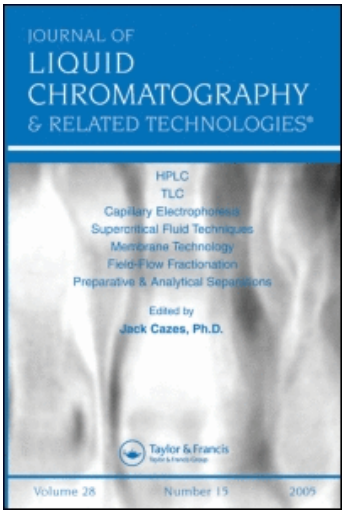

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Direct Enantiomeric Separation and Determination of Enantiomeric Purity of Methoxytetrahydro-Naphthalene Derivatives and Melatonin Ligands by HPLC using RSP-β-Cyclodextrin as Chiral Stationary Phase

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Direct Enantiomeric Separation and Determination of Enantiomeric Purity of Methoxytetrahydro-Naphthalene Derivatives and Melatonin Ligands by HPLC using RSP-β-Cyclodextrin as Chiral Stationary Phase

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Abstract: A series of N-[2-(7-methoxy-3-phenyl-1,2,3,4-tetrahydronaphtyl)ethyl] amides has been synthesized as new agonist and antagonist ligands for melatonin receptors. For those melatoninergic derivatives which contain two chiral carbons, a stereoselective high performance liquid chromatographic method, using cyclodextrin as chiral stationary phase, has been developed. Polar organic mode has been chosen as methodology, with a mixture of acetonitrile and methanol in different percentages as mobile phase and (R,S) hydroxypropyl-β-cyclodextrin (Cyclobond I 2000 RSP) as stationary phase. Limit of detection, limit of quantification, and enantiomeric purity have been calculated.

Keywords: Liquid chromatography, Chiral separation, β-Cyclodextrins, Melatoninergic ligands, Tetrahydronaphtalens, Enantiomeric purity

INTRODUCTION

Melatonin (Figure 1) is the main hormone secreted during the dark period by the pineal gland of mammals. It's structure (N-acetyl-5-methoxytryptamin)

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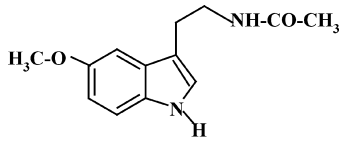
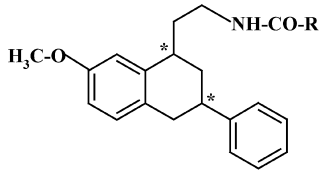


Melatonin



1	R = -CH_3	cis + trans
2	R = cyclo C_4H_7	cis + trans
3	R = cyclo C_3H_5	cis + trans

Figure 1. Chemical structures of melatonin and tetralins derivatives **1**–**3**.

was determined by Lerner in 1958.[1] It has been observed that this endogeneous hormone occurs in numerous and diversified physiological processes. Today, chronobiotic properties of melatonin have been demonstrated in humans.[2,3] In addition, melatonin is shown to be effective as a free-radical scavenger and an endogeneous antioxydant.[4] Furthermore, reactivity as a scavenger of nitric oxide[5] and lipoperoxy radical[6] has been noted for melatonin. For all these interesting biological properties, melatonin can be considered as a valuable ⟪lead compound⟫, but also perfectible, mainly because of its short half life (10–12 min) and its ubiquitous action. In order to gain improved structure activity relationships and better characterize the melatonin receptors, numerous analogs of melatonin have been synthetised. More specifically, the synthesis, the pharmacological and biochemical studies of tetrahydronaphthalenic derivatives (Figure 1) as potent and specific new agonist and antagonist melatoninergic ligands has been recently described.[7]

Many pharmacological studies have shown that enantiomers of numerous drugs differ in activity, in metabolism, or in toxicity. So, in the sight of the presence of two chiral centers in those compounds, resolution of the racemic mixtures seems advisable.

