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Review

On the Mechanisms of Enantiomer Separations by Chiral Thin-Layer Chromatography on Silica Gel, and Implications when Densitometric Detection is used. A Mini Review

M. SAJEWICZ AND T. KOWALSKA*

Institute of Chemistry, Silesian University,
9 Szkolna Street, 40-006 Katowice, Poland
E-mail: kowalska@us.edu.pl

Summary. Enantiomer separations have been one of the most important and, simultaneously, one of the most difficult to accomplish analytical (and technological) tasks, present at the top of separation scientists' agenda since the early sixties of the last century. Awareness of their importance has been awakened by an infamous case of the racemic drug thalidomide, a widely advertised sedative drug which had unexpected teratogenic activity in pregnant women that resulted in thousands of 'flipper babies' born in the late fifties and the early sixties in many countries around the world. Since that time, separation scientists have developed numerous methods for enantiomer separation, basically by use of gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE). In this respect, planar chromatography has remained to a large extent an undervalued enantiomer separation technique, despite separation performance sufficient to separate a pair of enantiomers. The large number of GC, HPLC, and CE enantiomer separation strategies and methods developed are evidence that – once confronted with this particular and no doubt very important challenge – instrumental chromatographic techniques have lost if not face, then, to a large extent, their reputation as robust, universal, and efficient separation tools. In these circumstances, planar chromatography on silica gel seems a very promising and tempting alternative, basically because of the advantageous properties of microcrystalline silica gel and the 2D effective diffusion available only in planar chromatographic mode. Enhancement of the enantiomer separating power of the silica gel by simple mechanical impregnation with a properly chosen chiral selector, and additional coupling of this with efficient instrumental detection (e.g. densitometric, DAD, or mass spectrometric) can yield in a simple, robust, and universal tool for separation of enantiomers comparable with the long-established chromatographic enantiomer-separation techniques. In this mini review, favourable preconditions for silica-gel-based planar chromatographic separation of enantiomers which can elevate planar chromatography to the status of leading tool for separation of enantiomers are discussed. Further improvements which can enhance the enantiomer separation performance of chiral planar chromatography are also indicated.

Key Words: 2D TLC enantiomer separation, 2D TLC effective diffusion, silica gel chirality, TLC enantioselectors

Background

Universality has always been one of the strongest, most vital, and most attractive assets of chromatographic separation techniques. Vast numbers of the separation tasks can be solved successfully by use of the same GC or HPLC column, carrier gas (GC) or mixed mobile phase (LC). This universality of chromatographic techniques has been severely challenged by the need to provide robust methods for enantiomer separation, which came as an urgent demand from pharmacology and other life sciences in the early sixties of the past century after the infamous teratogenic disaster caused by the racemic drug thalidomide, particularly advertised to pregnant women as a safe sedative. The pioneering role in direct enantiomer separations by GC was played by Gil-Av and his research group from the Weizmann Institute of Science in Rehovot, Israel [1–3], who first performed analytical separations of selected amino acid antimers derivatized to the respective esters to enhance their volatility. Later it became clear that HPLC would become the technique of choice for direct enantiomer separations, basically because of the limited volatility of a vast number of the enantiomers of interest.

In HPLC, direct enantiomer separations can be achieved either by use of chiral stationary phases (CSP) or chiral modifiers of mobile phases. For several reasons (important economic reasons included), application of CSPs has proved a more practical option than that of chiral mobile phases, although both strategies still co-exist in a parallel. Among pioneers of the rationally designed CSPs, Pirkle [5–8] and Welch [9, 10] should certainly be mentioned. Separation of enantiomers by HPLC soon proved to be a fairly complicated matter. It became evident that chiral selectors can either be chemically bonded with a solid support or can be used to coat the matrix owing to physical interactions. Naturally chiral carbohydrate matrices (preferably cellulose and amylose) have the serious disadvantages of swelling in some mobile phases, resulting in harmful back pressure in chromatographic columns. Alternative physical coating of solid supports obviously resulted in column bleed and, hence, irreproducibility of the results obtained. Many synthetic chiral stationary phases have proved brittle and easily crushed by the mobile phase pressure, which again generated harmful back pressure. As a result, chiral HPLC has lost much of its renowned universality as a chromatographic technique and has rather closely approached the point at which the tailor-made stationary phases are the best solutions for individual enantiomer separations. This is an understandably inconvenient situation which results in substantial cost, because of the need for a wide variety of chiral HPLC columns in any laboratory, without any guarantee of separation success.

A clear message about different complications and hardships related to successful enantiomer separations by GC, HPLC, and CE can be found between the lines of Wolf's monograph [11]. Similarly, another involuntary message about underestimation of the substantial potential of TLC for successful enantiomer separations can be found between the lines of the monograph by Kowalska and Sherma [12]. In these circumstances, it is the objective of this mini review to emphasize advantages of chiral planar chromatography for direct enantiomer separations, to point out the favourable preconditions thereof, and in that way to articulate the need for its further development.

