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ENANTIOMERIC SEPARATION OF HOMOCYSTEINE AND CYSTEINE BY ELECTROKINETIC CHROMATOGRAPHY USING MIXTURES OF γ-CYCLODEXTRIN AND CARNITINE-BASED IONIC LIQUIDS

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Abstract

Two new chiral ionic liquids based on the ester of the non-protein amino acid carnitine as cationic counterpart were synthesized, characterized and evaluated as chiral selectors for the enantiomeric separation of homocysteine and cysteine derivatized with 9-fluorenylmethoxycarbonyl chloride by electrokinetic chromatography. The use of both ionic liquids, L-carnitine methyl ester bis(trifluoromethane)sulfonimide and L-carnitine methyl ester L-(+)-lactate, as sole chiral selectors in the separation buffer, did not allow the enantiomeric separation of the studied amino acids. However, the combined use of L-carnitine methyl ester bis(trifluoromethane)sulfonimide ionic liquid with γ-CD forming a dual system led to the enantiomeric separation of both analytes and showed the existence of a strong synergistic effect. On the contrary, the dual system γ-CD plus L-carnitine methyl ester L-(+)-lactate did not improve the enantiomeric separations with respect to those obtained using the CD alone. The influence of different experimental variables such as buffer composition and pH, and ionic liquid concentration was investigated. Also, the nature of the anionic moiety was evaluated by comparing the results obtained with both ionic liquids when combined with γ-CD. The use of 2 mM γ-CD combined with 5 mM L-carnitine methyl ester bis(trifluoromethane)sulfonimide in a 50 mM phosphate buffer at pH 7.0 enabled to achieve the simultaneous enantiomeric separation of homocysteine and cysteine with high resolution values (>6.0) in analysis times close to 12 min.

1. Introduction

In last decades, chirality has been an important issue in pharmaceutical, environmental and food fields due to the different biological or toxicological activities of enantiomers [1-3]. In this sense, a challenge of analytical chemistry is focused on the development of new chiral methodologies for enantiomeric separations. Among all separation techniques, capillary electrophoresis (CE), mainly in the mode of electrokinetic chromatography (EKC), has been one of the most suitable for chiral separations owing to its great advantages such as low solvent consumption, high separation efficiency or high versatility to use different chiral selectors. Despite cyclodextrins (CDs), antibiotics or surfactants continue being the most employed chiral selectors, different limitations such as high cost, complicated synthesis or low solubility make the search of new compounds acting as chiral selectors a challenge in CE [4].

In the last years, ionic liquids have emerged as a promising alternative to conventional chiral selectors. These compounds are organic salts formed by bulky organic cations and organic or inorganic anions. If the cation/anion or both are chiral they are known as chiral ionic liquids (CILs). Ionic liquids have been used not only for chiral separations but also in different fields of chemistry such as synthesis, catalysis and extraction due to the fascinating properties they present (negligible vapor pressure, good thermal stability, high conductivity, etc.) [5, 6]. When CILs are used in CE to achieve chiral separations, they can be used as sole chiral selectors, in combination with other selectors or as ligands in ligand-exchange (LE)-CE. However, most works reported in literature employed CILs together with other selectors (mainly CDs) forming dual systems [7-9].

Biological thiols such as homocysteine (Hcy) and cysteine (Cys) are implied in different biological processes and metabolic pathways. Hcy is considered a biomarker of cardiovascular diseases [10-12] and Cys plays a crucial role in protein synthesis and as a

precursor of glutathione, which is an important natural antioxidant [13]. Due to the clinical importance of these compounds, different separation methods have been developed for their simultaneous (achiral) determination. These methodologies are mainly based on liquid chromatography with fluorescence detection [11, 14-17] and CE [18-21]. However, only two works have reported the enantiomeric separation of Hcy by CE, one of them by our research group [22, 23]. Regarding Cys, several works reporting the chiral separation of different amino acids, have described the separation of Cys enantiomers using different CE modes, such as EKC, LE-CE, capillary electrochromatography (CEC) or capillary array electrophoresis (CAE) [24-35]. One of these works was based on the use of a CIL (namely, 1-butyl-3-methylimidazolium L-Ornithine) as chiral ligand coordinated with Zn (II) in a LE-CE method for the chiral separation of eleven pairs of amino acid enantiomers (including Cys) previously derivatized with dansyl chloride [35]. In this work, Cys enantiomers were separated in ~ 40 min with a resolution value of 1.83. As far as we know, the simultaneous enantiomeric separation of Hcy and Cys by CE has never been reported.

The objective of this work was to synthesize and characterize two new chiral ionic liquids based on the non-protein amino acid carnitine as cationic counterpart and to evaluate their discrimination power towards Hcy and Cys when used as the sole chiral selectors in the separation buffer in CE and combined in dual systems with CDs.

2. Materials and methods

2.1 Reagents and samples

All the employed reagents were of analytical grade. Disodium hydrogen phosphate was obtained from Panreac Química S.A. (Barcelona, Spain). Sodium hydroxide, boric acid and pentane were provided by Sigma-Aldrich (Madrid, Spain). Acetonitrile was

purchased from Scharlau (Barcelona, Spain). The chiral selectors α-CD, methyl-β-CD (Me-β-CD) and heptakis(2,6-di-O-methyl)-β-CD (DM-β-CD) were provided by Sigma-Aldrich. β-CD, γ-CD, 2-Hydroxypropyl-β-CD (DS~3) (HP-β-CD) and heptakis(2,3,6-tri-O-methyl)-β-CD (TM-β-CD) were obtained from Fluka (Buchs, Switzerland). D,L-homocysteine (D,L-Hcy), L-homocysteine (L-Hcy) and the derivatization reagent 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) were supplied from Sigma-Aldrich. D,L-cysteine (D,L-Cys) and L-cysteine (L-Cys), were obtained from Fluka. L-carnitine hydrochloride, bis(trifluoromethane)sulfonimide lithium salt and 1.25 M HCl in methanol and methanol were purchased from Sigma-Aldrich. L-(+)-Lactic acid solution was purchased from Riedel de Haën (Seelze, Germany). Water used to prepare solutions was purified through a Milli-Q system from Millipore (Bedford, MA, USA).