To perform the enantioselective separation of those tetrahydronaphthalenic derivatives, we choose a native β-cyclodextrin and an (R,S)-hydroxypropyl ether β-cyclodextrin as stationary phases (Figure 2). First, because of the major development of derivatized CDs as chiral stationary phase occuring since 1990.[8] Indeed, today, those CDs stationary phases can be used in any of the three chromatographic modes : reversed phase, normal phase, and

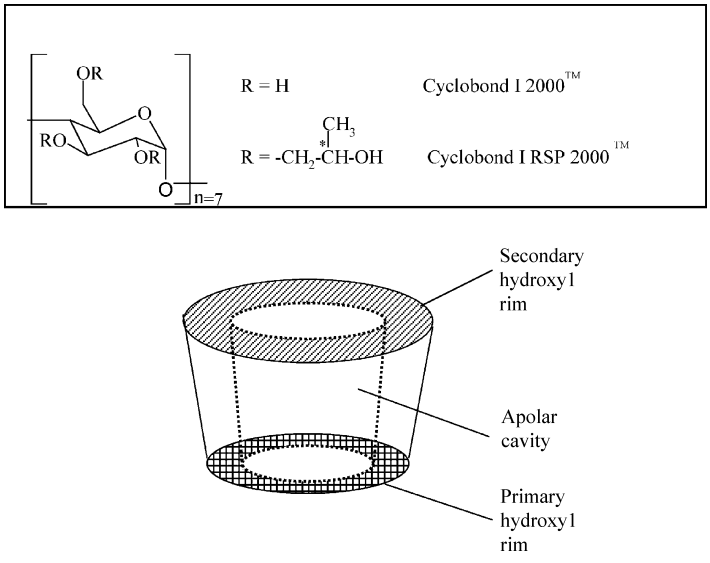


Figure 2. Chemical structures of cyclobond chiral stationary phases and ternary structure of a cyclodextrin.

polar organic mode to obtain the best separation, and to provide resolution for a large selection of molecules. Secondly, derivatized cyclodextrines, such as the new Cyclobond I 2000 RSP, present increased stability, reproducibility, and enantioselectivity regarding native β-cyclodextrin.[9]

In this paper, we described the results obtained with a native β-cyclodextrin and a (R,S) hydroxypropyl ether β-cyclodextrin as stationary phases. Limited data in literature are available on similar molecules.[10]

EXPERIMENTAL

Reagents and Materials

Compounds **1**–**3** (Figure 1) were prepared according to the same general synthetic pathway described previously,[7] leading to a mixture of the four diastereomers for tetralins **1**–**3**. The methanol and acetonitrile were HPLC grade obtained from Merck (Nogent sur Marne, France) or Baker (Noisy le Sec, France). All the mobile phases were filtered through a membrane (0.45 μm) and degassed with a Waters in-line degasser apparatus. The mobile phases used were A: methanol-acetonitrile:1/99; B: methanol-acetonitrile:2/98; C: methanol-acetonitrile:5/95; D: methanol-acetonitrile:10/90. Compounds were chromatographed by dissolving them in acetonitrile to a concentration of about 0.5 mM (which corresponds to

10 nmoles injected), and passed through a 0.45 μm membrane filter prior to loading the column.

Chromatography

Enantioselective separation was performed on a Cyclobond I 2000 (β-CD); 250 × 4.6 mm I.D.; 5 μm) and on a Cyclobond I 2000 RSP (R,S hydroxypropylether β-CD); 250 × 4.6 mm I.D.; 5 μm) (ASTEC, Whippany, NJ, USA) using a gradient Waters 600E metering pump model equipped with a Waters 996 photodiode array spectrophotometer. Chromatographic data were collected and processed on a Digital computer running with Millennium 2010. The column eluate was monitored at 200 and 210 nm. The sample loop was 20 μL (Rheodyne 7125 injector). Mixtures of methanol and acetonitrile, made isocratically, at various percentages, were used as mobile phases. The flow was $0.5\,mL \cdot min^{-1}$. The peak of the solvent front was considered to be equal to the dead time (t_o) and was taken from each particular run. It was about 3.80 min ($0.8\,mL \cdot min^{-1}$) for the Cyclobond I 2000 (β-CD) and 3.50 min ($0.8\,mL \cdot min^{-1}$) for the Cyclobond I 2000 RSP (R,S hydroxypropylether β-CD). Retention times were mean values of two replicate determinations. The retention factor k, was determined as $k = (t_R - t_0)/t_0$. The resolution was calculated as $R_s = [(1.18\ (t_{R2} - t_{R1}))/(\delta_1 + \delta_2)]$, where t_{Ri} and δ_i are the retention times and the half-widths of each enantiomer. R_s and α were determined from the two chromatograms of the two pure corresponding enantiomers. All separations were carried out at 25°C, unless noted otherwise, to determine the temperature dependence on the optical resolution.