The Advantage of Silica Gel as Solid Phase in Chiral TLC

In chromatographic circles it used to be believed that the silica gel used in planar and column liquid chromatography was amorphous. In contrast, the crystals of silicon dioxide (also known as quartz or rock crystal) used in optics for polarization of light are, chemically, the same material as the silica gel used in chromatography. Obviously, preparation of silica gel for chromatography is carried out in such a way that its precipitation is rapid and the particles obtained are, deliberately, minuscule. In other words, one cannot expect rock crystals on the surface of chromatographic plates, yet rapid precipitation does not necessarily need to be synonymous with preparation of an amorphous solid.

For this reason we decided to investigate more deeply the nature of the binder-free silica gel used in planar chromatography, by means of circular dichroism (CD) spectroscopy. Our results, reported elsewhere [13] and illustrated in *Fig. 1*, confirmed the microcrystalline nature of the material

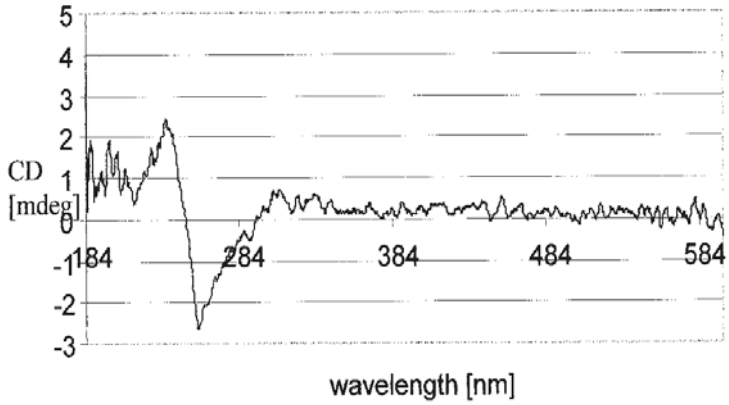


Fig. 1. CD spectrum of binder-free silica gel for TLC, as a Nujol suspension [13]

investigated. Obviously, one cannot expect precipitation of silica gel to be stereospecific, although it seems quite probable that the precipitate comprises right-handed and left-handed particles (which might give the false impression that silica gel is amorphous), with predominance of one chiral species over its mirror image. This predominance of one chiral microcrystalline species can prove invaluable in developing a powerful chiral planar chromatographic method.

The Advantage of 2D Effective Diffusion in Chiral TLC

Effective diffusion in planar chromatographic systems is two-dimensional, occurring both in the direction of mobile phase flow and in the direction perpendicular to it. Because of spatial limitations posed by chromatographic column walls, in column chromatography effective diffusion is one-dimensional, taking place in the direction of the mobile phase flow only. Effective diffusion is generally harmful to the separation process, because it results in band broadening and, therefore, reduction of the system's theoretical plate number and, hence, in overall deterioration of its resolving power. The phenomenon of 2D effective diffusion in planar chromatography is illustrated in *Fig. 2*.

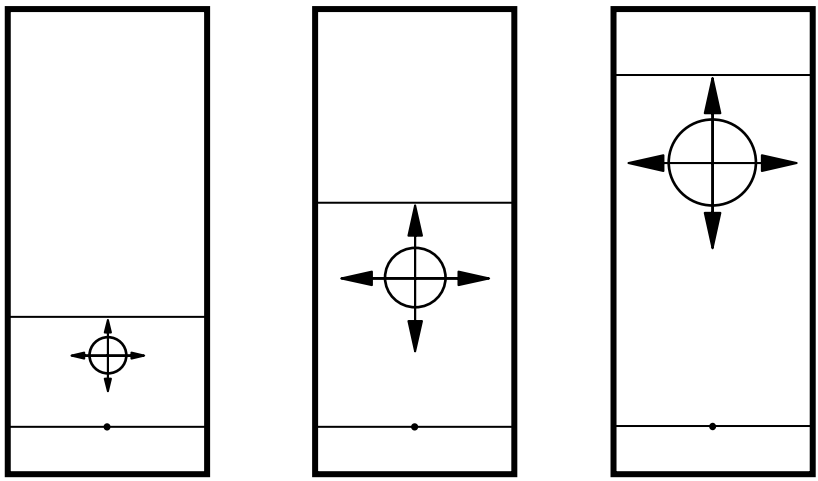


Fig. 2. Schematic representation of 2D effective diffusion in planar chromatography

Two-dimensional effective diffusion can prove very helpful in planar chromatographic enantiomer separation, however, as will be shown in the forthcoming sections. Combination of 2D effective diffusion with the native microcrystalline chirality of silica gel can result in 2D enantiomer separations in 1D chromatographic development mode.

