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Synthesis and characterization of carnitine-based ionic liquids and their evaluation as additives in cyclodextrin-electrokinetic chromatography for the chiral separation of thiol amino acids

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ABSTRACT

In this study, new chiral ionic liquids based on the non-protein amino acid L-carnitine as cationic chiral counterpart and several anions (bis(trifluoromethane)sulfonimide (NTf_2^-), L-lactate$^-$ or Cl^-) as counterions were synthesized. Moreover, three different salts based on L-carnitine were also synthesized and the other three were commercially acquired and used for comparison. The synthesized ionic liquids and salts were characterized by nuclear magnetic resonance, fourier transform infrared spectroscopy, high-performance liquid chromatography-mass spectrometry, and elemental analysis. Subsequently, they were used as additives to establish a γ-CD-based dual system for the enantiomeric separation of cysteine and homocysteine (previously derivatized with fluorenylmethoxycarbonyl chloride) by capillary electrokinetic chromatography. The effect of the nature of the anionic counterions and the presence of different substituents on the L-carnitine molecule on the chiral separation of both amino acids was investigated. The enantioseparations obtained with each dual system studied were compared in terms of the enantiomer effective mobilities (μ_{eff}) and the effective electrophoretic selectivity (α_{eff}). Practically, all the dual systems evaluated exhibited substantially improved enantioseparations for the two amino acids compared with the single γ-CD system.

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1. Introduction

The enantiomeric separation of chiral compounds is an important issue that has a great interest in several fields such as clinical, pharmaceutical, or food analysis due to the different pharmacological, toxicological, or biological activities of each enantiomer [1–4]. That is why the development of new chiral methodologies is so necessary. High-performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE) are the separation techniques most used to achieve chiral separations. Among them, CE has shown remarkable characteristics, mainly in the capillary electrokinetic chromatography mode (EKC). The low volume of samples and reagents used, the ease of changing the chiral selector added to the separation buffer, and the high separation efficiency in a short analysis time, make EKC a great alternative to perform chiral separations [5–7]. Currently, the search for new compounds that can be used as chiral selectors in EKC is of high interest since the classic chiral selectors (cyclodextrins, antibiotics, crown ethers, surfactants, etc.) could have some limitations such as low solubility, complicated synthesis, or a high cost. In some cases, satisfactory enantioseparations are not obtained using a single chiral selector so that the use of different additives or the combination of chiral selectors (dual systems) to enhance the enantioselectivity is needed [8–10].

The search for new environmentally sustainable chiral selectors came up with ionic liquids (ILs), which are organic molten salts with a melting point below 100 °C [11]. This group of compounds is characterized for having negligible vapor pressure, good thermal

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stability, high conductivity, and miscibility in different solvents. Also, their synthesis is simple from cheap and non-toxic compounds, so their use is spreading in areas such as sample preparation (extraction), electrochemical or chromatographic techniques [9,12,13]. Within the group of ILs, chiral ionic liquids (CILs) contain in their structure at least one chiral center (in the anion, cation, or both) which make them attractive as chiral selectors in EKC [8,13–15].

A simple and low-cost way to obtain CILs is the use of amino acids as anions or cations in their synthesis [16]. In the last years, several studies about the use of amino acid-based CILs in chiral EKC have been reported in the literature [17–30]. Most of these studies were based on the combined use of amino acid-based CILs with cyclodextrins. Usually, the employed CILs were composed of different lengths of tetraalkylammonium chains (such as tetramethylammonium or tetrabutylammonium) as cations and amino acids (arginine, lysine, aspartic and glutamic acids, etc.) as anions [18,19–23]. CILs composed of tetraalkylammonium or alkyl imidazolium chains as cations and amino acids as anions have also been employed in ligand exchange CE (LE-CE) [18,24,25]. Ren *et al.*, studied the synergistic effect between β-cyclodextrin and different tetraalkylammonium amino acid ionic liquids or the chiral recognition of the CILs as sole chiral selector by non-aqueous capillary electrophoresis (NACE) [26]. Several studies were carried out using CILs in which the cations are the amino acids, and the anion are lactate or bis(trifluoromethane)sulfonimide (NTf_2^-) and they are used as sole chiral selectors, and mainly, in dual systems (such as cyclodextrins) [18,27–30]. The most popular amino acids that are part of the CILs employed in CE are protein amino acids such as L-arginine, L-alanine, L-asparagine, L-lysine, or L-glutamic acid [18–28]. However, a few non-protein amino acids, such as L-ornithine, L-carnitine, or L-hydroxyproline, have been also used as CILs constituents [18,29,30].

Our research group has previously synthesized the chiral ionic liquid [2(2R)-2–hydroxy-4–methoxy-4-oxo–butyl]-trimethylammonium bis(trifluoromethane)sulfonamide ([L-carnOMe][NTf_2]) and evaluated its combination with γ-CD as chiral selector in EKC [29,30]. As a result of these studies, the enantiomeric separation of sulfur-containing amino acids such as cysteine (DL-Cys) and homocysteine (DL-Hcy) was achieved [29]. These amino acids have important implications in both biological and metabolic processes in the human body. The addition of the CIL ([L-carnOMe][NTf_2]) to the background electrolyte (BGE) with γ-CD significantly improved the enantiomeric resolution for both amino acids and reversed the migration order of both enantiomers [29]. Moreover, this dual system allowed to quantify DL-Cys in food supplements after improving the analysis time (faster analysis) through an optimization of the CE conditions [30]. In these previous works, [L-carnOMe][NTf_2] and [2(2R)-2–hydroxy-4–methoxy-4-oxo–butyl]-trimethyl-ammonium L-lactate ([L-carnOMe][L-lactate]) chiral ionic liquids were synthesized for the first time. Moreover, their use in EKC as chiral selectors or in combination with γ-CD for the enantioseparation of DL-Cys and DL-Hcy was described [29,30].

This work aimed to perform a comprehensive systematic study to evaluate the role of different L-carnitine-based ionic liquids and L-carnitine-derived salts as additives in a γ-CD-based dual system for the enantiomeric separation of two thiol amino acids (DL-Cys and DL-Hcy) of biological relevance by EKC. To do that, seven CILs, one achiral IL, and three different salts in which NTf_2^-, L-lactate$^-$ or Cl^- were employed as counterions, were originally synthesized and characterized. Subsequently, the effect of the nature of the anionic counterions as well as the type of substituent on the L-carnitine molecule on the chiral separation of DL-Cys and DL-Hcy when using dual systems constituted by each of the synthesized compound and γ-CD was investigated.