RESULTS AND DISCUSSION

In this work, cyclodextrin stationary phase has been run in one solvent mode: the polar organic mode. The mobile phase contains polar constituents and doesn't include water. Here, the eluent is composed of a mixture of acetonitrile and methanol. We choose this mode because of shorter retention times and higher resolution usually obtained. In addition, this kind of solvent is easier to remove compared to reversed-phase.[11]

Effects of Mobile Phase on Retention and Stereoselectivity

Direct stereoselective separations were first performed on native β-cyclodextrin (Cyclobond I 2000) stationary phases. Retention times observed with methanol/acetonitrile 1/99 (eluent A) were very short ($t_R = 8$ min) and baseline resolution obtained ($R_s > 1.2$) was satisfactory (Table 1, Figure 3).

Table 1. Chromatographic parameters: retention factors (k), enantioselectivity factor (α) and resolution (R_s) on Cyclobond I 2000

Compound	Eluent	k_1	k_2	α	R_s	First eluted enantiomer[a]
1a *cis*	A	1.26	1.40	1.11	2.03	[−]
1b *trans*	A	1.18	1.35	1.04	1.59	[+]
2a *cis*	A	0.67	0.80	1.19	1.92	[−]
2b *trans*	A	0.59	0.67	1.13	1.32	[+]
3a *cis*	A	0.78	0.93	1.18	2.05	[−]
3b *trans*	A	0.68	0.78	1.14	1.60	[+]

nr: Not resolved; Concentration *ca* 0.50 mM;

[a]Injection of the pure isomer.

Eluents A: methanol-acetonitrile: 1/99; B: methanol-acetonitrile: 2/98; C: methanol-acetonitrile: 5/95; D: methanol-acetonitrile: 10/90.

The flow-rate was 0.8 mL · min^{-1}. The temperature was 25°C unless noted otherwise.

In order to improve separation, we investigated a new of type of stationary phase. Literature reports that a great number of compounds partially resolved on native β-CD can be separated on β-RSP-CD.[12,13] Results obtained are summarized in Table 2.

Retention and enantioselectivity are both influenced by the percentage of organic modifier used. As expected, it can be seen that increasing the amount of methanol from 1 to 10%, (eluent A to D) leads to a decrease of retention factors (k) and resolution (R_s). This trend is observed for all compounds, for both *cis* and *trans* enantiomers. This behaviour can be explained by the higher capacity of the organic modifier to displace the solute from the cyclodextrin cavity.[14] Same observations have been made for similar compounds in HPLC[15] and in CE.[16]

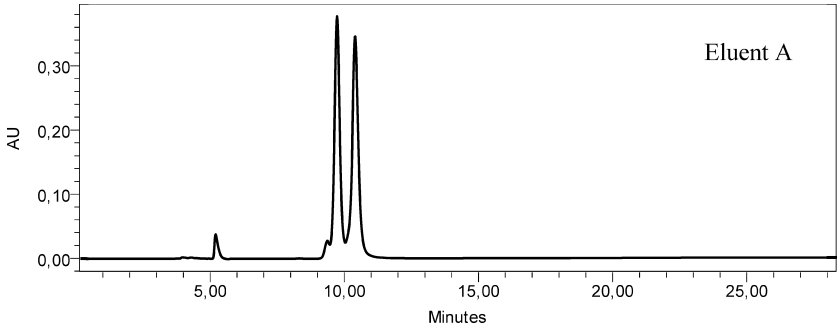


Figure 3. Typical chromatogram of compound **1a** (cis) obtained on Cyclobond I 2000 stationary phase, eluent A, flow rate 0.8 mL · min^{-1}, temperature 25°C.