2.2 Synthesis of ionic liquids

The ionic liquids L-Carnitine methyl ester bis(trifluoromethane)sulfonimide (L-CarC_1NTf_2), L-Carnitine methyl ester L-Lactate (L-CarnitineC_1Lac) were synthesized by the Center for Applied Chemistry and Biotechnology from the University of Alcalá.

A mixture of L-carnitine hydrochloride (0.40 g, 2.00 mmol, 1.0 eq.) in methanol (3 mL) was treated with a solution of 1.25 M HCl in methanol (1 mL). The resulting mixture was heated at 80ºC for 3 h. When the reaction was finished, the solvent was removed under vacuum affording the corresponding L-carnitine methyl ester chloride (420 mg, 98%) as a white oil-solid. L-carnitine methyl ester (358 mg, 1.68 mmol. 1.0 eq.) was dissolved in 2 mL of water. An equimolar amount of bis(trifluoromethane)sulfonimide lithium salt (482.8 g, 1.68 mmol, 1.0 eq.) was dissolved separately in water. Both solutions were mixed and stirred at room temperature for 3 h. After this time, the mixture resulted in two layers, of which the lower layer was separated and dried under vacuum overnight (45ºC,

150 mbar). This layer afforded 361 mg (47%) of L-carnitine methyl ester bis(trifluoromethane)sulfonimide ionic liquid as a colorless and very dense oil.

For the synthesis of L-carnitine methyl ester L-(+)-lactate, L-carnitine methyl ester (720 mg, 3.38 mmol, 1.0 eq.) was dissolved in 1 mL of distilled water. An equimolar amount of L-(+) lactic acid solution 88-92% (286 µL, 3.38 mmol, 1.0 eq.) was added dropwise. The reaction mixture was stirred at room temperature for 48 h. After that, the solvent was removed under reduced pressure, the residue was treated with toluene (2 x 5 mL), and the solvent was removed again under reduced pressure. The residue was dried under vacuum overnight (45 °C, 150 mbar). The ionic liquid was isolated as colorless and dense oil (700 mg, 78%).

The ionic liquids synthesized were characterized by nuclear magnetic resonance (NMR), LC-MS and elemental analysis. NMR spectra were recorded in either DMSO-d_6 or CD_3OD on a Varian-300 MHz or 500 MHz instrument with tetramethyl silane (TMS) as an internal standard. The multiplicities are presented as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broad). LC-MS analyses were carried out using a 1260 Infinity LC system coupled to a 6120 Quadrupole mass spectrometer (Agilent Technologies) equipped with an orthogonal electrospray ionization source (ESI). The analyses were performed using a SeQuant Zic-Hilic column (150 mm x 4.6 mm, particle size 5 µm) from Merck-Millipore. The mobile phase consisted of phase A (200 mM ammonium acetate, pH 5.2) and phase B (acetonitrile 80 % and solution of ammonium acetate 20%) in an isocratic method. The mobile phase flow rate was 1 mL/min, the column temperature was set to 25 °C, detection at 277.4 nm ± 16 nm.

The data obtained for the characterization of both ionic liquids by NMR, LC-MS and elemental analysis were the following:

L-CarnitineC_1NTf_2; ^{1}H-NMR (CD_3OD) δ (ppm) 4.25 (m, J = 6.0 Hz, 1H), 3.42 (s, 3H), 3.18 (d, J = 8.5 Hz, 2H), 2.95 (s, 9H), 2.30 (d, J = 8.5 Hz, 2H). Analysis by LC-MS (positive mode): t_R = 1.023 min (*m/z* 176.2 $(M+H)^+$). Analytical calculated for $C_{10}H_{18}N_2O_7S_2F_6$: C, 26.3; H, 4; F, 25; N, 6.1; S, 14.1. Found: C, 25.29; H, 4.14; N, 6.20; S, 14.40.

L-CarnitineC_1L-Lactate; ^{1}H-NMR (CD_3OD) δ (ppm) 4,63 (m, J = 6.2 Hz, 1H), 4.24 (c, J = 6.8 Hz, 1H), 3.74 (s, 3H), 3.49 (d, J = 6,2 Hz, 2H), 3.28 (s, 9H), 2.60 (dd, J = -10,0 Hz, J = 6.2 Hz, 2H), 1.40 (d, J = 6.8 Hz, 3H); ^{13}C-NMR (CDC_3OD) δ (ppm) 176.8, 172.8, 66.0, 62.5, 53.6, 53.5, 53.4, 50.9, 39.6, 19.4; Analysis by LC-MS (positive mode): t_R = 0.99 min (*m/z* 176.2 $(M+H)^+$; MS (negative mode): t_R = 1.59 min (*m/z* 89.2 $(M-H)^-$ (lactate ion). Analytical calculated for $C_8H_{18}NO_3$.HCl: C, 43.6; H, 8.3; N, 4.6. Found: C, 43.5; H, 7.74; N, 4.8.

2.3 CE conditions

All CE analyses were performed in an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) equipped with a DAD detector. The detection wavelength was set at 210 nm with a bandwidth of 4 nm. The instrument was controlled by the ChemStation software (B. 04. 03 SP1) from Agilent Technologies. Separations were performed in an uncoated fused silica capillary of 50 μm ID (362.8 μm OD) with a total length of 58.5 cm (50 cm effective length) provided by Polymicro Technologies (Phoenix, AZ, USA). Samples were injected in the CE system applying a pressure of 50 mbar for 4 s. The electrophoretic separation was achieved using a voltage of 20 kV and a working temperature of 20ºC.

New capillaries were conditioned with 1 M sodium hydroxide for 30 min, Milli-Q water for 5 min and buffer solution for 60 min. Before each working day the capillary was

168 flushed during 10 min with 0.1 M sodium hydroxide, followed by Milli-Q water for 5
169 min, buffer solution for 15 min and BGE for 10 min. Between injections, the capillary
170 was conditioned with 0.1 M sodium hydroxide (2 min), Milli-Q water (1 min) and BGE
171 (3 min).