Enantiomer Separations on Pure Silica Gel as Stationary Phase

In general, the microcrystalline chirality of silica gel is, alone, not usually sufficient to enable successful and complete enantiomer separation. Its intermolecular interaction with chiral species does, however, usually result in perceptible deviations of the analyte's track from the linear direction of mobile phase migration. Most probably such deviation was documented for the first time (although it passed rather unnoticed and not commented upon by the authors) in the planar chromatographic separation of the enantiomers of ibuprofen (as shown in Fig. 1 from Ref. [14]).

In our own studies we managed to demonstrate spectacular deviation from linearity of the route of migration of racemic ketoprofen on pure silica gel, although without proper enantiomer separation [15]. Such deviations can prove highly misleading when densitometric detection is used and the analyst assumes straight-line migration of the analyte, with this imaginary straight line anchored to the origin. The chromatogram is usually scanned in a single line and the deviating band of the analyte seems to have inexplicably disappeared. Because of the discussed deviation of the migration route of the analyte from linearity, it is then advisable:

(i) to scan the chromatogram bandwise at close (e.g. 1-mm) intervals with a track as wide as 2 cm (i.e. 1 cm to the left and 1 cm to the right of the expected straight-line migration route), and
(ii) to obtain an additional videoscan of the entire chromatogram for direct visual inspection of the deviated band's spatial arrangement.

In Ref. [16] we showed that although the enantiomers of racemic ibuprofen and racemic naproxen could not be fully separated on native silica gel, their respective chromatographic spots assumed a 'V' shape, with the skewed parts clearly marked by the two "V arms". These two "arms" most probably indicate the left-handed and the right-handed spatial orientation of the two antimers of ibuprofen and naproxen within the partially – and horizontally – separated chromatographic spots obtained *(Fig. 3)*.

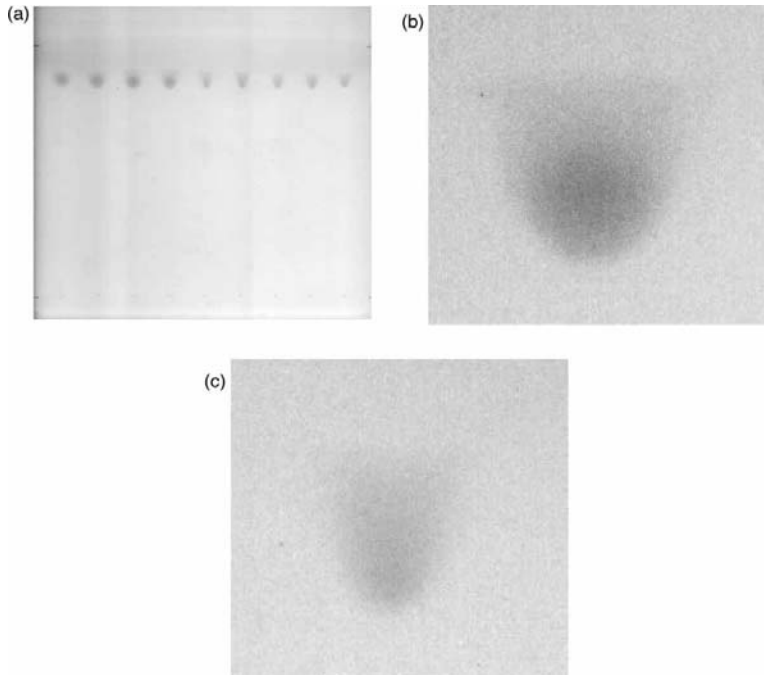


Fig. 3. Video images obtained from an unmodified silica gel 60 F_{254} plate developed with ethanol [16]: (a) the whole chromatogram (spots 1–5: *rac*-ibuprofen; spots 6–9: *rac*-naproxen); (b) enlargement of a typical chromatographic spot of *rac*-ibuprofen; (c) enlargement of a typical chromatographic spot of *rac*-naproxen

Enantiomer Separations on Silica Gel Impregnated with Chiral Selectors

The enantiomer separating performance of native silica gel can be substantially enhanced by impregnation with simple yet appropriately chosen chiral selectors. Many practical solutions are discussed in the literature devoted to enantiomer separations by planar chromatography; many of these are summarized in Ref. [12]. In this mini review, however, we discuss chiral selectors on the basis of examples taken exclusively from our own laboratory practice, in order to emphasize the two-dimensionality of such separations in 1D development mode, and to stress separate contributions to these separations originating from the silica gel and the chiral selector.

In the sub-sections below we focus on planar chromatographic separation of the enantiomers of three groups of low-molecular-weight chiral carboxylic acids, all of vital importance in different branches of the life sciences – profen drugs, α-amino acids, and hydroxy acids.