Table 2. Chromatographic parameters: retention factors (k), enantioselectivity factor (α) and resolution (R_s) on Cyclobond I 2000 RSP

Compound	Eluent	k_1	k_2	α	R_s	First eluted enantiomer[a]
1a *cis*	A	1.09	1.30	1.19	2.28	[−]
	B	0.95	1.12	1.18	2.13	[−]
	C	0.70	0.80	1.14	0.85	[−]
	D	0.43	0.49	1.14	<0.50	[−]
1b *trans*	A	1.03	1.19	1.15	1.19	[+]
	B	0.86	1.00	1.16	1.79	[+]
	C	0.63	0.73	1.16	0.90	[+]
	D	0.39	0.45	1.15	<0.50	[+]
2a *cis*	A	0.73	0.98	1.34	2.82	[−]
	B	0.65	0.83	1.27	2.00	[−]
	C	0.51	0.63	1.23	1.47	[−]
	D	0.32	0.39	1.21	<0.50	[−]
2b *trans*	A	0.65	0.73	1.12	1.02	[+]
	B	0.57	0.65	1.13	1.00	[+]
	C	0.45	0.51	1.14	<0.50	[+]
	D	0.27	0.32	1.18	<0.50	[+]
3a *cis*	A	0.77	0.99	1.28	2.20	[−]
	B	0.64	0.84	1.32	2.58	[−]
	C	0.48	0.62	1.29	1.96	[−]
	D	0.29	0.38	1.30	<0.50	[−]
3b *trans*	A	0.68	0.77	1.13	1.00	[+]
	B	0.57	0.64	1.12	0.98	[+]
	C	0.43	0.48	1.13	0.86	[+]
	D	0.25	0.29	1.14	<0.50	[+]

(See legend Table 1).

Effects of Solute Structure on Retention and Stereoselectivity

Capacity factors (k) and resolution values (R_s) are approximately the same for the three *cis* compounds, except for compound **2a** (R = $-C_3H_5$), which seems to be better resolved. For example, with eluent A, R_s values were 2.28 for the tetralin **1a**, 2.82 for the tetralin **2a**, and 2.20 for the tetralin **3a**. *Trans* compounds don't present this trend (Table 2). The bulkiness of the enantiomeric pairs doesn't seem to play any role. On the other hand, *cis*-enantiomers are better resolved than *trans*-enantiomers (Table 2). Concerning capacity factors (k), they are almost the same for *cis* and *trans* enantiomers (Figure 4a and 4b). The *cis*-enantiomers seem to have stronger enantioselective interactions than *trans*-enantiomers. Those observations could be explained by several facts well demonstrated today. It is established that in

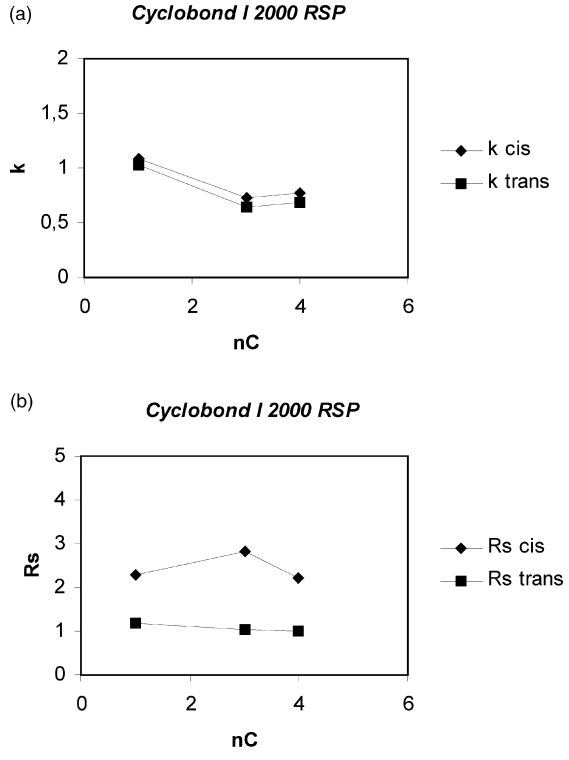


Figure 4. a) k vs. nC on Cyclobond I 2000 RSP stationary phase, flow rate $0.8\,\text{mL}\cdot\text{min}^{-1}$, temperature 25°C. b) R_s vs. nC on Cyclobond I 2000 RSP stationary phase, flow rate $0.8\,\text{mL}\cdot\text{min}^{-1}$, temperature 25°C.

organic polar mode inclusion complexation has a limited contribution. The enantioseparation is thought to occur via several interactions:[17] i) hydrophobic effects, which lead the apolar part of the solute to enter the cavity; ii) Van der Waals interactions; iii) hydrogen bonding between the polar part of the solute and the hydrophilic surface (secondary hydroxyl groups) of the CD; iv) steric effects (shape and size of the solute). The chiral center of the solute must be near and interacts with the rim of the cyclodextrin. Here, chiral separation is facilitated by hydroxypropylating the hydroxyl group, which certainly extends the cavity entrance.