172 **2.4 Preparation of solutions and samples**

173 The borate buffer (200 mM, pH 9.0) needed for the derivatization step was prepared
174 dissolving the suitable amount of boric acid in Milli-Q water and adding sodium
175 hydroxide until reaching pH 9.0. The separation buffers employed in CE were prepared
176 dissolving the appropriate amount of disodium hydrogen phosphate or boric acid in water
177 and adjusting the pH to the desired value with hydrochloric acid or sodium hydroxide
178 before completing the volume with water to achieve a concentration of 50 mM. The BGE
179 was obtained by dissolving the proper amount of the ionic liquid and/or the cyclodextrin
180 in the buffer solution.

181 Stock standard solutions of each amino acid were prepared by dissolving the amount
182 needed of amino acids in borate buffer (200 mM, pH 9.0). The stock solutions were stored
183 at 4 ºC until its derivatization with FMOC.

184 All solvents and samples were filtered before its use through 0.45 µm pore size disposable
185 nylon filters provided by Scharlau (Barcelona, Spain).

186 **2.5 Derivatization procedure**

187 Amino acids were derivatized following a method previously described in the literature
188 [36, 37]. A solution of 30 mM of FMOC-Cl in acetonitrile was freshly prepared each day,
189 considering that an excess of at least three times was necessary for the total derivatization
190 of the amino acids. In summary, 200 µL of standard amino acid solution (10 mM) were
191 mixed with 200 µL of FMOC-Cl solution. Then, the reaction was kept at room

192 temperature for 2 min and the excess of FMOC-Cl was extracted with 0.5 mL of pentane.
193 The resulting solution was 10 times diluted with Milli-Q water before injection in the CE
194 system.

195 **2.6 Data treatment**

196 Resolution values (Rs) for the enantiomers were obtained from their migration times and
197 peak widths at half height using the Chemstation software from Agilent Technologies.
198 Graphs composition with different electropherograms was carried out by using Origin 8.0
199 software.

200 **3. Results and discussion**

201 **3.1 Synthesis of L-carnitine ionic liquids**

202 The synthesis of L-CarnitineC_1NTf_2 was carried out following the synthetic scheme
203 shown in **Figure 1A** (see experimental part). Briefly, the intermediate compound (**2**) is
204 formed by the reaction of the carnitine halide in acidic-alcoholic medium to give the
205 corresponding ester derivative. Then, based on a metathesis reaction an anionic
206 interchange between the halide salt and the lithium salt with the desired anion was carried
207 out in order to obtain the ionic liquid. To perform the synthesis of L-CarnitineC_1L-Lactate
208 the anion L-Lactate was employed instead of bis(trifluoromethane)sulfonimide (NTf_2) as
209 observed in **Figure 1B**.

210 **3.2 L-CarnitineC_1NTf_2 and L-CarnitineC_1L-Lactate as chiral selectors for the**
211 **enantiomeric separation of FMOC-Hcy and FMOC-Cys**

212 As previously mentioned, CILs have demonstrated to have enantiomeric discrimination
213 ability towards several analytes and are an interesting alternative to the use of other
214 conventional selectors. Hence, L-CarnitineC_1NTf_2 and L-CarnitineC_1L-Lactate, two new
215 chiral ionic liquids, were tested as the sole chiral selectors for the enantiomeric separation

of FMOC-Hcy and FMOC-Cys in a 50 mM phosphate buffer at pH 7.0. However, none of these amino acids were enantiomerically separated with the use of the CILs alone in the separation buffer at concentrations of 20 and 40 mM.

3.3 Screening of neutral CDs as chiral selectors for the enantiomeric separation of FMOC-Cys

Although the discrimination power of different neutral CDs for the enantiomeric separation of FMOC-Hcy was evaluated in a previous work showing that only γ-CD enabled its chiral discrimination [23], the potential of these CDs for the enantioseparation of FMOC-Cys had not previously been investigated. For this reason, in the present work, a set of neutral CDs (α-CD, β-CD, γ-CD, Me-β-CD, DM-β-CD, TM-β-CD, HP-β-CD) was evaluated for the enantiomeric separation of this amino acid. All analyses were carried out using a concentration of 10 mM of each CD in 50 mM phosphate buffer at pH 7.0. These conditions were the same as those employed previously in the study of the enantiomeric separation of FMOC-Hcy with neutral CDs in which a resolution value of 1.9 was obtained using 10 mM γ-CD as optimum concentration [23]. Results obtained in the present work for FMOC-Cys showed that γ-CD was also the only CD, among the CDs investigated, allowing its enantiomeric separation. A resolution value of 1.8 was obtained for FMOC-Cys under the above-mentioned conditions. Then, considering that the concentration of the chiral selector affects the affinity of the enantiomers for the selector [38], the influence of γ-CD concentration on the enantiomeric resolution of FMOC-Cys was studied in the range 1-15 mM. As it can be observed in **Table 1**, the best enantiomeric separation (Rs = 3.0) was achieved employing a concentration of 2 mM γ-CD in the separation buffer. Regarding the enantiomeric migration order, it was established by spiking solutions of D,L-FMOC-Cys with the corresponding L-enantiomer which enabled to observe that the D-enantiomer was the first-migrating enantiomer. This migration order

241 was the same as that observed previously for FMOC-Hcy when γ-CD was used as chiral
242 selector [23].