2. Materials and methods

2.1. Reagents and samples

All the chemicals and reagents used in this work had analytical grades and were used as received without extra purification. Water employed was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Boric acid, sodium hydroxide, bis(trifluoromethane)sulfonamide lithium salt ($LiNTf_2$), 9-fluorenylmethoxycarbonyl chloride (FMOC), (3-carboxypropyl)trimethylammonium chloride (also named deoxy-carnitine hydrochloride ([Dx-carnOH]Cl)), *O*-acetyl-L-carnitine hydrochloride ([Ac- L-carnOH]Cl), 2 M HCl in diethyl ether, methanol (MeOH), ethanol (EtOH), propan-2-ol (*i*PrOH) and butanol (*n*BuOH), sodium L-(+)-lactate, DL- and L-cysteine, and DL- and L-homocysteine were purchased from Sigma (Saint-Louis, MO, USA). The structures of both amino acids are shown in Fig. S1A of supplementary material. Di-sodium hydrogen phosphate anhydrous and lithium chloride were obtained from Panreac (Barcelona, Spain). γ-Cyclodextrin (γ-CD) was from Fluka (Buchs, Switzerland). Hydrochloric acid (37%, v/v), *n*-pentane, and acetonitrile (ACN) were obtained from Scharlau (Barcelona, Spain). [(2R)-3-carboxy-2–hydroxy-propyl]-trimethyl ammonium chloride (L-carnitine hydrochloride, [L-carnOH]Cl) was from AcrosOrganics (Madrid, Spain).

2.2. Synthesis of L-carnitine-based ionic liquids and L-carnitine-derived salts

All the procedures employed to synthesize the different L-carnitine-based ionic liquids and L-carnitine-derived salts (whose structures are shown in Fig. S1B of supplementary material) were performed under N_2 atmosphere.

L-carnitine-derived salts were synthesized by treatment of 1.0 equimolar solutions of [L-carnOH]Cl in MeOH (3.8 mL), EtOH (4 mL), *i*PrOH (5 mL) or in *n*BuOH (3 mL) with a solution of 1.25 M HCl in MeOH (3.8 mL, 0.0076 mol, 1.0 eq.), EtOH (4.08 mL, 0.0051 mol, 1.0 eq.), *i*PrOH (4.08 mL, 0.0051 mol, 1.0 eq.), or with 3 M HCl in *n*BuOH (1.66 mL, 0.0051 mol, 1.0 eq.). The reaction mixtures were heated to reflux (80 °C) for 6 h. Then, the solvents were removed under reduced pressure obtaining [2(2R)−2–hydroxy-4–methoxy-4-oxo–butyl]-trimethyl-ammonium chloride ([L-carnOMe]Cl, 1.60 g, 100% [29]), [(2R)−4-ethoxy-2–hydroxy-4-oxo–butyl]-trimethyl-ammonium chloride ([L-carnOEt]Cl, 1.15 g, 100%), [(2R)−2hydroxy-4-isopropoxy-4-oxo–butyl]-trimethyl ammonium chloride ([L-carnO*i*Pro]Cl, 1.20 g, 100%), and [(2R)-2hydroxy-4–butyl–4-oxo–butyl]-trimethyl ammonium chloride ([L-carnO*n*Bu]Cl, 1.25 g, 96% [29]), as a white solids or a dense oil, respectively.

The synthesis of the chiral ionic liquids with L-lactate as counteranion was carried out by treatment of equimolar solutions of [L-carnOMe]Cl (1.455 g, 0.0068 mmol, 1.0 eq.) in distilled water (2.0 mL) or [L-carnO*n*Bu]Cl (1.0 g, 0.0039 mmol, 1.0 eq.) in distilled water (1.5 mL) with a solution of sodium lactate (761 mg, 0.0068 mmol, 1.0 eq.) in distilled water (2.0 mL); or a solution of sodium lactate (441.3 mg, 0.0039 mmol, 1.0 eq.) in distilled water (1.5 mL), respectively. Then, the reaction mixtures were stirred at room temperature until the oil formation was observed. After that, they were placed on extraction funnel isolating the dense oily compounds [L-carnOMe][L-lactate] (1.65 g, 91%) and [L-carnO*n*Bu][L-lactate] (0.980 g, 80%), as was described [29].

Equimolar solutions of [L-carnOH]Cl (1.0 g, 0.0051 mol, 1.0 eq.), [L-carnOMe]Cl (3.0 g, 0.014 mmol, 1.0 eq [29].), [L-carnOEt]Cl (0.711 g, 0.0031 mol, 1.0 eq.), [L-carnO*i*Pro]Cl (0.900 g, 0.00375 mol, 1.0 eq.), [L-carnO*n*Bu]Cl (1.0 g, 0.0039 mmol, 1.0 eq [29]) in distilled water (2 mL) were treated with a solution of

1.0 eq. $LiNTf_2$ in distilled water (2 mL). The reaction mixtures were stirred at room temperature until the oil formation was observed. After that, the mixtures were placed on extraction funnel isolating the compounds [L-carnOH][NTf_2] (0.988 g, 43%), [L-carnOMe][NTf_2] (3.65 g, 57% [29]), [L-carnOEt][NTf_2] (0.615 g, 42%), [L-carnO*i*Pro][NTf_2] (0.980 g, 53%) and [L-carnO*n*Bu][NTf_2] (0.886 g, 45% [29]) as a dense oily compounds.

Under the same reaction conditions described above, the treatment of equimolar solutions of 3-carboxypropyl(trimethyl)ammonium chloride ([Dx-carnOH]Cl, 0.388 g, 0.00198 mol, 1.0 eq.) or *O*-acetyl-L-carnitine hydrochloride ([Ac-L-carnOH]Cl, 0.5 g, 0.0021 mol, 1.0 eq.) in distilled water (1 mL) with an equimolar solution of $LiNTf_2$ in distilled water (1 mL) at room temperature afforded the oil formation. After that, the reaction mixtures were placed on extraction funnel isolating the compounds [Dx-carnOH][NTf_2] (0.365 g, 41%) and [Ac-L-carnOH][NTf_2] (0.322 g, 31%) as a dense oily compounds, respectively.

2.3. Characterization of L-carnitine-based ionic liquids and L-carnitine-derived salts

The NMR spectra were recorded in CD_3OD on Varian-300 MHz or 500 MHz instruments with tetramethyl silane (TMS) as an internal standard. The chemical shift (δ) is reported in parts per millions (ppm) and residual un-deuterated solvent peaks were used as internal references for proton (3.36 ppm for CD_3OD) and carbon (49.1 ppm for CD_3OD). ^{1}H NMR coupling constants (J) are reported in Herz (Hz) and the multiplicities are presented as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broad). The IR spectra were recorded on Agilent technologies Cary 630 FTIR spectrometer from 650 to 400 cm^{-1}.

An Agilent Technologies 1260 Infinity LC system coupled to a quadrupole mass spectrometer (Agilent Technologies) equipped with an orthogonal electrospray ionization source (ESI) was employed to carry out LC-MS analyses. A SeQuant ZIC-HILIC column (150 mm and 4.6 mm, 5 µm, Merck-Millipore) and a mobile phase consisting of phase A (200 mM ammonium acetate, pH 5.2) and phase B (acetonitrile: ammonium acetate (200 mM, pH 5.2), 80:20 v/v) in an isocratic method (flow rate of 1 mL/min) were used. The column temperature was 25 °C and the detector was set at 277.4 ± 16 nm.