Enantiomer Elution Order

In both β-cyclodextrin (Cyclobond I 2000) and (R,S) hydroxypropyl-β-cyclodextrin (Cyclobond I 2000 RSP) for trisubstituted tetralins **1a**, **2a**, and **3a** (*cis*), the levorotary (−) enantiomer elutes first, followed by the dextrorotary (+)

enantiomer; for **1b**, **2b**, and **3b** (*trans*) tetralin, it is the dextrorotary (+) enantiomer which elutes first (Tables 1 and 2). The enantiomer elution order of compound **3** is shown as an example in Figure 5 (Cyclobond I 2000 RSP, eluent B). This behaviour is obtained whatever eluent is used.

Limit of Detection, Limit of Quantification, and Determination of Enantiomeric Purity

After optimization, the chiral purity of each compound has been evaluated using the Cyclobond I 2000 and Cyclobond I 2000 RSP, with a mobile phase composed of methanol/acetonitrile 1/99 (eluent A). The chiral assay for each enantiomer was validated for detection and quantification limits. On Cyclobond I 2000, limit of detection (LOD) calculated at a signal-to-noise ratio equal to 3 was between 5.56 and 84.80 $\mu mol \cdot L^{-1}$, corresponding to 0.11 and 0.42% minor enantiomer for a major enantiomer target

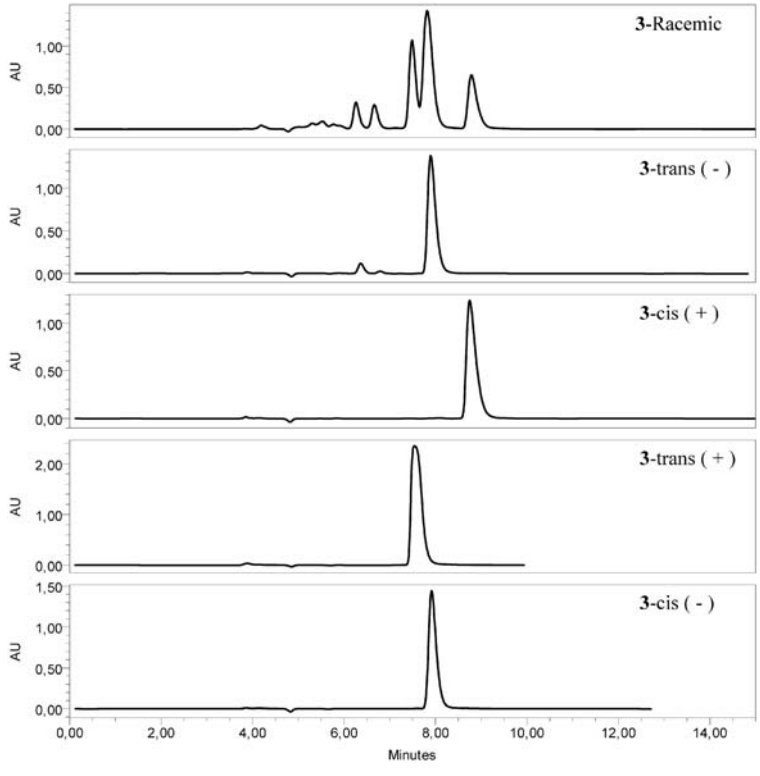


Figure 5. Stacked chromatograms of compound **3** (racemic) and its four pure enantiomers on Cyclobond I 2000 RSP stationary phase, eluent B, flow rate 0.8 $mL \cdot min^{-1}$, temperature 25°C.