243 **3.4 Evaluation of the combined use of γ-CD and L-CarnitineC$_1$NTf$_2$ as dual system**

244 As the combined use of CDs and ionic liquids has shown to be an interesting way to
245 improve the enantiomeric separation of several analytes [7], the potential of the ionic
246 liquid synthesized in this work, L-CarnitineC$_1$NTf$_2$, was investigated for the enantiomeric
247 separation of FMOC-Hcy and FMOC-Cys when combined with γ-CD in a dual system.
248 With this aim, the influence of the addition of different amounts of the CIL to a fixed
249 concentration (2 mM) of γ-CD was studied when using a 50 mM phosphate buffer at pH
250 7.0. Data shown in **Table 2** and the electropherograms depicted in **Figure 2** allow to
251 observe that as the concentration of L-CarnitineC$_1$NTf$_2$ in the separation buffer was
252 increased, higher Rs values were obtained reaching the maximum value when 5 mM of
253 the CIL was added (Rs = 6.1 and 6.4 for FMOC-Hcy and FMOC-Cys respectively)
254 showing a strong synergistic effect resulting from the combination of γ-CD and the CIL.
255 This synergistic effect can be explained taking into account that different dynamic CIL-
256 CD interactions can give rise to alterations between the selectors and the analytes which
257 in turn can modify the chiral recognition process in terms of enantiomeric resolutions and
258 migration times [39-43]. However, higher concentrations of L-CarnitineC$_1$NTf$_2$ caused a
259 decrease in the Rs and led to a worse separation (see **Table 2**) suggesting that the presence
260 of high concentrations of CIL ions can negatively affect the CD-analyte interactions.
261 An interesting result to be highlighted is that the combined use of both chiral selectors
262 led to a reversal in the enantiomeric migration order. As above-mentioned, when γ-CD
263 was used as sole chiral selector, the D-amino acids were the first-migrating enantiomers
264 while the L-enantiomers were the second. However, the opposite order was observed

when using the dual system formed by γ-CD and L-CarnitineC_1NTf_2 being the L-enantiomers the first migrating ones for both amino acids studied (see **Figure 3**).

The influence of buffer composition and pH was investigated by using a 50 mM borate buffer at pH 9.0. Results showed that, as expected, analysis times were shorter than at pH 7.0 but Rs values also decreased when increasing the pH to 9.0 (see **Table 2**). **Figures 4A** and **4B** show the comparison of the electropherograms corresponding to the enantiomeric separation of FMOC-Hcy and FMOC-Cys at both pH values under the conditions giving rise to the maximum resolution (2 mM γ-CD plus 5 mM ionic liquid). Regarding the enantiomeric migration order at pH 9.0, this was the same as that obtained at pH 7.0 and the L-enantiomer was the first-migrating one for both amino acids. The reversal in the enantiomeric migration order observed in this work with the dual system formed by γ-CD and L-CarnitineC_1NTf_2 was in agreement with the phenomenon described for the first time in a previous work by our research group when using the ionic liquid (R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethane-sulfon)imidate ($EtCholNTf_2$) combined with γ-CD in the separation buffer [23]. In this case, the interactions between the analyte (FMOC-Hcy) and the CD in absence and presence of $EtCholNTf_2$ were investigated by NMR [42]. According to the results obtained, $EtCholNTf_2$ seemed to be partially inserted into the γ-CD cavity when both selectors were used together in the separation buffer modifying the interactions present in the system formed by Hcy and γ-CD. Bearing in mind these previous results and considering that other works have also suggested that the presence of the CIL ions can alter the analytes-CD interactions due to the own interactions of CILs ions with the CD [39-43], it could be expected that the presence of the CIL L-CarnitineC_1NTf_2 in the separation buffer can modify the chiral recognition of the CD towards the analytes in the dual system compared to that existing in a single system. As a consequence, all these arguments could justify

290 the reversal observed in the enantiomeric migration order with the new CIL L-
291 CarnitineC_1NTf_2.
292
293 **3.5 Evaluation of the combined use of γ-CD and L-CarnitineC1L-Lactate as dual**
294 **system**
295 The combined use of γ-CD and the ionic liquid L-CarnitineC_1L-Lactate synthesized in
296 this work was also evaluated for the enantiomeric separation of FMOC-Hcy and FMOC-
297 Cys. When this CIL was added at concentrations in the range from 2 to 20 mM to a 50
298 mM phosphate buffer at pH 7.0 containing a fixed concentration (2 mM) of γ-CD, the Rs
299 did not improve for Hcy with respect to the use of γ-CD as the sole chiral selector in the
300 separation buffer (Rs 1.0). In fact, as shown in **Table 2,** even the enantiomeric migration
301 order for Hcy was the same as that observed when using γ-CD as the sole chiral selector
302 (the D-form was the first-migrating enantiomer) [23] suggesting that the presence of the
303 CIL does not alter the enantiorecognition process between γ-CD and FMOC-Hcy. In the
304 case of FMOC-Cys, Rs slightly improved (from Rs 3.0 to 3.8) with the addition of L-
305 CarnitineC_1L-Lactate (see **Table 2**) and similar results as those observed for L-
306 CarnitineC_1NTf_2 combined with γ-CD were obtained (L-FMOC-amino acids were the
307 first-migrating enantiomers). Thus, a reversal in the enantiomeric migration order was
308 observed for FMOC-Cys with respect to the results obtained when γ-CD was employed
309 as the sole chiral selector in the separation buffer (see **Figure 4C**) but this was not the
310 case for FMOC-Hcy.
311 These results show that the anionic part of the CIL took an important role in the
312 recognition mechanism. In this way, NTf_2 appears to be a very interesting anionic
313 counterpart in amino acid-based chiral ionic liquids to generate synergistic effects.
314

315 **3.6 Simultaneous enantiomeric separation of FMOC-Hcy and FMOC-Cys**

316 Bearing in mind that the two thiols amino acids analyzed in this work are implied in the
317 metabolic pathway of biothiols, it is interesting to investigate the possibility to carry out
318 their simultaneous enantiomeric separation. As it can be deduced from the data shown in
319 **Table 2**, under the experimental conditions employed, the simultaneous separation of the
320 four enantiomers was not possible as their migration times were too close. However, a
321 change in the temperature (15ºC instead of 20ºC) and applied voltage (30 kV instead of
322 20 kV) allowed a variation in the migration times enabling to carry out the simultaneous
323 enantioseparation of FMOC-Hcy and FMOC-Cys (see **Figure 5)**. This is the first time
324 that the enantiomers of these two thiols amino acids are simultaneously separated
325 showing the potential of the analytical methodology developed using the new ionic liquid
326 synthesized in this work.

