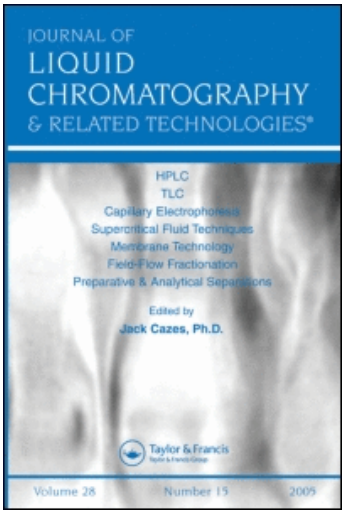

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TLC AND POLARIMETRIC INVESTIGATION OF THE OSCILLATORY *IN VITRO* CHIRAL CONVERSION OF *R*-β-HYDROXYBUTYRIC ACID

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TLC AND POLARIMETRIC INVESTIGATION OF THE OSCILLATORY *IN VITRO* CHIRAL CONVERSION OF *R*-*β*-HYDROXYBUTYRIC ACID

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□ *Earlier we have shown that chiral aliphatic hydroxy acids, i.e.,* L*-lactic acid,* R-α*-hydroxybutyric acid, and* S-α*-hydroxybutyric acid, can undergo oscillatory* in vitro *chiral conversion, exhibiting oscillatory changes in the chromatographic retention parameter* (R_F) *and the specific rotation* ($[\alpha]_D$) *for the periods of two or even many more weeks after preparation of the solutions in aqueous ethanol. As the phenomenon of spontaneous oscillatory chiral conversion with low molecular weight carboxylic acids dissolved in the abiotic aqueous media seems to be of a relatively general nature, it made us curious to examine steric limitations of this process. One essential question that we posed in this study regards the impact of the distance between the carboxylic group of the acid and the asymmetry center in the chiral molecule on its ability to undergo chiral conversion. With the earlier examined lactic acid and two* α*-hydroxybutyric acids, the hydroxyl group was placed in position* α*, hence the asymmetry center was directly neighboring the carboxylic group. It was the aim of this study, to examine one more low molecular weight hydroxy acid with the hydroxyl group placed in position* β*, hence with the asymmetry center separated by one methylene unit from carboxyl functionality acting as spacer. To this effect, we selected* R-β*-hydroxybutyric acid and investigated its ability to undergo a spontaneous oscillatory* in vitro *chiral conversion in the abiotic aqueous medium, for this purpose using thin layer chromatography and polarimetry as the best suiting analytical techniques. It was experimentally established that* R-β*-hydroxybutyric acid – similar to the earlier scrutinized* α*-hydroxy acids – can undergo the oscillatory chiral conversion and the methylene spacer between the carboxyl and the hydroxyl functionality cannot prevent this particular compound from undergoing steric conversion.*

Keywords chiral TLC, enantioseparation of *β*-hydroxybutyric acid, oscillatory chiral conversion, polarimetry

INTRODUCTION

The remarkable phenomenon of spontaneous *in vitro* oscillatory chiral conversion of selected profens, amino acids, and hydroxy acids has been in

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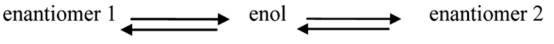
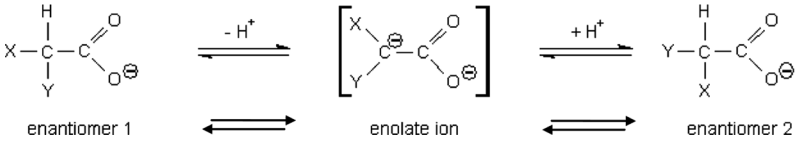
the focus of our attention for some time now. In papers,[1–3] we reported on the chiral conversion of *S*-(+)-ibuprofen, *S*-(+)-naproxen, *S,R*-(±)-2-phenylpropionic acid, *S*-(+)-flurbiprofen, *R*-(-)-flurbiprofen, and *S,R*-(±)-ketoprofen. In papers,[4–6] we reported on the analogous behavior of *L*-alanine, *L*-α-phenylalanine, and *L*-tyrosine.[4–6] In papers,[7,8] the oscillatory chiral conversion of the selected hydroxy acids, i.e., *L*-lactic acid, *R*-α-hydroxybutyric acid, *S*-α-hydroxybutyric acid, *R*-mandelic acid, and *S*-mandelic acid, was confirmed. Most of the reported cases of chiral conversion took place with acid samples dissolved in aqueous ethanol.