Table 3. Limit of detection, limit of quantification and enantiomeric purity of compounds **1–3** on Cyclobond I 2000, eluent A

Compound	Enantiomer (%)	LOD (mM)	C limit (%)	LOQ (mM)	C limit (%)	Enantiomeric purity
1a	(−)[a]	0.61	$3.06 \cdot 10^{-5}$	0.020	$10.20 \cdot 10^{-5}$	>99.46
	(+)	0.54	$2.72 \cdot 10^{-5}$	0.018	$9.09 \cdot 10^{-5}$	>99.39
1b	(+)[a]	0.45	$2.26 \cdot 10^{-5}$	0.015	$7.58 \cdot 10^{-5}$	>99.34
	(−)	0.66	$3.30 \cdot 10^{-5}$	0.022	$11.00 \cdot 10^{-5}$	>99.55
2a	(−)[a]	0.20	$1.00 \cdot 10^{-5}$	0.007	$3.36 \cdot 10^{-5}$	>99.58
	(+)	0.42	$8.48 \cdot 10^{-5}$	0.056	$8.30 \cdot 10^{-5}$	>99.80
2b	(+)[a]	0.11	$0.56 \cdot 10^{-5}$	0.004	$1.85 \cdot 10^{-5}$	>99.68
	(−)	0.32	$1.60 \cdot 10^{-5}$	0.011	$5.33 \cdot 10^{-5}$	>99.89
3a	(−)[a]	0.14	$1.68 \cdot 10^{-5}$	0.011	$5.64 \cdot 10^{-5}$	>99.63
	(+)	0.37	$1.84 \cdot 10^{-5}$	0.012	$6.13 \cdot 10^{-5}$	>99.86
3b	(+)[a]	0.26	$1.31 \cdot 10^{-5}$	0.009	$4.37 \cdot 10^{-5}$	>99.54
	(−)	0.46	$2.31 \cdot 10^{-5}$	0.015	$7.71 \cdot 10^{-5}$	>99.74

[a]First eluted enantiomer.

concentration of approximately 0.50 mM corresponding to 100%. Higher results (Table 3) are obtained on Cyclobond I 2000 RSP; limit of detection (LOD) was between 1.27 and 6.16 $mmol \cdot L^{-1}$ corresponding to 0.25 and 1.23% minor enantiomer, for a major enantiomer target concentration of 0.50 mM corresponding to 100%. The chiral purity was determined by the relative percentages of peaks areas (Table 4).

Table 4. Limit of detection, limit of quantification and enantiomeric purity of compounds **1–3** on Cyclobond I 2000 RSP, eluent A

Compound	Enantiomer (%)	LOD (mM)	C limit (%)	LOQ (mM)	C limit (%)	Enantiomeric purity
1a	(−)[a]	1.28	$3.06 \cdot 10^{-3}$	4.20	0.021	>98.82
	(+)	1.18	$2.72 \cdot 10^{-3}$	4.00	0.020	>98.72
1b	(+)[a]	0.78	$2.26 \cdot 10^{-3}$	2.60	0.013	>99.04
	(−)	0.96	$3.30 \cdot 10^{-3}$	3.20	0.016	>99.22
2a	(−)[a]	0.68	$3.40 \cdot 10^{-3}$	2.20	0.011	>99.20
	(+)	0.80	$3.98 \cdot 10^{-3}$	2.65	0.013	>99.32
2b	(+)[a]	0.25	$1.27 \cdot 10^{-3}$	0.84	0.004	>99.15
	(−)	0.85	$4.27 \cdot 10^{-3}$	2.80	0.014	>99.75
3a	(−)[a]	0.75	$3.76 \cdot 10^{-3}$	2.50	0.012	>98.77
	(+)	1.23	$6.16 \cdot 10^{-3}$	4.00	0.020	>99.25
3b	(+)[a]	0.62	$3 \cdot 10 \cdot 10^{-3}$	2.06	0.010	>98.96
	(−)	1.04	$5.20 \cdot 10^{-3}$	3.46	0.017	>99.38

[a]First eluted enantiomer.

CONCLUSION

It can be observed that *cis*-enantiomers have shorter retention times and are better resolved than *trans*-enantiomers on the two cyclobond stationary phases. In conclusion, those CSPs seem to be better suited for separation of enantiomers and, particularly *cis*-enantiomers, than of diastereoisomers. This behaviour is not reported usually for this kind of CSP.

Moreover, greater values of LOD and LOQ are obtained on Cyclobond I 2000 and are under 0.66 and 0.022%, respectively.

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