2.4. CE conditions

A CE 7100 system from Agilent Technologies (Palo Alto, CA, USA) equipped with a spectrophotometric diode array detector (DAD) was employed to carry out all the analyses. The CE system was controlled by the Agilent ChemStation software. Uncoated fused-silica capillaries with a total length of 58.5 cm (effective length of 50 cm) and 50 μm I.D. (capillary O.D. of 360 μm) were employed to perform the chiral separations. Analyses were performed using 20 kV as separation voltage maintaining a working temperature of 20 °C, and a hydrodynamic injection (50 mbar during 4 s). Detection was at 210 ± 4 nm.

New capillaries were rinsed with 1 M NaOH (30 min), Milli-Q water (15 min), and running buffer (1 h) (applying 1 bar) to be conditioned. At the beginning of each working day, the capillaries were conditioned with 0.1 M NaOH (5 min), Milli-Q water (5 min), and running buffer (30 min). Between different BGEs, the capillaries were washed with 0.1 M NaOH (10 min), Milli-Q water (5 min), running buffer (15 min), and BGE (10 min). Between injections, the capillary was conditioned with 0.1 M NaOH (2 min), Milli-Q water (1 min), and BGE (2 min).

2.5. Preparation of BGEs and solutions

Phosphate buffer solution (50 mM) was prepared by dissolving the adequate amount of di-sodium hydrogen phosphate anhydrous in Milli-Q water and adjusting the pH to 7.0 with an aqueous 1 M HCl solution. BGEs containing 2 mM γ-CD were prepared by dissolving the appropriate amount of γ-CD in 50 mM phosphate buffer, whereas the BGEs containing L-carnitine-derived salts, $LiNTf_2$, or CILs were prepared by dissolving the adequate amount of each of these compounds in phosphate buffer solution or buffer solution containing γ-CD. The pH values of each BGE were checked after the preparation, and all the values fitted with the buffer pH of 7.0.

Borate buffer solution (200 mM) was prepared dissolving the appropriate amount of boric acid in Milli-Q water and adjusting the pH to a value of 9.0 with an aqueous 1 M NaOH solution. Stock standard solutions of each amino acid were prepared dissolving each one in 200 mM borate buffer (pH 9.0) to obtain a final concentration of 10 mM (namely 7 mM of L-enantiomer and 3 mM of D-enantiomer). All these standard solutions were stored at −20 °C.

Before CE analysis, solutions of amino acids were derivatized using a protocol previously described in the literature [29]. Briefly, 200 μL of 30 mM FMOC (prepared in ACN) were mixed with 200 μL amino acid stock standard solution. After 2 min, 0.5 mL *n*-pentane was added to the solution to stop the derivatization process and remove the excess of FMOC. Then, 60 μL of the aqueous phase were diluted to a final volume of 600 μL with MilliQ-water.

Before CE analysis, all working solutions were filtered through 0.45 µm Nylon syringe filters (GVS, Sanford, ME, USA) and sonicated to avoid air bubbles.

2.6. Data treatment

Migration times and resolution values of each peak were obtained using the ChemStation software from Agilent Technologies. Origin 8.0 software was employed in the composition of electropherograms figures and ChemDraw Professional 15.0 was used to draw the structures of all the compounds used in this work. The AlogP values have been calculated by using "BioviaDraw" 2018 (Dassault System), which calculates AlogP from atomic methods taking into account the contribution of each atom present in the molecule and is used as an orientation to establish a scale of hydrophobic compounds. Some figures and all calculated data were treated with Microsoft Excel 365.

The real mobilities for the EOF, enantiomers 1 and 2 (μ_{rEOF}, μ_{r1} and μ_{r2}) were obtained with the Eq. (1):

$$\mu_r = \frac{l \cdot L}{t \cdot V} \tag{1}$$

where l is the effective length (m) and L is the total length (m) of the capillary, t is the migration time (s) of each enantiomer and the EOF, and V is the applied voltage (V).

The effective mobilities (μ_{eff1} and μ_{eff2}) of each enantiomer were calculated using Eq. (2):

$$\mu_{eff} = \mu_r - \mu_{rEOF} \tag{2}$$

and the effective electrophoretic selectivity (α_{eff}) was calculated according to Eq. (3):

$$\alpha_{eff} = \frac{\mu_{eff1}}{\mu_{eff2}} \tag{3}$$

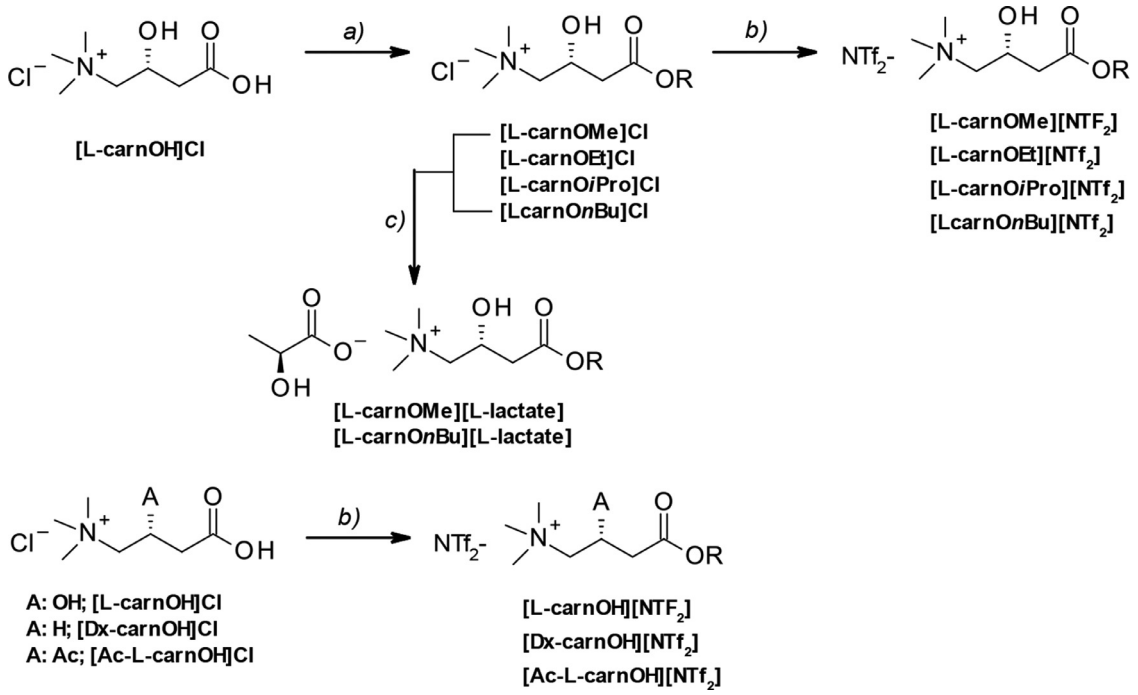

Fig. 1. Synthetic procedure employed for L-carnitine derivatives ionic liquids and salts. Reagents and conditions: a) HCl in alcoholic media, 80 °C, 6 h; b) $LiNTf_2$ (1.0 eq.), distilled water H_2O, room temperature, 4 h; c) [L-lactate]Na (1.0 eq.), distilled water H_2O, room temperature, 4 h.