327

328 **4. Conclusions**

329 The ionic liquid L-CarnitineC_1NTf_2 synthesized in this work has demonstrated its
330 potential to generate a synergistic effect when used in combination with γ-CD for the
331 enantiomeric separation of FMOC-Hcy and FMOC-Cys. Enantiomeric resolutions of 6.1
332 and 6.4 were obtained for FMOC-Hcy and FMOC-Cys respectively. The reversal in the
333 enantiomeric migration order observed for both amino acids when this CIL was combined
334 with γ-CD, with respect to the use of the CD as the sole chiral selector in the separation
335 buffer, suggested that the CIL is implied in the recognition mechanism. Moreover, the
336 fact that no or small synergistic effect was observed for FMOC-Hcy and FMOC-Cys
337 when using the CIL L-CarnitineC_1L-Lactate combined with γ-CD and that no reversal in
338 the enantiomeric migration order took place for FMOC-Hcy when this CIL was combined
339 with γ-CD (compared to the use of γ-CD alone in the separation buffer), suggested that

340 NTf_2, as anionic counterpart, could play and important role in the interactions involved
341 in the chiral recognition. The use of 2 mM γ-CD combined with 5 mM L-CarnitineC_1NTf_2
342 in a 50 mM phosphate buffer at pH 7.0 at a temperature of 15 ºC and an applied voltage
343 of 30 kV enabled to achieve for the first time the simultaneous enantiomeric separation
344 of both FMOC-amino acids with high Rs (5.9 for Hcy and 4.1 for Cys) and short analysis
345 time (9 min).
346 The results obtained in this work are in agreement with those previously obtained by our
347 research group when using another amino acid based CIL such as Et$CholNTf_2$ for the
348 enantiomeric separation of FMOC-Hcy since a synergistic effect and a reversal migration
349 order were also observed in that case suggesting the important role that the anionic
350 counterpart NTf_2 can play in the chiral recognition mechanism of these thiol amino acids.
351

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Figure captions.

Figure 1. A) Synthesis of L-carnitine methyl ester bis(trifluoromethane)sulfonimide (L-CarC_1NTf_2). B) Scheme of the synthesis of L-carnitine methyl ester L-Lactate (L-CarC_1L-Lactate). 1, L-carnitine hydrochloride; 2, L-carnitine methyl ester; 3, L-carnitine methyl ester Bis(trifluoromethane)sulfonimide; 4, L-carnitine methyl ester L-(+) lactate.

Figure 2. Electropherograms corresponding to the separation of (A) FMOC-Hcy and (B) FMOC-Cys using a dual system based on the combination of γ-CD (2 mM) and increasing concentrations of L-CarnitineC_1NTf_2. Experimental conditions: 50 mM phosphate buffer (pH 7.0); uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) x 50 μm ID; UV detection at 210 nm; applied voltage, 20 kV; temperature 20°C; injection by pressure, 50 mbar for 4s.

Figure 3. Electropherograms corresponding to the enantiomeric separation of (A) FMOC-Hcy and (B) FMOC-Cys in 50 mM phosphate buffer pH 7.0 containing 2 mM γ-CD plus different concentrations of L-CarnitineC_1NTf_2. Other experimental conditions as in Fig 2.

Figure 4. Electropherograms corresponding to the enantiomeric separation of FMOC-Hcy and FMOC-Cys under the best experimental conditions. A) BGE: 2 mM γ-CD + 5 mM L-CarnitineC_1NTf_2 in phosphate buffer pH 7.0. B) BGE: 2 mM γ-CD + 5 mM L-CarnitineC_1NTf_2 in borate buffer pH 9.0. C) BGE: 2 mM γ-CD + 5 mM L-CarnitineC_1L-Lactate in phosphate buffer pH 7.0. Other experimental conditions as in Fig 2.

Figure 5. A) Electropherogram corresponding to the enantiomeric separation of FMOC-Hcy and B) Electropherogram corresponding to the simultaneous separation FMOC-Hcy and FMOC-Cys. Experimental conditions: BGE: 2 mM γ-CD + 5 mM L-CarnitineC_1NTf_2 in 50 mM phosphate buffer (pH 7.0); uncoated fused-silica capillary, 58.5 cm (50 cm to

520 the detector window) x 50 μm ID; UV detection at 210 nm; applied voltage, 30 kV;
521 temperature 15°C; injection by pressure, 50 mbar for 4s.
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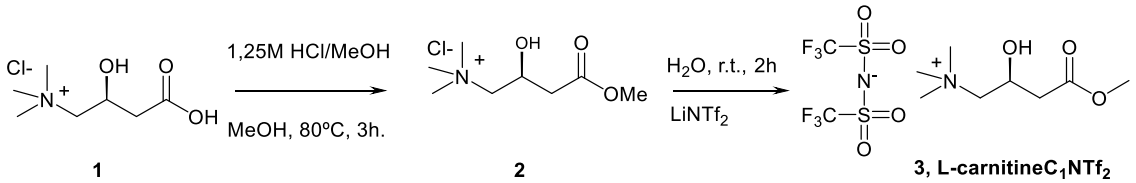
Figure 1.