The general scheme of the discussed chiral conversion can simply be given by Eq. (1).



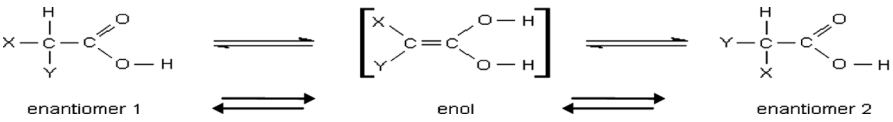
(1)

If we, however, consider chiral conversion of the discussed carboxylic acids in aqueous solutions more closely, then Eq. (1) can be replaced by Eq. (1a):[9]



(1a)

In the anhydrous media and in the presence of the trace amounts of water, the probable mechanism of chiral conversion can be given by Eq. (1b):[10]



(1b)

where X: $-NH_2$, $-OH$, $-Ar$, etc., and Y: $-R$, etc.

Owing to the results of our earlier studies, nowadays the phenomenon of the spontaneous oscillatory *in vitro* chiral conversion of the low-molecular weight carboxylic acids seems relatively common, which made us curious to know the scope of its universality and the structural limits thereof. All cases of the oscillatory chiral conversion so far described in papers,[1–8] referred to carboxylic acids with the asymmetry center in position α, hence neighboring carboxylic functionality. It was the aim of this study, to examine one more low molecular weight hydroxy acid, this time with the hydroxyl group placed in position *β*, hence with the asymmetry center separated with one methylene unit from carboxyl functionality acting as spacer. To this effect, we selected *R*-*β*-hydroxybutyric acid and

investigated its ability to undergo spontaneous oscillatory *in vitro* chiral conversion in the abiotic aqueous medium. We were interested to check, if an extended distance between carboxylic functionality and the asymmetry center can hamper the discussed oscillatory process.

EXPERIMENTAL

R-β-Hydroxybutyric Acid

The structural formula of *R*-β-hydroxybutyric acid is given in Fig. 1. In our study, we used the samples of *R*-β-hydroxybutyric acid, manufactured by Fluka (Buchs, Switzerland; cat. # 54920).

For the TLC experiment, we used a solution of *R*-β-hydroxybutyric acid in ethanol – water (7:3, *v*/*v*), its concentration equal to $50\,g\,L^{-1}$ ($4.80 \times 10^{-1}\,mol\,L^{-1}$). For the polarimetric experiment, we used a solution of *R*-β-hydroxybutyric acid in ethanol – water (7:3, *v*/*v*), its concentration equal to $9.3\,g\,L^{-1}$ ($8.93 \times 10^{-2}\,mol\,L^{-1}$).

Methanol and dioxane used in our experiments were of HPLC purity grade (Merck KGaA, Darmstadt, Germany), ethanol was of analytical purity grade (POCh, Gliwice, Poland), and water was double distilled and deionized in our laboratory.

Thin-Layer Chromatography (TLC)

Development of the chromatograms was carried out on commercial chromatographic plates precoated with 0.25 mm thick silica gel layer (20 cm × 20 cm, manufactured by Merck; cat # 1.05715). Prior to the development, all plates were predeveloped with methanol – water (9:1, *v*/*v*) and dried at ambient temperature for 3 h, in order to wash out any possible impurities adsorbed on the solid silica gel layer in the course of the plate storage. Then the plates were impregnated by dipping them for 2s in the $5.01 \times 10^{-2}\,mol\,L^{-1}$ water – methanol (4:6, *v*/*v*) solution of $Cu(CH_3COO)_2$ (analytical purity grade, manufactured by POCh).

Thin-layer chromatographic development of the *R*-β-hydroxybutyric acid samples stored for certain amount of time was carried out at $22 \pm 1°C$, using dioxane – water (9:1, *v*/*v*) as mobile phase.

The samples of *R*-β-hydroxybutyric acid were spotted on to the chromatographic plates with use of an AS 30 model automatic applicator

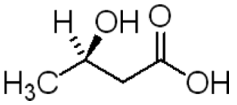


FIGURE 1 The chemical structure of *R*-β-hydroxybutyric acid.