3. Results and discussion

3.1. Synthesis and characterization of L-carnitine-based ionic liquids and L-carnitine-derived salts

In this work, different L-carnitine-based ionic liquids and L-carnitine-derived salts were synthesized using [L-carnOH]Cl, [Dx-carnOH]Cl, and [Ac-L-carnOH]Cl as starting materials (Fig. 1).

The corresponding L-carnitine esters were obtained in a good yield (> 90%) by refluxing of [L-carOH]Cl with an acidic solution of MeOH, EtOH, *i*PrOH, and *n*BuOH. Each isolated compound is treated with equimolecular amounts of bis(trifluoromethane)sulfonyl imide lithium salt under anion methathesis reactions conditions affording the CILs with moderate yields (40–50%) [31–33]. The reaction of [L-carnOMe]Cl and [L-carnO*n*Bu]Cl with sodium L-lactate in aqueous media yielded the CILs [L-carnOMe][L-lactate] and [L-carnO*n*Bu][L-lactate] in a good yield (80–90%). When the salts [Dx-carnOH]Cl and [Ac-L-carnOH]Cl, used as starting materials, were treated with equimolecular amounts of bis(trifluoromethane)sulfonyl imide lithium salt under the experimental conditions described above, the ionic liquid [Dx-carnOH][NTf_2] and the chiral ionic liquid [Ac-L-carnOH][NTF_2] were obtained with 41% and 31% yield, respectively. All the synthesized compounds were characterized by their physical-chemical properties (Table 1) as well as by MS, FTIR, NMR (^1H and ^{13}C), and elemental analysis. Results indicated that the proposed structures were consistent with their obtained chemical structure. The analytical data are reported in Table S1 of supplementary material.

As can be seen in Table 1, all the compounds in which NTf_2^- or L-lactate$^-$ were used as anion gave rise to the formation of CILs whereas when Cl^- was employed, all the compounds must be considered salts (except [L-carnO*n*Bu]Cl) since their melting points were above 100 °C. From the calculated AlogP values, which is a measure of how hydrophilic or hydrophobic a molecule is, different trends in the solubility of the compounds in water could be observed. On the one hand, an increase in the chain length of the ester group (methyl < ethyl < propyl < butyl) afforded compounds with higher AlogP indicating that these compounds prefer the organic phase instead aqueous phase, decreasing their solubility in the second phase. On the other hand, the anion used in the formation of the different L-carnitine-based ionic liquids and L-carnitine-derived salts had also an influence on the polarity. In this case, as it can be observed in Table 1, the presence of NTf_2^-made the salts and ionic liquids more insoluble than those obtained using L-lactate$^-$ or Cl^- (AlogP < 0). These AlogP values will have an impact on the behavior of such compounds as chiral selectors in dual systems as it will be indicated later.

3.2. Evaluation of the potential of L-carnitine-based ionic liquids and L-carnitine-derived salts as additives in cyclodextrin-electrokinetic chromatography

Previous results obtained by our research team demonstrated a synergistic effect between γ-CD and two L-carnitine-based ionic liquids, namely [L-carnOMe][NTf_2] and [L-carnOMe][L-lactate], in the chiral separation of DL-Cys and DL-Hcy (previously derivatized with FMOC–Cl) by EKC [29]. In the present work, the research is focused on studying the effect that different cationic or anionic substituents of L-carnitine-based ionic liquids may have on the enantiomeric separation of these two thiol amino acids. Thus, a considerable group of ionic liquids and salts formed by the combination of L-carnOH$^+$ derivatives with three different anions (Cl^-, L-lactate$^-$ and NTf_2^-) were investigated as additives in EKC. All these experiments were performed using dual systems in which 2 mM γ-CD was combined with 5 mM of each of the ionic liquids or salts in 50 mM phosphate buffer (pH 7.0). Other CE conditions have been described in Section 2.4.

The results obtained in all the analyses performed using the different duals systems were compared with the enantiomeric resolution reached when γ-CD was employed as the sole chiral selector in the separation media. In this scenario, cysteine enantiomers were separated with a resolution value of 2.67 (Fig. 2A and Table 2)

Table 1
Physical-chemical properties of all ionic liquids and salts investigated in this work.

Compound	Yield (%)	AlogP	Melting point (°C)	Type of compound
[L-carnOH]Cl [a)]	–	−4.4894	138–142	Salt
[L-carnOMe]Cl [b)]	100	−4.2638	107	Salt
[L-carnOEt]Cl [c)]	100	−3.915	116	Salt
[L-carnO*i*Pro]Cl [c)]	100	−3.5375	122	Salt
[L-carnO*n*Bu]Cl [b)]	96	−2.9351	< RT	CIL
[Dx-carnOH]Cl [a)]	–	−3.6575	220	Salt
[Ac-L-carnOH]Cl [a)]	–	−3.8847	194	Salt
[L-carnOH][NTf_2] [c)]	43	1.2998	< RT	CIL
[L-carnOMe][NTf_2] [b)]	49	1.5254	< RT	CIL
[L-carnOEt][NTf_2] [c)]	42	1.8742	< RT	CIL
[L-carnO*i*Pro][NTf_2] [c)]	53	2.2517	< RT	CIL
[L-carnO*n*Bu][NTf_2] [b)]	45	2.8541	< RT	CIL
[Dx-carnOH][NTf_2] [c)]	41	2.8541	< RT	IL
[Ac-L-carnOH][NTf_2] [c)]	31	1.6789	< RT	CIL
[L-carnOMe][L-lactate] [b)]	91	−3.4564	< RT	CIL
[L-carnO*n*Bu][L-lactate] [b)]	80	−2.1277	< RT	CIL
NTf_2^- (as Li^+ salt) [a)]	–	3.1155	234–238	Salt
Cl^- (as Li^+ salt) [a)]	–	−2.6737	605	Salt
L-lactate$^-$ (as Na^+ salt) [a)]	–	−1.8663	163–164	Salt

CIL, chiral ionic liquid; IL, ionic liquid; RT, room temperature.
a) Commercial product.
b) Previously synthesized in our research group [25].
c) Synthesized in this work.

