable'' inversion of elution order when the chromatogram of the racemate is monitored by sole polarimetric detection. 2-Phenoxypropanoic acid methyl ester showed irregular elution order on CHIRALCEL OB in 1.3 *M* alcohol in *n*-hexane [25]. Nineteen alcohols were tested, nine of them gave the (−) form first and 10 of them the reverse. Even if the existence of two chiral recognition processes on the OB-CSP with opposite enantioselectivities is reasonable and if the inversion of sign of the rotatory power is less probable when using a modifier of the same nature as on going from ethanol to acetonitrile, the inversion was solely based on the sign of the polarimetric detection. The confidence in reported elution order by polarimetric detector decreases as soon as the difference in solvent properties increases. Geiser et al. have reported several separations using lower alcohols or CH_3CN [26–28]. Reversal of elution order based on the sole polarimetric detection was claimed for an α-hydroxyglycine derivative on going from CH_3CN–TFA (99:1, v/v) to EtOH–TFA (99:1, v/v) [27,29]. In the absence of reported experimental checks on the elution order using enriched samples, this separation might be a good candidate for apparent reversal of elution order. The same might hold for the reported inversion of elution order for nicotine separated on CHIRALCEL OD with *n*-heptane–ethanol–TFA (70:30:0.2, v/v) and *n*-heptane–ethanol (99:1, v/v), respectively [30], the sign of the optical rotatory power of nicotine being known to be strongly solvent dependent [15].

3-(2-Methoxyphenyl)-4-methyl-1,3-thiazole-2(3H)-thione atropisomers, **2**, which were under study in an unrelated project on barriers to atropisomerisation [31] present opposite rotation angles in CH_3CN and EtOH at 589 nm (Na lamp) and thus were prone to provide another example. These atropisomers were available long ago through semi-preparative chromatography on microcrystalline cellulose triacetate and the separation was monitored using a Perkin-Elmer 241 MC 1-ml cell at 589 nm [32]. They were baseline separated on CHIRALCEL OJ using pure EtOH as mobile phase (t_1=6.4 min, t_2=15.78 min). On-line polarimetric detection using a Jasco OR-1590 indicated that the first eluted enantiomer (t_1= 6.4 min) presents a positive rotation angle in the solvent of elution. Since the absolute configuration has not been determined yet, the first eluted enantiomer on CHIRALCEL OJ using EtOH as mobile phase will be labelled **2A** whereas the second eluted one will be labelled **2B**. Collection of the pure enantiomers using a semi-preparative column under the same eluting conditions provided the two enantiomers **2A** and **2B** in optically pure forms for the determination of rotation angles in various solvents. For **2A**: $[\alpha]_D^{25}$ +6.1 (EtOH, *c* 0.54); $[\alpha]_D^{25}$ −24.0 (CH_3CN, *c* 0.075); $[\alpha]_D^{25}$ −3.8 ($CHCl_3$, *c* 0.21). For **2B**: $[\alpha]_D^{25}$ −5.9 (EtOH, *c* 0.425); $[\alpha]_D^{25}$ +24.7 (CH_3CN, *c* 0.15); $[\alpha]_D^{25}$ +2.8 ($CHCl_3$, *c* 0.4).

We were thus highly confident when pure enantiomer **2A** and pure enantiomer **2B** were, respectively, injected on CHIRALCEL OJ using CH_3CN as eluent to provide the chromatographic data under these conditions. Enantiomers **2A** and **2B** are eluted at t_1=3.25 min and t_2=3.66 min, respectively, thus the same elution order was observed on going from EtOH to CH_3CN whereas the measurement at 589 nm of the sign of the rotation angle of the collected enantiomer solutions could have granted an apparent inversion of order of elution. Example **2** is not as illustrative as example **1** since the separation is rather poor in CH_3CN compared to the one observed in EtOH, but example **2** was rewarding in terms of serendipity.

Monitoring the sign of the rotation angle with an on-line Jasco OR-1590 detector during independent chromatography of **2** enantiomers with CH_3CN as eluent, we were expecting a negative rotation angle for enantiomer **A** and a positive one for **B** according to the optical rotatory power we measured at 589 nm; we observed the same signs as in EtOH! These observations were very confusing until the rotation angles in EtOH and CH_3CN were measured for the

pure enantiomers at various wavelengths: in EtOH the rotation angle increases as the wavelength decreases (plain or normal ORD curve [33]) whereas in CH_3CN the rotation angle decreases and then inverts at low wavelengths (complex or anomalous ORD curve [33]). The Jasco OR polarimeter, which was used for on-line detection, is *simultaneously* operating the full range of wavelengths from 350 to 900 nm, the sign of the resulting signal is thus the balance between the positive contribution and the negative contribution in case of an anomalous ORD curve. To the best of our knowledge such an observation has not yet been documented.

Summing up, compounds **1** and **2** provide examples in which the same order of elution is verified in EtOH and CH_3CN whereas an apparent inversion might have been advocated on the basis of polarimetric detection. Our new observations on the response of a Jasco OR-1590 detector in the case of enantiomers presenting an anomalous ORD curve reinforces our statement that one has to be very careful when monitoring the order of elution in different mobile phases by polarimetric on-line detection.