A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O
OH
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MeOH, 80ºC, 3h.
OMe
H2O, r.t., 2h
LiNTf2
F3C
S
N-
1
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3, L-carnitineC1NTf2

B

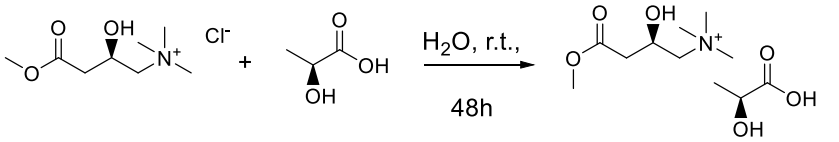
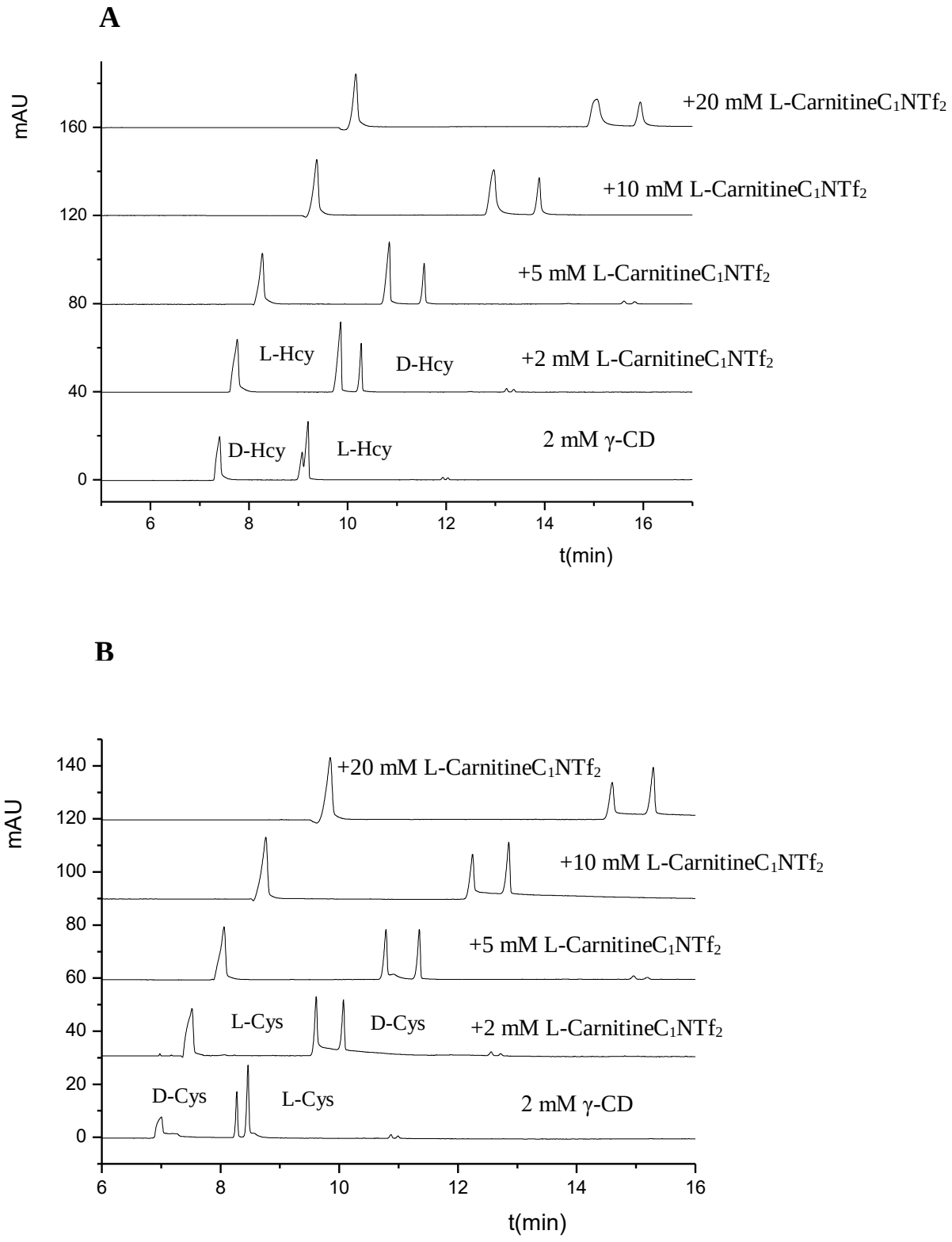

O
OH
Cl-
+
H2O, r.t.,
48h
2
L-(+) lactic acid
4, L-carnitineC1-L-(+)-lactate

Figure 2.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mAU
160
120
80
40
0
+20 mM L-CarnitineC1NTf2
+10 mM L-CarnitineC1NTf2
+5 mM L-CarnitineC1NTf2
L-Hcy
D-Hcy
+2 mM L-CarnitineC1NTf2
D-Hcy
L-Hcy
2 mM γ-CD
6
8
10
12
14
16
t(min)
B
mAU
140
120
100
80
60
40
20
0
+20 mM L-CarnitineC1NTf2
+10 mM L-CarnitineC1NTf2
+5 mM L-CarnitineC1NTf2
L-Cys
D-Cys
+2 mM L-CarnitineC1NTf2
D-Cys
L-Cys
2 mM γ-CD
6
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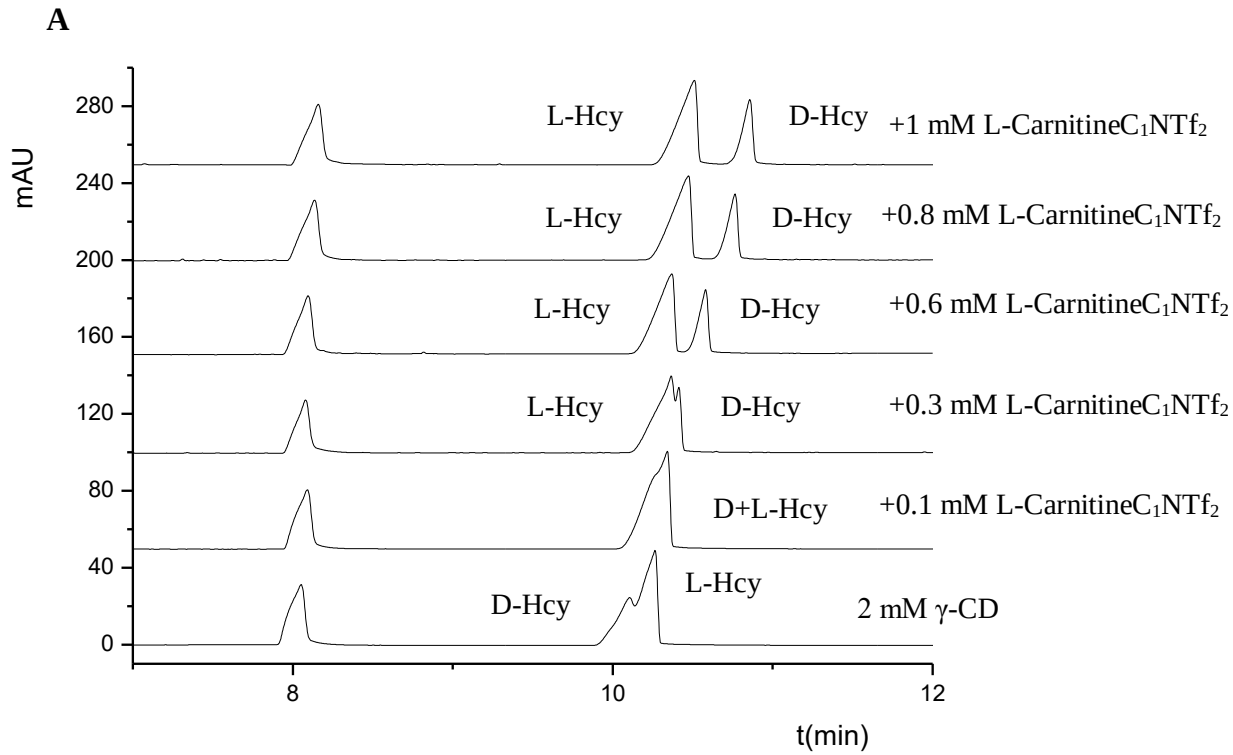
t(min)