Separation of the Enantiomers of Profen Drugs

The pioneering work on the separation of the enantiomers of profen drugs by planar chromatography was performed by Bhushan and coworkers in the Indian Institute of Technology in Roorkee, India. His important paper on the separation of the enantiomers of *rac*-ibuprofen has already been cited [14]; his general suggestion was that an efficient way of separating the enantiomers of profen drugs was on native silica gel impregnated with amino acids (one of the best performing being L-arginine). Stationary phase layers should be prepared in such a way as to provide the amino acid selector in the cationic form and the separated profen enantiomers in the anionic form. The mechanism of such enantiomer separations can be summarized by eqs (1) and (2), which show recombination of the two oppositely charged ions to form two different diastereoisomeric salts:

$$\text{L-arginine}^+ + \textit{S}\text{-(+)-profen}^- \rightleftharpoons \text{L-arginine}^+ \cdot \textit{S}\text{-(+)-profen}^-;\ K_1 \qquad (1)$$

$$\text{L-arginine}^+ + \textit{R}\text{-(−)-profen}^- \rightleftharpoons \text{L-arginine}^+ \cdot \textit{R}\text{-(+)-profen}^-;\ K_2 \qquad (2)$$

where the enantiomer separation condition is given by the inequality $K_1 \neq K_2$.

Yet it was only in our laboratory – equipped with densitometric and videoscanning detection – that we started exposing the migration tracks of the separated profen enantiomers and their deviations from the expected straight-line course. Initially we visualized such deviations diagrammatically, as shown, e.g., in *Fig. 4* (taken from Ref. [17]). This diagram is the simplest representation of the 2D separation of the enantiomers of an aryl-

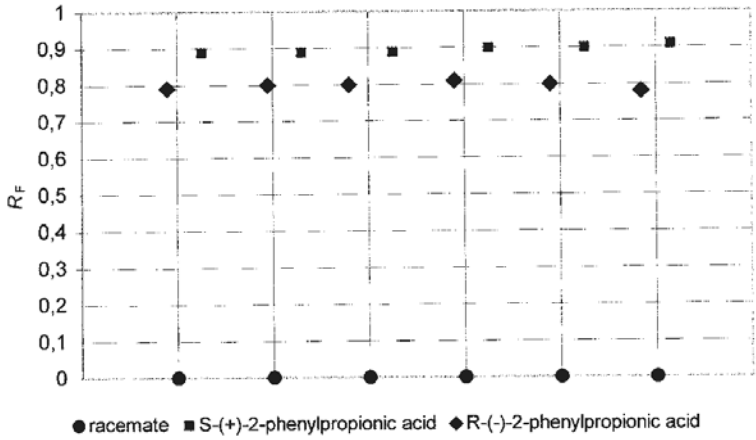


Fig. 4. Schematic representation of the deviation from the vertical of the migration tracks of the enantiomers of 2-phenylpropionic acid on silica gel 60 F_{254} impregnated with L-arginine. The mobile phase was ACN–MeOH–H_2O 5:1:0.75 (*v*/*v*) [17]

propionic acid in the 1D development mode. It emphasizes that the vertical component of this separation is most probably because of the action of the chiral selector (i.e., L-arginine), and the horizontal component is most probably because of the combined action of the microcrystalline chirality of silica gel and the 2D effective diffusion, freely available in the planar chromatographic systems.

An alternative means of representation is to demonstrate specific skewness of the 2D enantiomer separations in form of the 3D densitograms, generated from linear densitometric scans of the chromatogram, performed densely enough, with small intervals between neighbouring scans. Typical examples are shown in *Figs 5* and *6* (also taken from Ref. [17]).

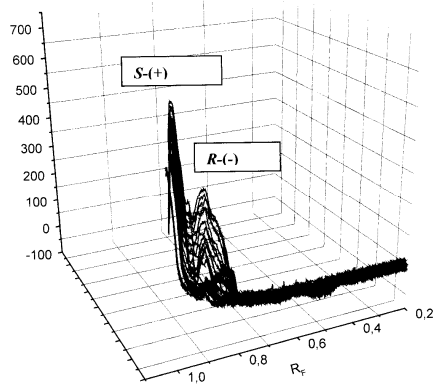


Fig. 5. Three-dimensional representation of the skewed chromatographic peak shapes of *S*-(+)-2-phenylpropionic acid (right-handed) and *R*-(−)-2-phenylpropionic acid (left-handed). The stationary phase was silica gel 60 F_{254} impregnated with L-arginine, and the mobile phase was ACN–MeOH–H_2O 5:1:0.75 (*v*/*v*) [17]