4. Conclusion

The generalization of the use of all chromatographic modes (i.e. reversed-phase, normal-phase and polar organic modes) to optimize an analytical or preparative separation or to enlarge the application field of a given CSP, on one hand, and the generalization of the use of polarimetric detection on the other hand might result in more frequent description of apparent inversion of elution order. Reciprocally, conservation of the monitored sign of rotation may mask true inversion of elution order when different chromatographic modes are used. The order of elution should be checked on enriched samples. In another method, the polarimetric responses of enriched samples can be checked for instance on an achiral column for different mobile phases.

Acknowledgements

We thank Clariant company for funding. We acknowledge generous funding from the ‘‘Conseil Général des BdR’’, ‘‘Conseil Régional Provence-Alpes-Côte d’Azur’’ and ‘‘Fond Européen de Développement Régional (FEDER)’’ for the chiral chromatography screening unit.

References

[1] B.-A. Persson, S. Andersson, J. Chromatogr. A 906 (2001) 195.
[2] M. Okamoto, J. Pharm. Biomed. Anal. 27 (2002) 401.
[3] C. Roussel, C. Suteu, J. Chromatogr. A 761 (1997) 129.
[4] J. Dingenen, in: G. Subramanian (Ed.), A Practical Approach to Chiral Separations by Liquid Chromatography, VCH, Weinheim, 1994, Chapter 6.
[5] E.R. Francotte, J. Chromatogr. A 906 (2001) 379.
[6] T. Hanai, I. Suzuki, K. Nakanishi, Anal. Sci. 1 (1985) 483.
[7] D.M. Goodall, Trends Anal. Chem. 12 (1993) 177.
[8] F. Brandl, N. Pustet, A. Mannschreck, J. Chromatogr. A 909 (2001) 147.
[9] A. Mannschreck, Chirality 4 (1992) 163.
[10] F. Geiser, in: 221st ACS National Meeting San Diego, 2001.
[11] D.R. Bobbitt II, S.W. Linder, G.W. Yanik, in: 221st ACS National Meeting San Diego, 2001.
[12] W. Vetter, U. Klobes, B. Luckas, G. Hottinger, G. Schmidt, J. AOAC Int. 81 (1998) 1245.
[13] B. Chankvetadze, I. Kartozia, C. Yamamoto, Y. Okamoto, J. Pharm. Biomed. Anal. 27 (2002) 467.
[14] E.L. Eliel, S.H. Wilen (Eds.), Stereochemistry of Organic Compounds, John Wiley, New York, 1994.
[15] C. Winther, Z. Physik. Chem 60 (1907) 563;
C. Winther, Z. Physik. Chem 60 (1907) 590;
C. Winther, Z. Physik. Chem 60 (1907) 641;
C. Winther, Z. Physik. Chem 60 (1907) 756.
[16] G.G. Lyle, R.E. Lyle, in: J.D. Morrison (Ed.), Asymmetric Synthesis, Analytical Methods, Vol. 1, Academic Press, 1983, p. 13.
[17] M. Serradeil-Albalat, PhD thesis n°01AIX30102, Université Aix-Marseille III, Marseille, 2002.
[18] T. Wang, Y.W. Chen, A. Vailaya, J. Chromatogr. A 902 (2000) 345.
[19] T. Wang, Y.W. Chen, J. Chromatogr. A 855 (1999) 411.
[20] T.L. Xiao, B. Zhang, J.T. Lee, F. Hui, D.W. Armstrong, J. Liq. Chromatogr. Relat. Technol. 24 (2001) 2673.
[21] A. Karlsson, A. Nyström, Chromatographia 53 (2001) 135.
[22] M. Okamoto, H. Nakazawa, J. Chromatogr. 588 (1991) 177.
[23] M. Okamoto, H. Nakazawa, Anal. Sci. 7 (1991) 147.
[24] P. Macaudière, M. Lienne, M. Caude, R. Rosset, A. Tambuté, J. Chromatogr. 467 (1989) 357.
[25] M.H. Gaffney, R.M. Stiffin, I.W. Wainer, Chromatographia 27 (1989) 15.
[26] F. Geiser, T. Yun, W.L. Champion, J. Lee, in: Poster Presentation Prep’98, Washington, 1998.
[27] F. Geiser, R.J. Bopp, K.B. Prickett, W.L. Champion Jr., T. Yun, in: Poster Presentation D6 21-M, HPLC’98, St. Louis, 1998.

[28] Brochure, Chiral Technologies, Exton-Pa, USA, 1998.
[29] F. Geiser, J.K. Lee, R.J. Bopp, in: Poster Presentation 1186 PittCon'98, New Orleans, 1998.
[30] F. Geiser, R.J. Bopp, J.K. Lee, in: Poster Presentation 09/A HPLC'97, Birmingham, 1997.
[31] M. Bouchekara, A. Djafri, N. Vanthuyne, C. Roussel, ARKIVOC x (2002) 72, http://www.arkat-usa.org/ark/journal/2002/Part(x)_General/2-612h/612H.pdf.
[32] A. Chemlal, PhD thesis, University Aix-Marseille III, Marseille, 1990.
[33] P. Salvadori, F. Ciardelli, in: F. Ciardelli, P. Salvadori (Eds.), Fundamental Aspects and Recent Developments in Optical Rotatory Dispersion and Circular Dichroism, Heyden, 1973, p. 3.