(Desaga, Heidelberg, Germany). The 3 μL aliquots of the samples were spotted 1 cm above the lower edge of the plate, eight samples per one plate, in the 2 cm distance from one another. The development distance was 8.5 cm and, thereafter, the plates were dried for 3 h at 22 ± 1°C. Finally, each lane was densitometrically scanned in the direction of the development and then the plates were sprayed with 5% (*v*/*v*) conc. H_2SO_4 in ethanol, and heated at 100–110°C for ca. 10 min, to reveal the characteristic black spots of the hydroxy acid on a white background. This experiment was carried out for 4 days and the chromatograms of the gradually ageing *R*-*β*-hydroxybutyric acid solutions were developed in one day intervals.

The anticipated mechanism of enantioseparation of the *R* and *S*-*β*-hydroxybutyric acid antimers (due to the chiral conversion, the latter antimer is spontaneously generated in the sample) was through formation of the bidentate complexes between copper(II) cations deposited on the silica gel surface on the one hand and each of the two *β*-hydroxybutyric acids on the other (with the two different stability constants, K_1 and K_2). This mechanism can be summarized by the following scheme:

$$Cu^{2+} + 2\ R\text{-HBA} \rightleftharpoons [Cu(R\text{-HBA})_2]^{2+};\ K_1 \quad (2a)$$

$$Cu^{2+} + 2\ S\text{-HBA} \rightleftharpoons [Cu(S\text{-HBA})_2]^{2+};\ K_2 \quad (2b)$$

where *R*-HBA and *S*-HBA hold for *R*-*β*-hydroxybutyric acid and *S*-*β*-hydroxybutyric acid, respectively. The physicochemical condition of the enantioseparation is given by the inequality $K_1 \neq K_2$.

Densitograms were acquired with the aid of a CD 60 model densitometer (Desaga) equipped with the Windows-compatible ProQuant data acquisition and processing program. For scanning, the wavelength $\lambda = 326$ nm was selected from the mercury lamp and the geometry of the scanning beam was equal to 0.1 mm × 1.0 mm. The R_F values of the analytes were calculated from their respective concentration profiles.

The chromatograms were also scanned in visible light by use of a Chromimage flat-bed videoscanner (AR2i, Le Plessis Robinson, France), to save the pictures of the whole chromatograms and those of the individual chromatographic spots.

Polarimetry

Measurements of specific rotation ($[\alpha]_D$) of the investigated samples were carried out in the continuous manner. Continuous registration was carried out using a 341 model Perkin-Elmer precision polarimeter. Measurements were carried out for almost 14 days (331 h) at 22°C and

the wavelength used was 589 nm, which corresponds to the sodium D line. The cell length and volume were equal to, respectively, 1 cm and 3 mL. Specific rotation ($[\alpha]_D$) of the investigated samples was calculated from the below given equation:

$$[\alpha]_D = 100\alpha/cd \tag{3}$$

where α holds for the measured rotation angle (in angle degrees), D holds for the applied wavelength, λ (equal to 589 nm), c denotes concentration of the investigated compound in g 100 mL^{-1} solution, and d is the optical path length in dm.

As reported in the literature, the specific rotation ($[\alpha]_D$) of *R*-*β*-hydroxybutyric acid measured at 25°C in the ethanol solution equals to −17.5°[11] and in the aqueous solution it is equal to −24.7°.[12] In our own study, specific rotation of the freshly prepared solution of *R*-*β*-hydroxybutyric acid in aqueous ethanol at 22°C was equal to −16.5°.

RESULTS AND DISCUSSION

From the experience gained from our earlier investigations on the spontaneous oscillatory *in vitro* chiral conversion of the low molecular weight carboxylic acids dissolved in the abiotic aqueous media it shows that one of the best performing analytical techniques, able to trace steric lability of chiral compounds is thin layer chromatography. This statement is founded upon abundant experimental evidence presented in papers,[1–8] despite the fact that an important and up to the date reference book[13] focused on tracing steric conversion of multiple compound classes mentions exclusively high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE), as the adequate measuring techniques, and it fails to draw the researchers' attention to the advantages of a simple yet very efficient planar chromatography.