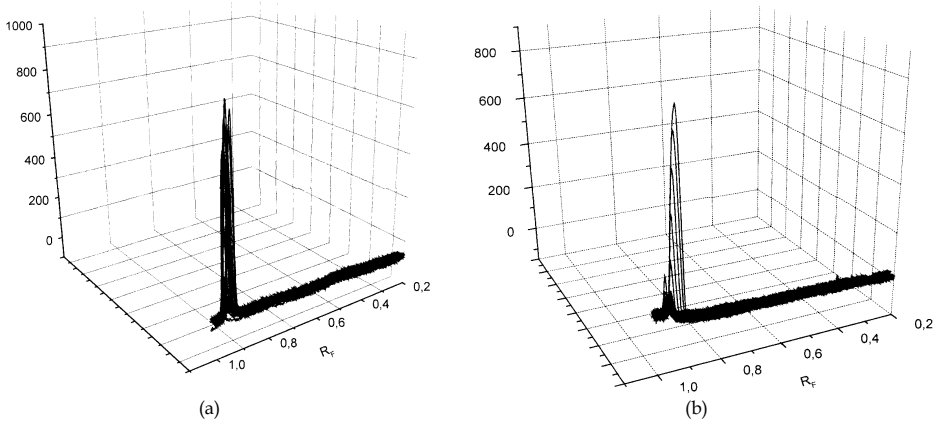


Fig. 6. Three-dimensional representation of the skewed chromatographic peak shapes of (a) *S*-(+)-naproxen (left-handed) and (b) *R*-(−)-naproxen (right-handed). The stationary phase was silica gel 60 F_{254} impregnated with L-arginine, and the mobile phase was ACN–MeOH–H_2O 5:1:1.5 (*v*/*v*) [17]

Separation of the Enantiomers of α-Amino Acids

One of the most convenient methods of planar chromatographic separation of the enantiomers of α-amino acids is by enhancing the separating power of native silica gel with a chiral selector in form of a complex cation deposited on its surface. Among the best-performing complex cations are those based on copper(II) chelated by optically pure ligands (e.g., by the two L-proline ligands, $[Cu(L\text{-}Pro)_2]^{2+}$), although within this general approach other bivalent transition metal cations (e.g., Co(II), Ni(II), or Mn(II)), and the other optically pure ligands can also be tested and used. This approach is just one variant of ligand-exchange chromatography (LEC) and the LEC separation of the enantiomers of amino acids by planar chromatography has been comprehensively discussed in Ref. [18]. It is worthy of note that the only commercially available chromatographic plates intended for enantiomer separations are those manufactured by Macherey Nagel, known under their commercial name of Chiralplates. These plates are precoated with silica gel impregnated with the L-proline derivatives of the copper(II) cation.

The mechanism of enantiomer separation involving the $[Cu(L\text{-}Pro)_2]^{2+}$ cation as chiral selector can be represented schematically with separation of *rac*-alanine as example:

$$[Cu(L\text{-}Pro)_2]^{2+} + L\text{-}Ala \rightleftharpoons [Cu(L\text{-}Pro)(L\text{-}Ala)]^{2+} + L\text{-}Pro;\ K_1 \qquad (3)$$

$$[Cu(L\text{-}Pro)_2]^{2+} + D\text{-}Ala \rightleftharpoons [Cu(L\text{-}Pro)(D\text{-}Ala)]^{2+} + L\text{-}Pro;\ K_2 \qquad (4)$$

where the enantiomer separation condition is given by the inequality $K_1 \neq K_2$. This mechanism consists in displacement of one L-proline ligand by an α-amino acid molecule.

In *Fig. 7*, we give an example of the separation of the enantiomers of *rac*-tyrosine, taken from Ref. [19]. A classical densitogram depicting successful, almost baseline, separation of L and D-tyrosine is shown in *Fig. 7a*. In *Fig. 7b* we show a 2D picture of these two separated tyrosine antimers, constructed by assembly of densitometric scans and resembling a videoscan of the two separated chromatographic spots. An advantage of this kind of representation is its emphasis of the two-dimensionality of the enantiomer separation, vertical and horizontal. It seems true that for the vertical enantiomer separation, intermolecular interactions with the copper(II)–L-Pro complex and replacement of one L-Pro molecule with one tyrosine molecule is responsible. Horizontal enantiomer separation can best be seen from the fact that the band maxima of the two separated species are not arranged along the line indicating the direction of the eluent flow, but are shifted sidewise in the two opposite directions. It also seems true that the horizon-