563 **Figure 3.**



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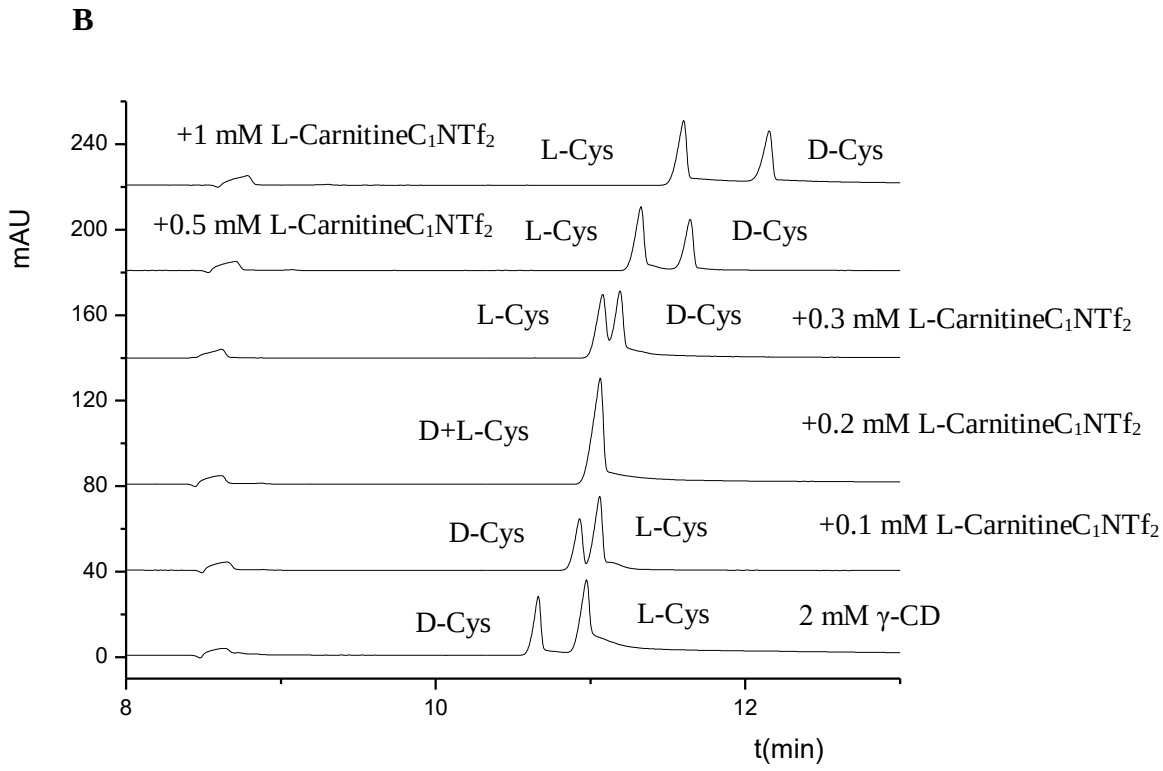

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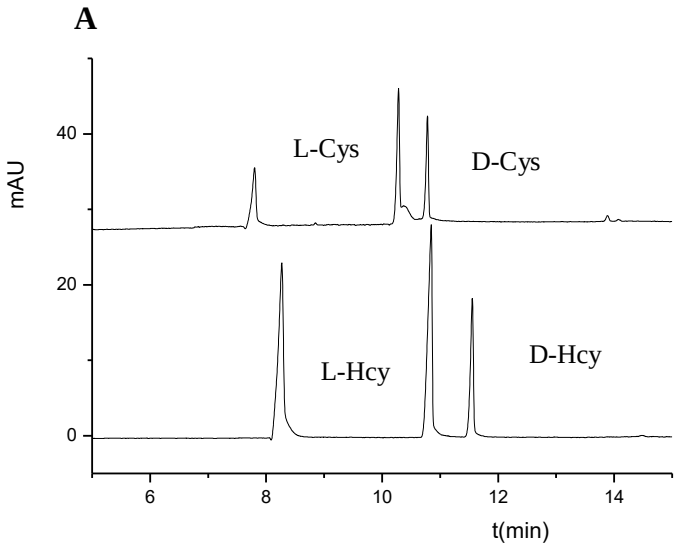
567 **Figure 4.**