There are two major advantages of thin layer chromatography as a working tool in studying steric lability of chiral compounds. Due to these advantages, in certain cases TLC outperforms HPLC, GC, and CE, and in most cases, its performance is equivalent to those of the instrumental column techniques.

The first advantage of TLC is its ability to provide the two dimensional enantioseparations, which is inherent of the planar chromatography system alone, and it certainly cannot be obtained with any column chromatographic technique. Due to the fact that enantioseparations still remain one of the most sensitive separation tasks, 2D enantioseparations (performed either in 1D, or 2D development mode) certainly are a valuable bonus and this issue was extensively discussed in papers.[14,15]

The second advantage of TLC is that the enantioseparated antimers remain preserved on the chromatographic plate and can be examined off-line by different instrumental analytical techniques (e.g., by UV and MS spectrometry applied *in situ*).

Another measuring technique very important for chiral research and traditionally used in this area is polarimetry. For the above reasons, the results presented in this study originate from TLC and polarimetry.

Thin-Layer Chromatography (TLC)

There were two goals of the research presented in this paper. The first one was to provide experimental evidence that *R*-*β*-hydroxybutyric acid can undergo a spontaneous oscillatory chiral conversion upon dissolution in ethanol – water, followed by storage of the obtained solution for a certain amount of time at ambient temperature. The second goal was to enantioseparate two antipodes of *β*-hydroxybutyric acid, obtained as an outcome of the oscillatory chiral conversion.

To this effect, we first prepared a solution of the optically pure *R*-*β*-hydroxybutyric acid in ethanol – water and stored it for the period of four days. After four days, the stored sample was enantioseparated, as earlier described in Experimental, and the obtained chromatograms were densitometrically scanned. Up to our best knowledge, this was the first attempt to enantioseparate *β*-hydroxybutyric acid by means of TLC.

In Fig. 2, we showed four videoscans of the chromatograms visualized by spraying them with the sulphuric acid solution. Each chromatogram represents the investigated sample after a different storage period. In the first three chromatograms, i.e., those obtained for the *R*-*β*-hydroxybutyric acid solution stored for 1, 20, and 47 hours, a single chromatographic spot is visible. In the fourth chromatogram valid for the sample stored for 69 hours, two chromatographic spots appear namely the left-handed and the right-handed one. Thus, from Fig. 2d it can be deduced, that after 69 h storage period chiral conversion of the *R* compound yielded in the mixture quantitatively close to the racemate, which enabled good visualization of the enantioseparation effect. This result can even better be seen from Fig. 3, which represents the 3D picture of the enantioseparated *β*-hydroxybutyric acid. Moreover, from Figs. 2a-c, it can be deduced that the left-handed spot visible on these videoscans (and on the 3D chromatogram) represents enantiomer *R*, and the right-handed spot holds for enantiomer *S*. The separation outcome shown in Figs. 2 and 3 is convincing enough to prove that in spite of the applied 1D development mode, the obtained enantioseparation turns up two-dimensional. Combining our earlier experience[14–16] with the present one it can be concluded that Cu(II) contributes to vertical enantioseparation (i.e., to that in the direction of the