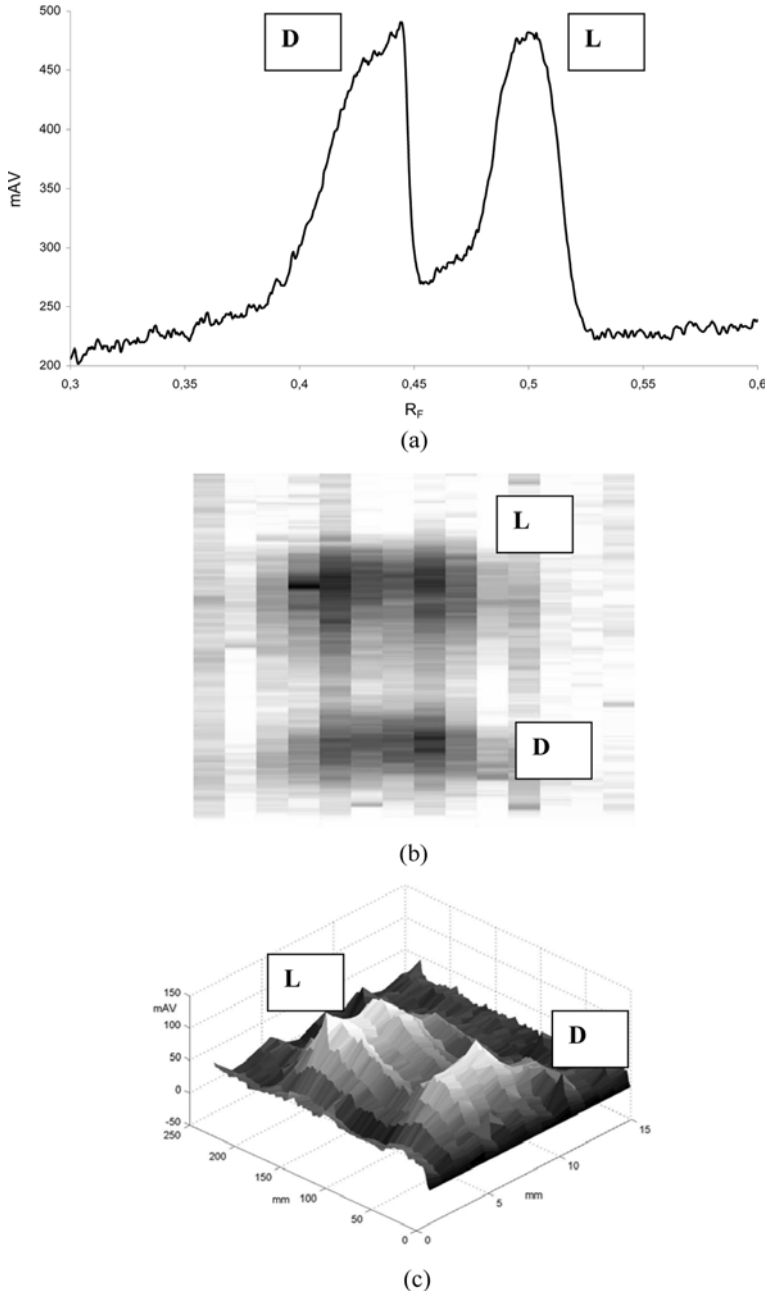


Fig. 7. (a) Densitogram, (b) 2D representation, and (c) 3D representation of the two separated spots of the enantiomers of L and D-tyrosine. The 2D and 3D representations were constructed by assembly of densitometric scans of the separated tyrosine antimers taken at 1-mm intervals. The stationary phase was silica gel 60 F_{254} impregnated with Cu(II) cations and L-proline. The mobile phase was *n*-BuOH–ACN–H_2O 6:2:3 (*v*/*v*) [19]

tal contribution to separation of the enantiomers is because of the microcrystalline chirality of silica gel combined with 2D effective diffusion in the planar system.

Separation of the Enantiomers of Hydroxy Acids

Hydroxy acids are the third group of low-molecular-weight carboxylic acids of vital importance in the life sciences, so separation and quantification of their enantiomers is necessary. Few separations of their enantiomers by chiral planar chromatography have yet been achieved, however. Perhaps one of the most interesting and most successful enantiomer separations before our own investigations was that of lactic acid by the Italian diary industry. This happened as long ago as in 1991 (i.e., without a modern instrumental detection) [20]. The mechanism of enantiomer separation proposed by the Italian scientists makes still another variant of LEC. In this case, the silica gel layer was impregnated with copper(II) cations only (in this case copper acted as a non-chiral selector) with the separated lactic acid antimers acting as bidentate chelating ligands. This mechanism of enantiomer separation can be briefly summarized by the equations:

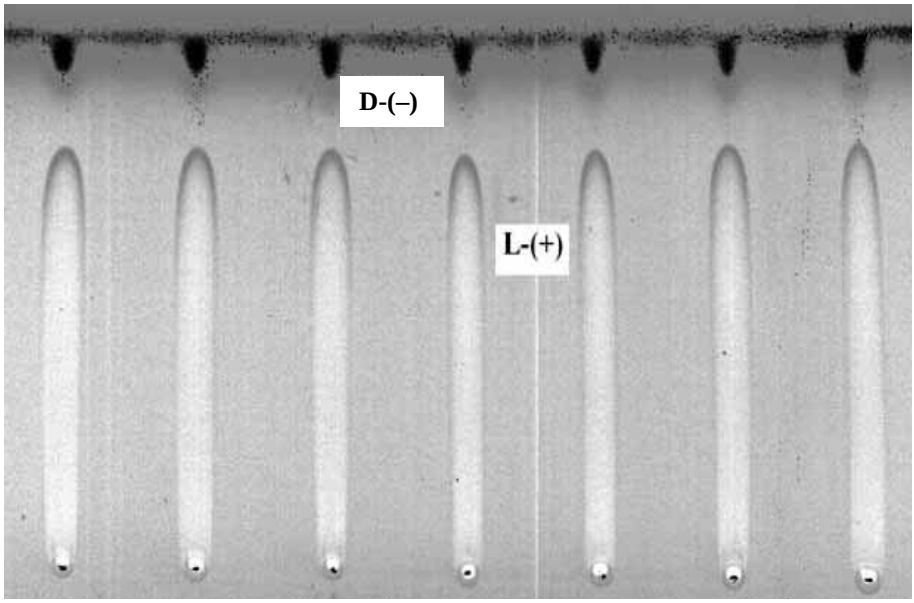
$$Cu^{2+} + 2\ \text{L-(+)-LA} \rightleftharpoons [Cu(\text{L-(+)-LA})_2]^{2+};\ K_1 \quad (5)$$

$$Cu^{2+} + 2\ \text{D-(−)-LA} \rightleftharpoons [Cu(\text{D-(−)-LA})_2]^{2+};\ K_2 \quad (6)$$

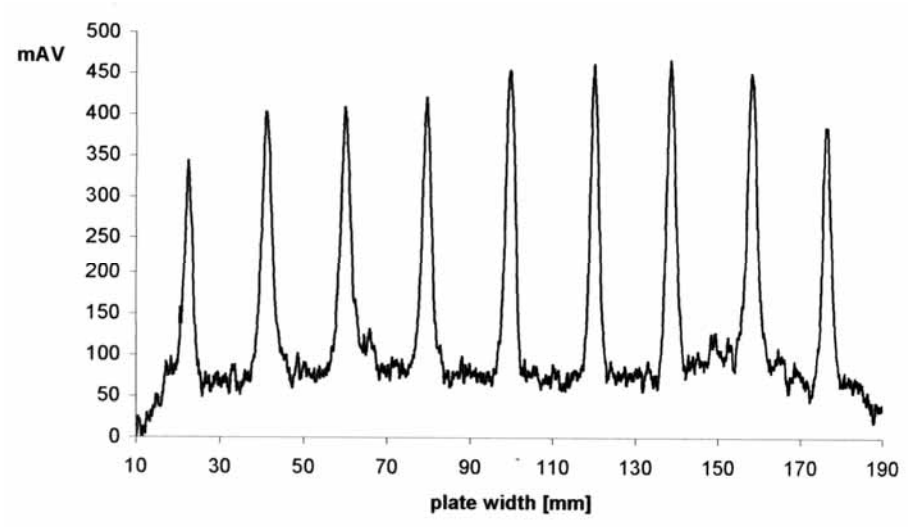
where the enantiomer separation condition is given by the inequality $K_1 \neq K_2$.

We repeated the procedure proposed in Ref. [20] and obtained the same results instrumentally [21]. The very highly efficient separation of the enantiomers of racemic lactic acid is shown in *Fig. 8*.

The enantiomer separation procedure proposed in Ref. [20] for lactic acid has proved successful with *rac*-β-hydroxybutyric acid also [22]. We studied the spontaneous oscillatory in-vitro chiral conversion of *R*-β-hydroxybutyric acid stored in 70% aqueous ethanol by checking the progress of conversion at one-day intervals. As can be seen from *Fig. 9A*, after storage for ca. 69 h both antimers appeared side by side on the chromatogram in the form of the two opposite crescents. In this particular case, the horizontal contribution to separation of the enantiomers was clearly predominant. There was, however, also a vertical contribution to this separation, as demonstrated by densitometric scanning of the two separated β-hydroxybutyric acid bands in such a way as to expose the best maxima of the respective concentration profiles. From *Fig. 9B* it can be seen that the retardation factors (i.e., R_F values) of the two antimers differed substantially.



(A)



(B)

Fig. 8. A. Chromatogram obtained from D and L-lactic acid dissolved in ethanol–water 7:3 (*v*/*v*). The stationary phase was silica gel impregnated with Cu(II) acetate and the mobile phase dioxane–water 9:1 (*v*/*v*). B. Densitogram scanned just below the mobile-phase front of the above chromatogram in a direction perpendicular to that of development, showing the concentration profiles of D-(+)-lactic acid without interference from the contaminants on the mobile-phase front [21]