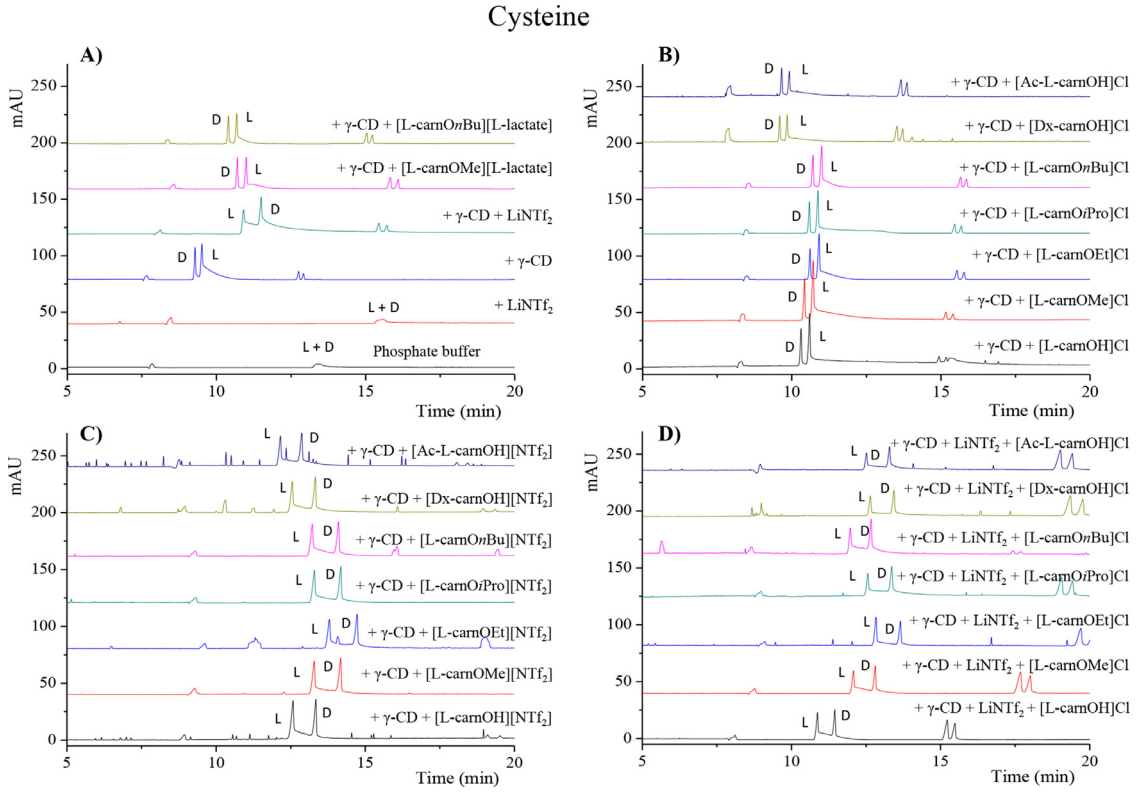

Fig. 2. Electropherograms corresponding to the enantioseparation of DL-Cys using different BGEs. A) γ-CD single systems and dual systems based on the use of γ-CD plus CILs of [L-lactate] as anions, B) dual systems of γ-CD plus salts or CIL with Cl^- as anion, C) dual systems of γ-CD plus CILs with NTf_2^- as anion, D) dual systems of γ-CD plus salts or CIL with Cl^- as anion and the salt $LiNTf_2$. CE conditions: 50 mM phosphate buffer (pH 7.0); uncoated fused-silica capillary, 50 μM I.D. (360 μm O.D.) x 58.5 cm (50 cm of effective length); UV detection at 210 ± 4 nm; temperature 20 °C; applied voltage, 20 kV; injection by pressure, 50 mbar for 4 s.

whereas homocysteine enantiomers were partially separated with a resolution value < 0.7 (Fig. 3A and Table 3).

3.2.1. Effect of the substituents nature on L-carnitine molecule

To evaluate the effect that could cause different L-carnitine cations on the discrimination power of the dual system, two different studies were performed. In the first study, the influence of the alkyl chain length of the ester group in the cationic part, such as in $[L\text{-carnOMe}]^+$, $[L\text{-carnOEt}]^+$, $[L\text{-carnO}i\text{Pro}]^+$, $[L\text{-carnO}n\text{Bu}]^+$ in comparison with $[L\text{-carnOH}]^+$, was investigated. This study was performed both, in L-carnitine-based ionic liquids wearing NTf_2^- counterion and in L-carnitine-derived salts, containing Cl^- as counterion. The second study was focused on establishing the influence of the hydroxyl group present on L-carnitine. For this purpose, two different strategies were carried out. In the first one, the chiral OH group present in $[L\text{-carnOH}]^+$ was protected with an acetyl-

Table 2
Average values for resolutions, migration times, and electrophoretic mobilities ($n = 3$) of cysteine enantiomers by EKC using different chiral selector systems.

BGE	t_{EOF} (min)	t_1 (min)	t_2 (min)	μ_{rEOF} (x10^{-9} $m^2s^{-1}V^{-1}$)	μ_{eff1} (x10^{-9} $m^2s^{-1}V^{-1}$)	μ_{eff2} (x10^{-9} $m^2s^{-1}V^{-1}$)	Resolution	α_{eff}
50 mM phosphate buffer (pH 7.0)	7.89	13.33	–	30.89	−12.61	−12.61	–	1.000
+ $LiNTf_2$	8.45	15.52	–	28.85	−13.14	−13.14	–	1.000
+ γ-CD	7.69	9.31	9.54	31.70	−5.52	−6.15	2.67	1.114
+ γ-CD + $LiNTf_2$	8.13	10.91	11.51	29.98	−7.64	−8.80	4.29	1.152
+ γ-CD + [L-carnOMe][L-lactate]	8.58	10.70	10.99	28.41	−5.63	−6.23	3.47	1.107
+ γ-CD + [L-carnO*n*Bu][L-lactate]	8.52	10.61	10.90	28.61	−5.64	−6.25	3.27	1.108
+ γ-CD + [L-carnOH]Cl	8.30	10.32	10.61	29.37	−5.75	−6.39	3.25	1.112
+ γ-CD + [L-carnOMe]Cl	8.37	10.41	10.69	29.13	−5.71	−6.34	3.11	1.110
+ γ-CD + [L-carnOEt]Cl	8.50	10.60	10.90	28.68	−5.68	−6.31	3.34	1.111
+ γ-CD + [L-carnO*i*Pro]Cl	8.48	10.57	10.86	28.74	−5.68	−6.30	3.21	1.108
+ γ-CD + [L-carnO*n*Bu]Cl	8.56	10.70	10.99	28.48	−5.70	−6.30	3.14	1.106
+ γ-CD + [Dx-carnOH]Cl	7.87	9.58	9.83	30.97	−5.53	−6.18	3.22	1.117
+ γ-CD + [Ac-L-carnOH]Cl	7.96	9.69	9.94	30.62	−5.47	−6.10	3.26	1.116
+ γ-CD + [L-carnOH][NTf_2]	8.93	12.56	13.33	27.30	−7.89	−9.01	5.52	1.142
+ γ-CD + [L-carnOMe][NTf_2]	9.27	13.26	14.14	26.29	−7.91	−9.06	6.10	1.145
+ γ-CD + [L-carnOEt][NTf_2]	9.51	13.59	14.49	25.63	−7.69	−8.81	6.21	1.145
+ γ-CD + [L-carnO*i*Pro][NTf_2]	9.30	13.26	14.14	26.21	−7.83	−8.97	6.17	1.146
+ γ-CD + [L-carnO*n*Bu][NTf_2]	9.29	13.19	14.06	26.24	−7.76	−8.90	6.18	1.147
+ γ-CD + [Dx-carnOH][NTf_2]	8.93	12.51	13.28	27.30	−7.81	−8.94	5.76	1.145
+ γ-CD + [Ac-L-carnOH][NTf_2]	8.76	12.12	12.84	27.83	−7.71	−8.84	5.64	1.146
+ γ-CD + [L-carnOH]Cl + $LiNTf_2$	8.14	10.93	11.52	29.94	−7.64	−8.79	5.95	1.149
+ γ-CD + [L-carnOMe]Cl + $LiNTf_2$	8.79	12.10	12.83	27.73	−7.59	−8.73	6.55	1.151
+ γ-CD + [L-carnOEt]Cl + $LiNTf_2$	9.09	12.79	13.61	26.82	−7.76	−8.91	6.54	1.148
+ γ-CD + [L-carnO*i*Pro]Cl + $LiNTf_2$	8.93	12.46	13.25	27.30	−7.73	−8.90	6.61	1.151
+ γ-CD + [L-carnO*n*Bu]Cl + $LiNTf_2$	8.65	11.94	12.63	28.18	−7.76	−8.88	5.75	1.144
+ γ-CD + [Dx-carnOH]Cl + $LiNTf_2$	9.05	12.73	13.54	26.93	−7.79	−8.93	7.04	1.147
+ γ-CD + [Ac-L-carnOH]Cl + $LiNTf_2$	9.02	12.64	13.43	27.02	−7.74	−8.87	7.10	1.147