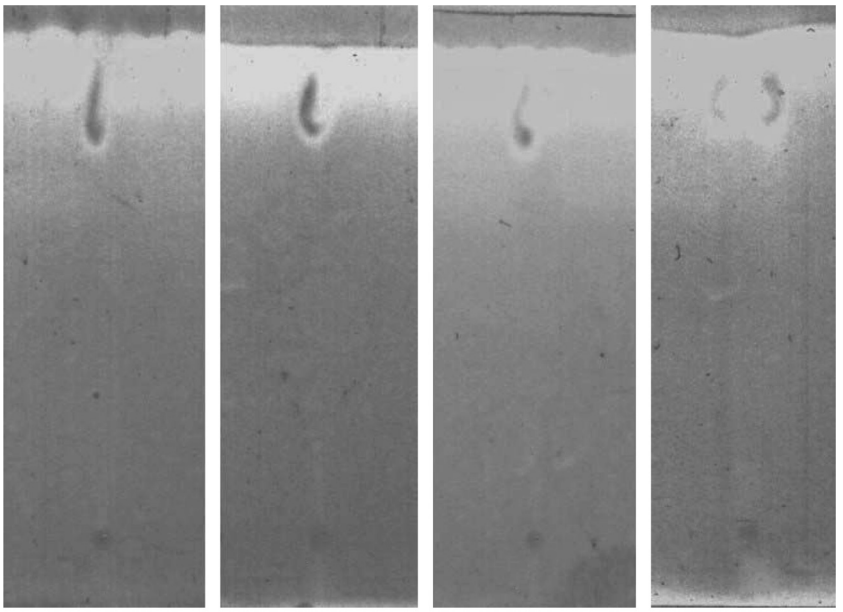


FIGURE 2 Videoscans of the chromatograms obtained for *R*-*β*-hydroxybutyric acid dissolved in ethanol – water 7:3 (*v*/*v*) and stored in the solution for (a) 1 h, (b) 20 h, (c) 47 h, and (d) 69 h.

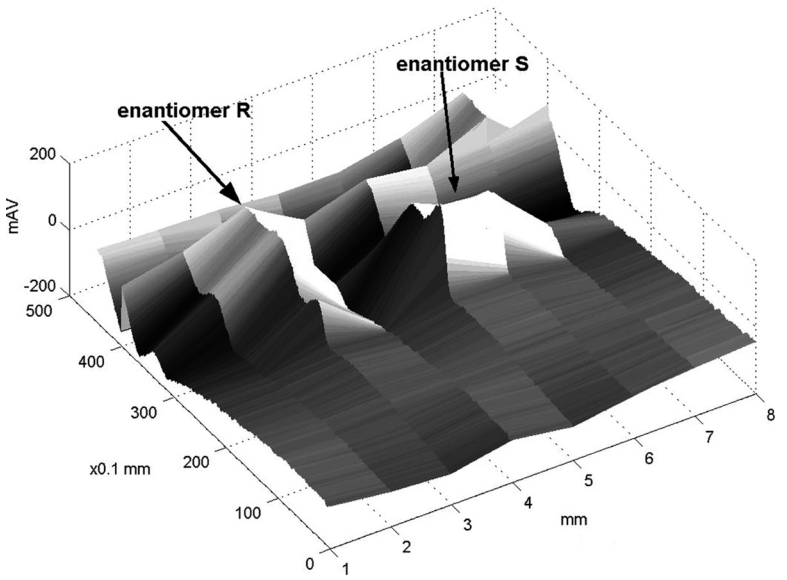


FIGURE 3 Three-dimensional representation of the chromatogram of *β*-hydroxybutyric acid after development of *R*-*β*-hydroxybutyric acid solution stored for 69 hours. Solution was in ethanol – water 7:3 (*v*/*v*) and stored at 22 ± 1°C. The 3D picture was based on densitometric scans taken at 1-mm intervals.

mobile phase flow), and microcrystalline silica gel is responsible for horizontal enantioseparation (i.e., that perpendicular to the former one).

In Figs. 4a,b, we presented densitograms of *R*-*β*-hydroxybutyric acid and *S*-*β*-hydroxybutyric acid, scanned in a parallel way along the two different tracks, in order to better visualize positions of their respective concentration maxima. From Fig. 4, it can be seen that the retardation factors (i.e., the R_F values) of the two antimers substantially differ. R_F is valid for the *R* species equals 0.86, while that valid for the *S* species equals to 0.78 (so the difference between these two considerably surpasses the experimental error of the individual R_F values equal to ± 0.02).

Polarimetry

The main goal of polarimetric experiment was to collect evidence on the occurrence of the oscillatory chiral conversion of *β*-hydroxybutyric acid. To this effect, we have for the first time used in our studies polarimeter with