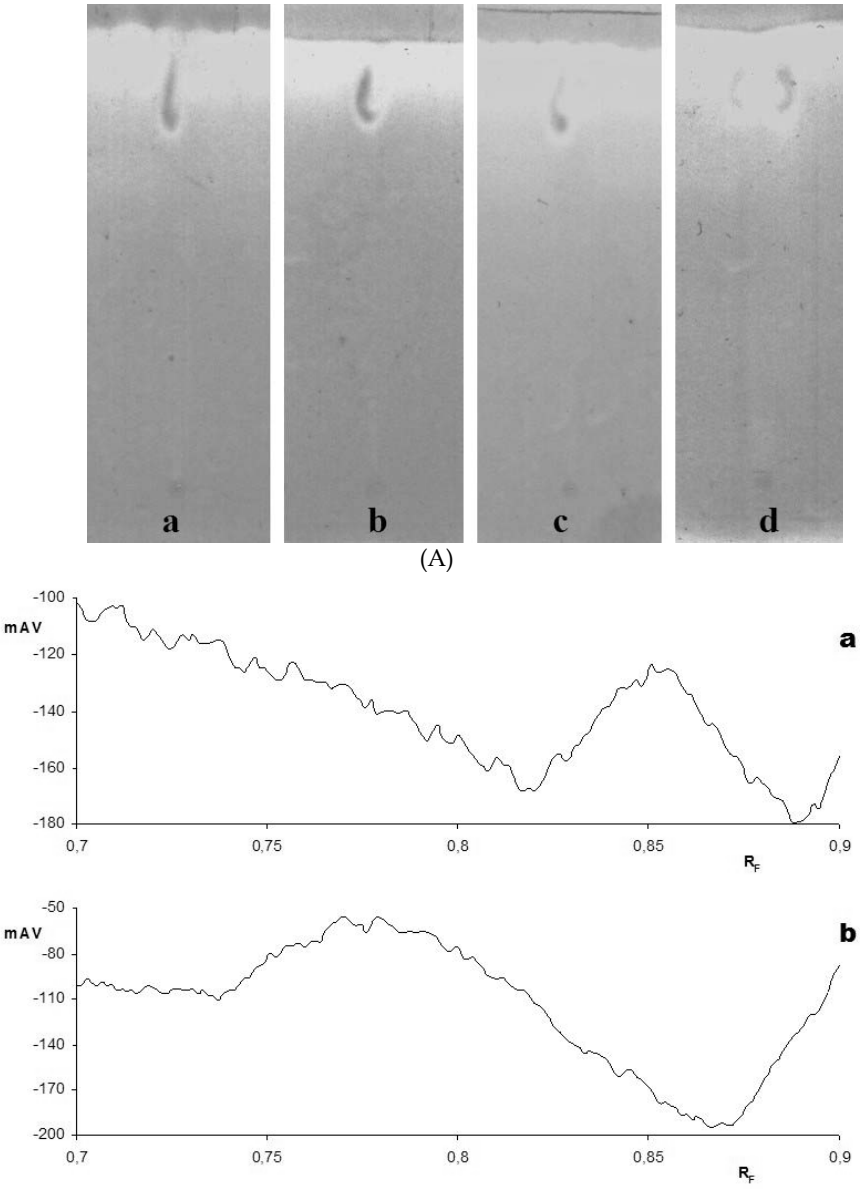


Fig. 9. A. Videoscans of chromatograms obtained from *R*-β-hydroxybutyric acid dissolved in ethanol–water 7:3 (*v*/*v*) and stored for (a) 1 h, (b) 20 h, (c) 47 h, and (d) 69 h. Visualization was carried out by spraying the chromatogram with 5% (*v*/*v*) conc. H_2SO_4 in ethanol, and heating at 100–110°C for ca. 10 min. B. Densitometrically scanned concentration profiles of (a) *R*-β-hydroxybutyric acid and (b) *S*-β-hydroxybutyric acid. Because of the 2D separation of the enantiomers of the β-hydroxybutyric acid in 1D development mode, the demonstrated concentration profiles represent densitometric scans for the tracks where the *R* and the *S* species have their respective maxima [22]

The R_F of the *R* species was 0.86 whereas that of the *S* species was 0.78 (so the difference between the two substantially exceeds the experimental error of the individual R_F values, which was ±0.02).

Conclusions

- Two major assets of thin-layer chromatography on silica gel are:
  (i) the microcrystalline chirality of the silica gel used in TLC; and
  (ii) the 2D effective diffusion in planar chromatography.

For these reasons, enantiomer separations by TLC on silica gel benefit from an important advantage absent from column chromatography.

- Impregnation of silica gel with a chiral (or non-chiral) component additionally enhances the enantiomer separation potential of the planar layers.
- Because silica gel alone is sometimes enough to separate a pair of enantiomers, planar chromatography on unmodified silica gel should be regarded as a mildly chiral TLC system.
- The microcrystalline chirality of silica gel and the 2D effective diffusion contribute substantially to the universal nature of the enantiomer separations performed with such systems.
- Choice of an appropriate chiral selector for impregnation and use of densitometric, DAD, or MS instrumental detection can elevate chiral planar chromatography to the status of one of the most flexible and best-performing analytical tools in research on chirality.

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