The different BGE additives were employed in the following concentrations: 2 mM γ-CD, 5 mM $LiNTf_2$ and 5 mM of CIL, IL or derivatives of L-carnitine salts.
RSD < 4.0% for migration times; RSD < 3.4% for electrophoretic mobilities; RSD < 4.8% for Rs; RSD < 0.4% for α_{eff}.

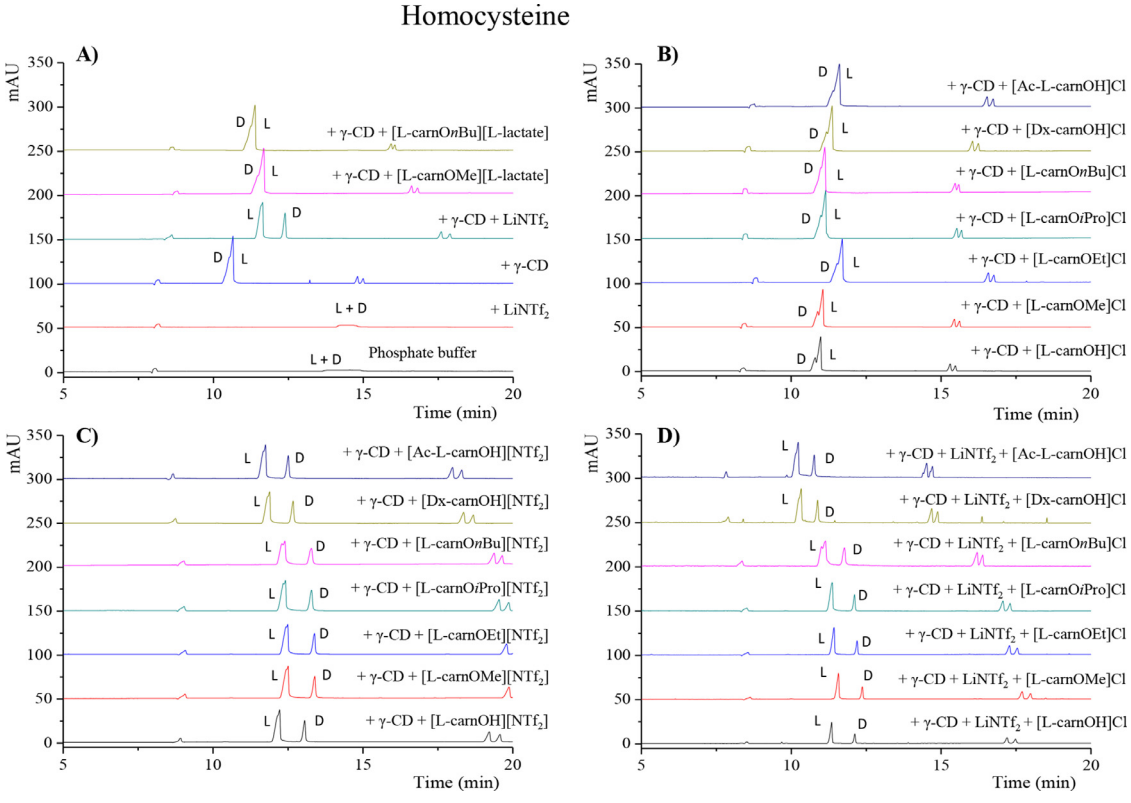

Fig. 3. Electropherograms corresponding to the enantioseparation of DL-Hcy using different BGEs. A) γ-CD single systems and dual systems based on the use of γ-CD plus CILs of [L-lactate] as anions, B) dual systems of γ-CD plus salts or CIL with Cl^- as anion, C) dual systems of γ-CD plus CILs with NTf_2^- as anion, D) dual systems of γ-CD plus salts or CIL with Cl^- as anion and the salt $LiNTf_2$. Other CE conditions as in Fig. 2.

Table 3
Average values for resolutions, migration times, and electrophoretic mobilities ($n = 3$) of homocysteine enantiomers by EKC using different chiral selector systems.