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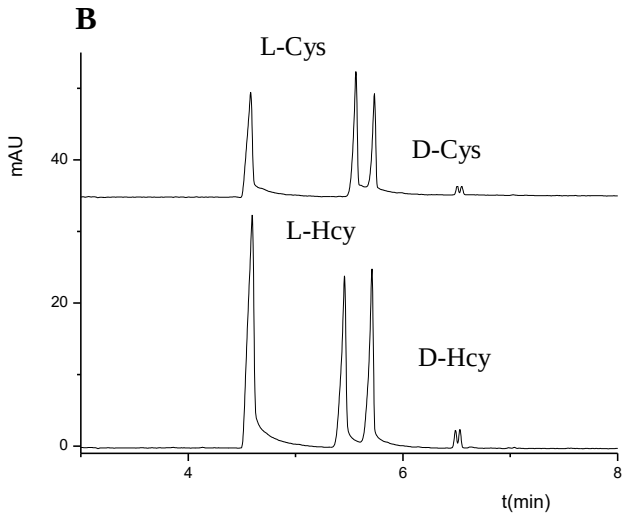

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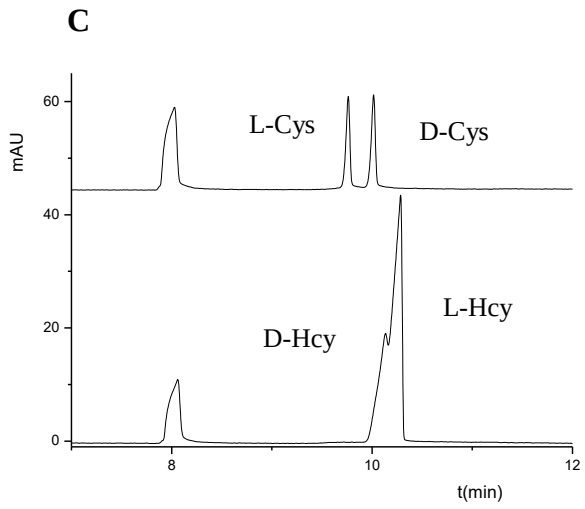

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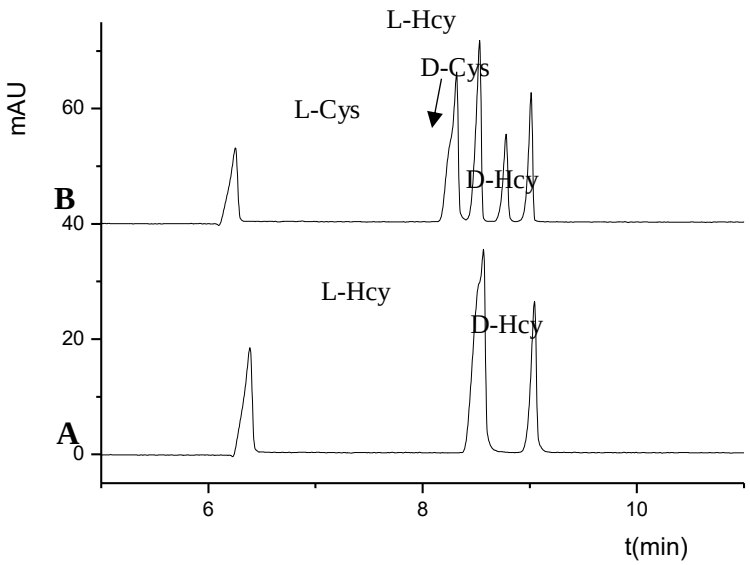
572 **Figure 5.**



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586 **Table 1.** Influence of γ-CD concentration on the migration time and the enantiomeric
587 resolution for FMOC-Cys.

	γ-CD (mM)	t_1 (min)	t_2 (min)	R_s	Enantiomer Migration Order
50 mM Phosphate buffer pH 7.0	1	9.53	9.74	2.3	D - L
	2	9.26	9.49	3.0	D - L
	5	8.76	8.95	2.6	D - L
	10	8.27	8.39	1.8	D - L
	15	7.79	7.88	1.3	D - L

588 Other CE conditions: UV detection at 210 nm; temperature, 20ºC; applied voltage, 20 kV;
589 injection by pressure, 50 mbar for 4 s.
590 Rs: resolution; t_1: time of the first-migrating enantiomer. t_2: time of the second-migrating
591 enantiomer.
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Table 2. Migration times, resolution and enantiomeric migration order for homocysteine and cysteine using different dual chiral systems.

Analyte		Homocysteine					Cysteine				
	Chiral selector	**[CS](mM)**	**t_1 (min)**	**t_2 (min)**	**R_s**	**Enantiomer Migration order**	**[CS](mM)**	**t_1 (min)**	**t_2 (min)**	**R_s**	**Enantiomer Migration order**
50 mM Phosphate buffer pH 7.0	**Dual system 2 mM γ-CD + L-CarnitineC$_1$NTf$_2$**	2	9.86	10.28	3.8	L - D	2	9.61	10.07	6.1	L - D
		5	10.85	11.55	6.1		5	10.79	11.35	6.4	
		10	12.97	13.89	5.2		10	12.25	12.86	5.6	
		20	15.06	15.94	3.4		20	14.60	15.29	5.4	
	Dual system 2 mM γ-CD + L-CarnitineC$_1$L-Lactate	2	10.39	10.55	0.9	D - L	2	9.97	10.24	3.6	L - D
		5	10.13	10.29	0.8		5	9.76	10.02	3.5	
		10	10.38	10.54	0.8		10	9.97	10.22	3.5	
		20	11.70	-	-		20	11.11	11.43	3.8	
50 mM Borate buffer pH 9.0	**Dual system 2 mM γ-CD + L-CarnitineC$_1$NTf$_2$**	2	5.01	5.20	2.2	L - D	2	5.07	5.24	3.0	L - D
		5	5.46	5.71	3.6		5	5.56	5.73	2.9	
		10	6.12	6.41	2.0		10	6.26	6.44	2.4	
		20	7.33	7.62	1.3		20	7.57	7.78	2.6	

Other CE conditions: UV detection at 210 nm; temperature, 20ºC; applied voltage, 20 kV; injection by pressure, 50 mbar for 4 s.
Rs: resolution; t_1: time of the first-migrating enantiomer. t_2: time of the second-migrating enantiomer.