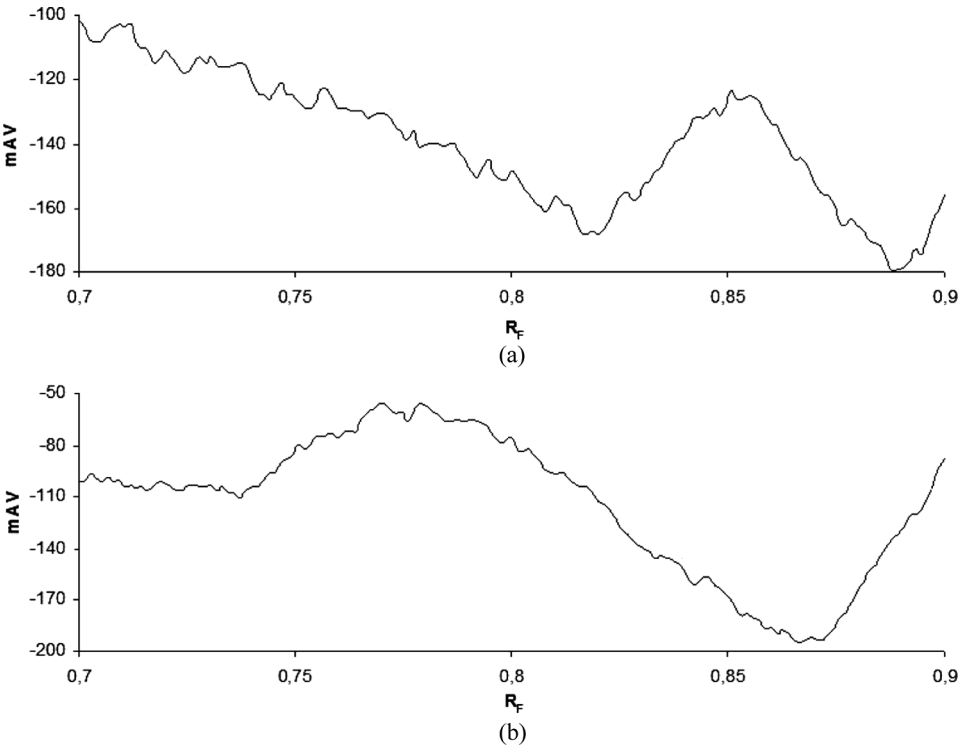


FIGURE 4 Densitometrically scanned concentration profiles of (a) *R*-*β*-hydroxybutyric acid and (b) *S*-*β*-hydroxybutyric acid. Because of the 2D enantioseparation of the *β*-hydroxybutyric acid antimers in the 1D development mode, the demonstrated concentration profiles represent densitometric scans for the tracks where the *R* and the *S* species obtain their respective maxima.