BGE	t_{EOF} (min)	t_1 (min)	t_2 (min)	μ_{rEOF} (x10^{-9} $m^2s^{-1}V^{-1}$)	μ_{eff1} (x10^{-9} $m^2s^{-1}V^{-1}$)	μ_{eff2} (x10^{-9} $m^2s^{-1}V^{-1}$)	Resolution	α_{eff}
50 mM phosphate buffer (pH 7.0)	8.05	14.45	–	30.28	−13.41	−13.41	–	1.000
+ $LiNTf_2$	8.22	14.77	–	29.65	−13.15	−13.15	–	1.000
+ γ-CD	8.13	10.45	10.57	29.98	−6.66	−6.92	0.66	1.040
+ γ-CD + $LiNTf_2$	8.59	11.61	12.35	28.38	−7.38	−8.64	3.72	1.170
+ γ-CD + [L-carnOMe][L-lactate]	8.79	11.47	11.65	27.73	−6.48	−6.81	0.78	1.051
+ γ-CD + [L-carnO*n*Bu][L-lactate]	8.62	11.25	11.37	28.28	−6.61	−6.84	0.55	1.035
+ γ-CD + [L-carnOH]Cl	8.40	10.78	10.96	29.02	−6.41	−6.78	0.97	1.058
+ γ-CD + [L-carnOMe]Cl	8.45	10.87	11.04	28.85	−6.42	−6.77	0.86	1.054
+ γ-CD + [L-carnOEt]Cl	8.83	11.50	11.68	27.60	−6.41	−6.74	0.81	1.051
+ γ-CD + [L-carnO*i*Pro]Cl	8.47	10.97	11.13	28.78	−6.56	−6.88	0.77	1.049
+ γ-CD + [L-carnO*n*Bu]Cl	8.44	10.98	11.10	28.88	−6.68	−6.92	0.60	1.036
+ γ-CD + [Dx-carnOH]Cl	8.58	11.15	11.33	28.41	−6.55	−6.90	0.82	1.053
+ γ-CD + [Ac-L-carnOH]Cl	8.75	11.36	11.57	27.86	−6.40	−6.79	0.83	1.061
+ γ-CD + [L-carnOH][NTf_2]	8.90	12.20	13.03	27.39	−7.41	−8.68	3.99	1.172
+ γ-CD + [L-carnOMe][NTf_2]	9.05	12.48	13.37	26.93	−7.40	−8.70	3.93	1.176
+ γ-CD + [L-carnOEt][NTf_2]	9.03	12.46	13.36	26.99	−7.43	−8.75	4.10	1.177
+ γ-CD + [L-carnO*i*Pro][NTf_2]	9.01	12.39	13.26	27.05	−7.38	−8.67	3.72	1.175
+ γ-CD + [L-carnO*n*Bu][NTf_2]	9.01	12.37	13.25	27.05	−7.35	−8.66	3.15	1.178
+ γ-CD + [Dx-carnOH][NTf_2]	8.73	11.86	12.64	27.92	−7.37	−8.64	3.69	1.172
+ γ-CD + [Ac-L-carnOH][NTf_2]	8.66	11.73	12.49	28.15	−7.37	−8.63	3.59	1.172
+ γ-CD + [L-carnOH]Cl + $LiNTf_2$	8.52	11.35	12.12	28.61	−7.13	−8.50	7.13	1.191
+ γ-CD + [L-carnOMe]Cl + $LiNTf_2$	8.62	11.55	12.35	28.28	−7.17	−8.54	6.65	1.191
+ γ-CD + [L-carnOEt]Cl + $LiNTf_2$	8.58	11.48	12.26	28.41	−7.18	−8.53	6.41	1.188
+ γ-CD + [L-carnO*i*Pro]Cl + $LiNTf_2$	8.52	11.37	12.11	28.61	−7.17	−8.48	4.89	1.183
+ γ-CD + [L-carnO*n*Bu]Cl + $LiNTf_2$	8.36	11.15	11.77	29.16	−7.30	−8.45	2.34	1.158
+ γ-CD + [Dx-carnOH]Cl + $LiNTf_2$	7.89	10.31	10.86	30.89	−7.25	−8.45	3.11	1.165
+ γ-CD + [Ac-L-carnOH]Cl + $LiNTf_2$	7.82	10.21	10.75	31.17	−7.30	−8.50	3.07	1.164

The different BGE additives were employed in the following concentrations: 2 mM γ-CD, 5 mM $LiNTf_2$ and 5 mM of CIL, IL or derivatives of L-carnitine salts.
RSD < 1.8% for migration times; RSD < 2.4% for electrophoretic mobilities; RSD < 5.0% for Rs; RSD < 0.6% for α_{eff}.

protecting group. In the second one, the elimination of the chirality of the asymmetric carbon was investigated Fig. 2B and C show the electropherograms obtained using DL-Cys as model compound whereas Fig. 3B and C report the electropherograms corresponding to the analysis of DL-Hcy.

From the obtained results, it can be established, as a general trend, that an increase in the alkyl chain of L-carnitine ester derivatives (either in NTf_2^- or Cl^-counterions) leads to a slight decrease of the enantiomeric resolutions values and α_{eff}, and an insignificant increase of the migration times (see data in Tables 2 and 3). Comparing the chiral separation obtained which those achieved using a single γ-CD system, it is clear that the addition of these CILs and salts enabled to improve the chiral separation of both amino acids with one exception; the use of the dual system γ-CD + [L-carnO*n*Bu]Cl resulted in a partial resolution (Rs = 0.60) for homocysteine similar to that achieved using only γ-CD. It should be noted that between the two studied groups (i.e. those CILs in which the anion is NTf_2^- and those salts in which Cl^- is used as anion) there is a considerable difference in the values obtained not only for the enantiomeric resolution but also in the α_{eff} (Fig. 4). This fact highlights the role of NTf_2^- in the discrimination of homocysteine and cysteine enantiomers.

The results obtained using as additives ILs and salts in which the asymmetric carbon of $[L\text{-carnOH}]^+$ was modified so that the chirality was lost $[Dx\text{-carnOH}]^+$ or the OH of the chiral group was protected by an acetyl group $[Ac\text{-L-carnOH}]^+$ are shown in Figs. 2B,C, 3B,C, and 4. In general terms, the combined use of [Ac-L-carnOH][NTf_2] or [Ac-L-carnOH]Cl (in which the hydroxyl group is protected by an acetyl group) with γ-CD slightly increased the resolution and the α_{eff} values obtained in the separation of cysteine enantiomers compared to those in which the hydroxyl group is un-protected, [L-carnOH][NTf_2] or [L-carnOH]Cl. Conversely, in the case of DL-Hcy, the resolution values decreased maintaining the α_{eff} practically constant. From these results, it seems that the acetylation of the OH group is not a key parameter for the chiral separation of these two amino acids. Regarding the use of [Dx-carnOH]Cl or [Dx-carnOH][NTf_2], which implies the elimination of the asymmetric carbon from the structure of L-carnitine, both the resolution values and the α_{eff} were very similar to those obtained using L-carnitine as the cationic counterpart of the CILs (see Figs. 2B,C, 3B,C, and 4 as well as Tables 2 and 3). This fact could demonstrate that effectively the chiral separation in all these systems is due to the amino acid-γ-CD interaction and the variation of the separation parameters can be attributed to the presence of the CIL ions which can modify the chiral recognition in the dual system compared to that achieved in the single system. This idea was previously supported by other works suggesting that the interaction of CILs ions with CD can alter the analyte-CD interactions [22,34–37].

Other results that are worthy to be mentioned are related to the enantiomer migration order obtained for both amino acids when their chiral separation is performed using the single CD system or dual systems in which the CD is combined with L-carnitine-based ionic liquids and L-carnitine-derived salts. As it can be observed in the different electropherograms shown in Figs. 2 and 3, the D-enantiomers of both amino acids were the first-migrating enantiomers when the single chiral separation system was employed. This order was kept when the anion Cl^- was used as anionic counterpart in the formation of ionic liquids or salts from L-carnitine. However, a reversal in the enantiomeric migration order was observed (i.e. L-enantiomers migrated first) when γ-CD was combined with CILs based on the use of NTf_2^- as anionic counterpart.

To a more in-depth investigation of the role of the anion NTf_2^-in the chiral separation of DL-Cys and DL-Hcy, different experiments were carried out. First, the addition of 5 mM $LiNTf_2$ to phosphate buffer in absence of γ-CD did not enable to observe defined peaks corresponding to DL-Cys or DL-Hcy. However, when γ-CD was also present in the separation media, enantiomeric resolution improved up to values of 4.29 and 3.72 for DL-Cys and DL-Hcy, respectively. Moreover, no significant increase in the migration times compared with those achieved employing γ-CD as a chiral selector was observed (see Figs. 2A, 3A, Tables 2, and 3).