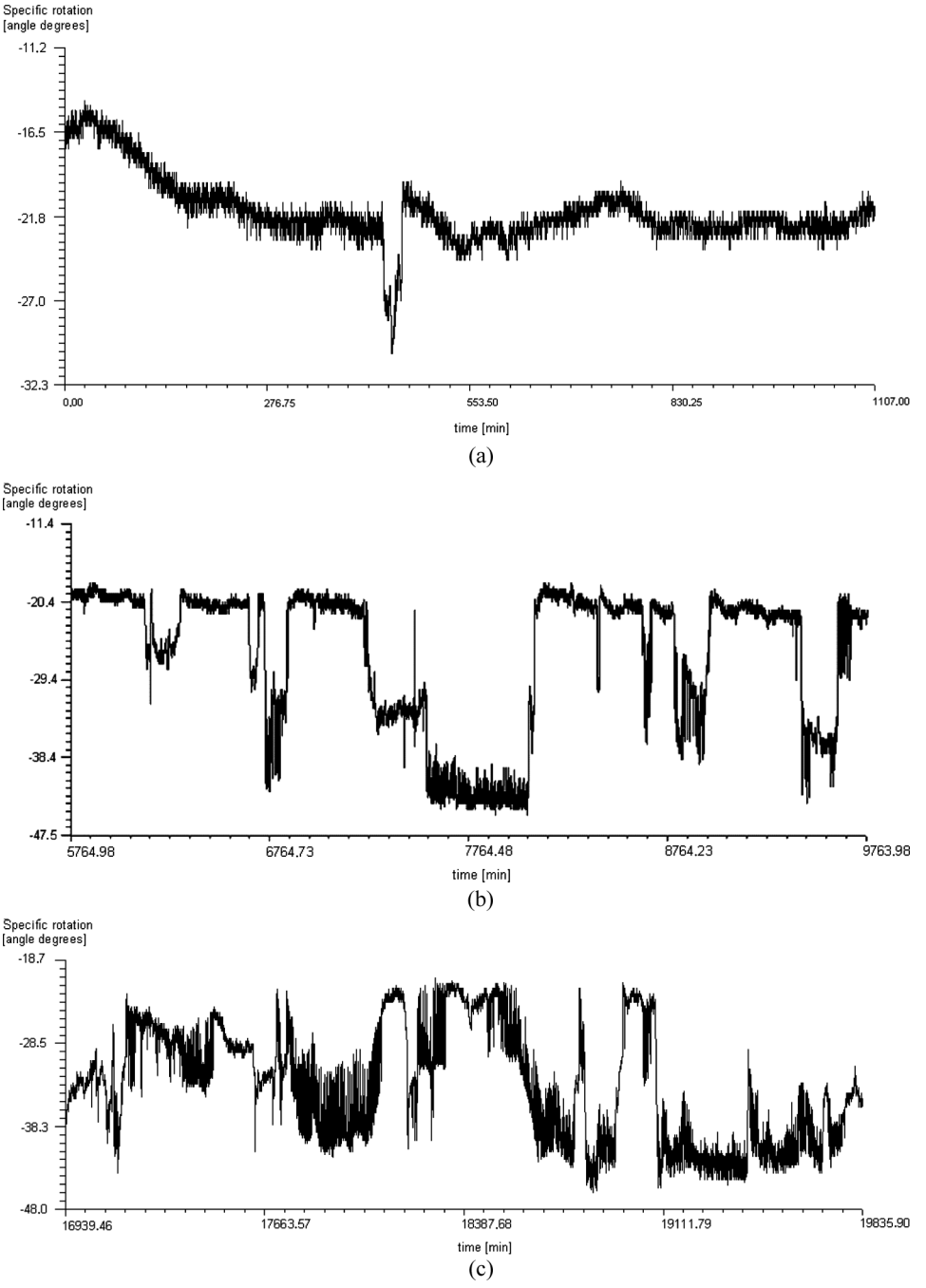


FIGURE 5 Continuous polarimetric registration of specific rotation ($[\alpha]_D$) in the function of time for *R*-*β*-hydroxybutyric acid dissolved in ethanol–water, 7:3 (v/v) and stored (a) from 0 to 19 h, (b) from 96 to 163 h, and (c) from 282 to 331 h at $22 \pm 1°C$.

continuous registration. The obtained results are shown in Fig. 5. From the plots shown in this figure it clearly comes out that with β-hydroxybutyric acid, the observed changes of specific rotation ($[\alpha]_D$) are considerable and the amplitude can embrace even 20 angle degrees. This result is a convincing polarimetric proof of the occurrence of the oscillatory chiral conversion with the investigated carboxylic species.

Further, it has to be said that the polarimetric results witness an enhanced change of the specific retention values after a more stable period of the several initiatory days. Namely, much more vigorous oscillations can be seen in Figs. 5b and 5c, than in Fig. 5a. This observation coincides well with the thin-layer chromatographic results and more specifically, with the fact that the two β-hydroxybutyric acid antimers were first visualized for the sample stored for 69 hours (as shown in Fig. 2d).

CONCLUSIONS

It can be concluded that the 2D thin-layer chromatographic enantioseparation of the two β-hydroxybutyric acids in the applied 1D chromatographic development mode is possible. In our study, this enantioseparation was achieved owing to the joint effect of copper(II) cation (which acts as central ion for the bidentate complexes involving the two enantioseparated antimers) and to the microcrystalline chirality of silica gel. Further – based on our earlier experience – we believe that copper(II) cation is responsible for the vertical separation of the two investigated β-hydroxybutyric acid antimers (which is expressed by the two different R_F values for the two maxima of the respective concentration profiles, as shown in Fig. 4), whereas microcrystalline chirality of silica gel is responsible for the horizontal enantioseparation (as presented the best in Fig. 2d). Thus, TLC has provided direct proof that *R*-β-hydroxybutyric acid undergoes spontaneous chiral conversion, when dissolved in aqueous ethanol and stored for certain period of time.

Polarimetrically, we confirmed that – similar to the other previously investigated chiral carboxylic acids – spontaneous chiral conversion of β-hydroxybutyric acid is oscillatory in nature.

ACKNOWLEDGMENT

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