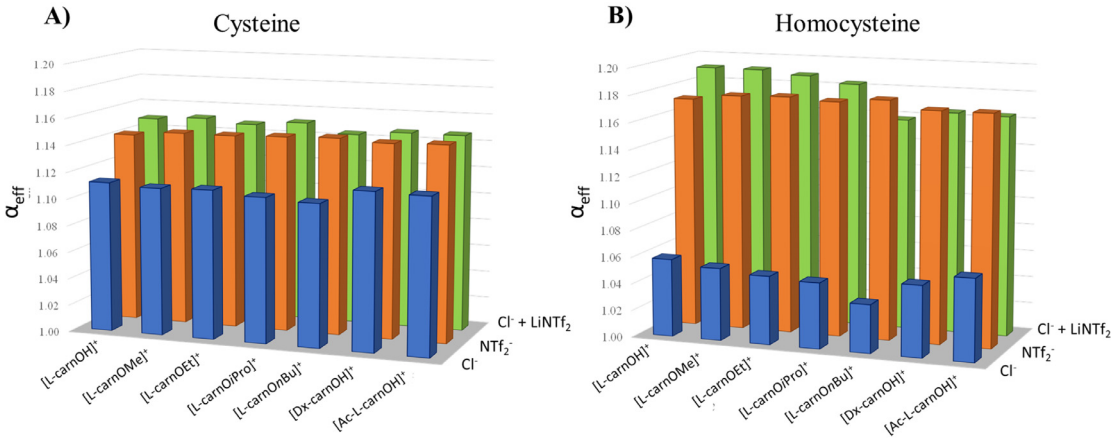

Fig. 4. Effective electrophoretic selectivities (α_{eff}) of DL-Cys and DL-Hcy obtained using dual systems based on the combination of γ-CD plus: salts in which Cl^- is used as anion, CILs in which the anion is NTf_2^-, and salts in which Cl^- is used as anion + $LiNTf_2$. CE conditions as in Fig. 2.

Since $LiNTf_2$ is an achiral salt and the variations in the electroosmotic flow obtained when γ-CD was used alone or in combination with $LiNTf_2$ are not significant, the results obtained are difficult to explain but are in agreement with the observations made previously by other authors [38,39]. Anyway, these results demonstrated the relevance of NTf_2^- to achieve an adequate enantioseparation of the studied compounds since its presence in the separation media improved the chiral separation. At this point, it is also important to establish a comparison between the chiral separation obtained using the systems γ-CD + $LiNTf_2$ and γ-CD + L-carnitine CILs based on the use of the anion NTf_2^-. As it can be seen in Table 2, the use of any of these CILs in combination with γ-CD improved the separation parameters (resolution value and α_{eff}) for DL-Cys with respect to those obtained using γ-CD + $LiNTf_2$. In the case of DL-Hcy, although the resolution was in some cases lower than that obtained using γ-CD + $LiNTf_2$, the α_{eff} increased in all cases (Table 3). These results may demonstrate that also the cationic part of the CILs plays an important role in the separation mechanism.

Finally, the last experiments were focused on evaluating the enantioresolution obtained for DL-Cys and DL-Hcy when the chiral analyses were carried out using tertiary systems based on the combined use of γ-CD + $LiNTf_2$ + salts (or the CIL) in which Cl^- was used as the anion. For both amino acids, the use of these systems allowed to considerably improve the results obtained with dual systems in which $LiNTf_2$ was not present without a substantial change in the migration times (see Figs. 2D, 3D, Tables 2, and 3).

3.2.2. Effect of the nature of the ILs or salts anion

As discussed in the previous section, the influence of the nature of the anion was studied with [L-carnOMe]$^+$ and [L-carnO*n*Bu]$^+$ as cations and using Cl^-, L-lactate$^-$ or NTf_2^- as anions.

Following an increasing order in the hydrophobic nature of the anions, Cl^- < L-lactate$^-$ < NTf_2^- (Table 1), a general trend was observed in the electropherograms of DL-Cys and DL-Hcy (Fig. 2 and Fig. 3, respectively). In general, a decrease in EOF (longer EOF times), higher migration times, higher enantiomeric resolution, and higher α_{eff} values were observed.

That is, when comparing the use of [L-carnOMe]Cl and [L-carnO*n*Bu]Cl with [L-carnOMe][L-lactate] and [L-carnO*n*Bu][L-lactate] small differences were observed. This fact can be explained by the slight decrease in the solubility of the compounds in aqueous media which can modify the EOF and therefore, the migration times. Regarding the interactions between γ-CD and the enantiomers of both amino acids, it does not seem that significant variations of interactions can occur when adding the salts or ILs derived from [L-carnOH]$^+$ since there is no great variation in the times or the resolutions. Moreover, the use of CILs containing L-lactate as counterion produced broad peaks with the corresponding decrease of the resolution in the case of DL-Hcy.

On the other hand, when comparing the above results with [L-carnOMe][NTf_2] or [L-carnO*n*Bu][NTf_2], a great change is observed in the enantiomeric resolutions of both amino acids (with higher α_{eff} values), as well as the inversion in the enantiomer migration orders. These two CILs have higher values of Alog P (Table 1) so that they have a lower solubility in water than those compounds containing Cl^- or L-lactate as anions. This fact could affect the enantioseparation of amino acids by EKC since a slight decrease in the electroosmotic flow could be produced, and as result, longer migration times. However, the change in the electroosmotic flow is not enough to explain the resolution values, α_{eff} values, and enantiomer migration orders obtained. Therefore, the use of L-carnitine-based ionic liquids containing NTf_2^- seems to play a relevant role in the chiral recognition of the γ-CD toward DL-Cys and DL-Hcy.

4. Concluding remarks

New chiral ionic liquids and salts derived from the non-protein amino acid L-carnitine have been synthesized and characterized for the first time in this work. All of them, along with others previously described by our research group, have been employed to perform a systematic study to evaluate their role as additives in a γ-CD-based dual system for the enantiomeric separation of DL-Cys and DL-Hcy by EKC. Different scenarios were considered in this study. On the one hand, it was possible to demonstrate that an increase in the alkyl chain of L-carnitine ester derivatives leads to a slight decrease in the enantiomeric resolution values and α_{eff}. On the other hand, the results obtained revealed that the acetylation of the OH group or the elimination of the asymmetric carbon from the structure of L-carnitine were not relevant for the discrimination power of the dual system. An important point that can be deduced from the obtained results in this systematic study is that the presence of NTf_2^- as anion counterpart is an important factor in the discrimination of homocysteine and cysteine enantiomers. The use of L-carnitine-based ionic liquids containing NTf_2^- in the separation buffer not only improved the chiral separation but also modified the chiral recognition of the γ-CD toward the thiol amino acids (a reversal in the enantiomeric migration order was observed

with the dual system compared to that achieved when using the single system).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Sandra Salido-Fortuna: Investigation, Formal analysis, Validation, Data curation, Visualization, Writing – original draft. **M. Isabel Fernández-Bachiller:** Investigation, Data curation, Visualization, Writing – review & editing. **María Luisa Marina:** Conceptualization, Methodology, Visualization, Resources, Supervision, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **María Castro-Puyana:** Conceptualization, Methodology, Formal analysis, Visualization, Resources, Supervision, Writing – original draft, Writing – review & editing, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.462955.

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