Universidad de Alcalá
BIBLIOTECA

Document downloaded from the institutional repository of the University of Alcala: https://ebuah.uah.es/dspace/

This is a postprint version of the following published document:

Greño, M. et al., 2019. Nuclear magnetic resonance to study the interactions acting in the enantiomeric separation of homocysteine by capillary electrophoresis with a dual system of γ-cyclodextrin and the chiral ionic liquid EtCholNTf2. ELECTROPHORESIS, 40(15), pp.1913–1920,

which has been published in final form at DOI:10.1002/elps.201800483

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

© 2019 John Wiley & Sons, Inc.

(Article begins on next page)

This work is licensed under a

Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

CAPILLARY ELECTROPHORESIS AND NUCLEAR MAGNETIC RESONANCE TO STUDY THE ENANTIOMERIC SEPARATION OF HOMOCYSTEINE WITH A DUAL SYSTEM OF (R)-N,N,N-TRIMETHYL-2-AMINOBUTANOL-BIS(TRIFLUOROMETHANESULFON)IMIDATE AND γ-CYCLODEXTRIN

Maider Greño[1], Antonio Salgado[2], María Castro-Puyana[1,3], María Luisa Marina[1,3]

[1]Departamento de Química Analítica, Química Física e Ingeniería Química. Universidad de Alcalá. Ctra. Madrid-Barcelona Km. 33.600, 28871, Alcalá de Henares (Madrid), Spain.

[2]Centro de Espectroscopía de Resonancia Magnética Nuclear (CERMN), Centro de Apoyo a la Investigación en Química, Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, Alcalá de Henares, 28871 Madrid, Spain.

[3]Instituto de Investigación Química Andrés M. del Río. Universidad de Alcalá. Ctra. Madrid-Barcelona Km. 33.600, 28871, Alcalá de Henares (Madrid), Spain.

Abbreviations: **CIL**, chiral ionic liquid; **EtCholNTf$_2$**, (R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethanesulfon)imidate; **FMOC**, 9-fluorenylmethoxycarbonyl chloride; **Hcy**, homocysteine; **IL**, ionic liquid.

Keywords: Capillary electrophoresis, NMR**,** chiral ionic liquids, homocysteine.

Correspondence: Dr. María Luisa Marina, Departamento de Química Analítica, Química Física e Ingeniería Química. Facultad de Ciencias. Universidad de Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871, Alcalá de Henares (Madrid), Spain.

E-mail: mluisa.marina@uah.es

Tel.: 0034 918854935

Abstract

The enantiomeric separation of FMOC-homocysteine by CE was investigated using γ-CD and the chiral ionic liquid (R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethane-sulfon)imidate (EtCholNTf$_2$) as chiral selectors in a 50 mM borate buffer at pH 9.0. The separation of the enantiomers was achieved in 5 min with a resolution value of 0.9 when using 10 mM γ-CD. However, the enantiomeric separation did not take place when the ionic liquid was employed as sole the chiral selector. Thus, the combination of both selectors was studied, obtaining higher Rs values for FMOC-homocysteine and a reversal in the enantiomer migration order in comparison with the use of γ-CD alone in the separation buffer. Then, NMR experiments were carried out in order to explain the experimental results obtained. The NMR analyses showed the formation of an inclusion complex, being the hydrophobic group of FMOC-homocysteine inserted into the γ-CD cavity. Interactions between EtCholNTf$_2$ and γ-CD were also observed, suggesting that the ionic liquid would also enter the cavity of the γ-CD.

1. Introduction

Ionic liquids (ILs) have attracted much attention in the last years in different fields of analytical chemistry [1, 2]. In particular, there is an increasing interest in the use of these compounds in extraction and separation techniques [3, 4]. Regarding separation techniques, they have been widely used in gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE), mainly as stationary phases, additives in mobile phase and modifiers of background electrolytes [5-7].

Ionic liquids are organic salts with melting points below 100 °C or usually closer to room temperature. They are formed by bulky organic cations (pyridinium, pyrrolidinium, imidazolium, tetraalkylammonium) and organic (triflate, bistrifluoromethanesulfonimide) or inorganic (chloride, bromide, hexafluorophosphate) anions [6]. The ability to interchange different anions and cations makes possible to obtain multiple combinations of ionic liquids. In fact, their chemical composition influences properties such as thermal stability, negligible vapor pressure and high conductivity.

Among the different types of ILs, there is a subtype including the so-called chiral ionic liquids (CILs) which show a chiral moiety in their structure. These CILs are nowadays considered a promising alternative in the field of chiral separations by chromatographic techniques [8]. In CE they have been used as ligands in ligand-exchange CE, as sole chiral selectors and in dual systems with other selectors (mainly cyclodextrins (CDs)) [9, 10]. Until now, there are just a few examples were CILs were used on their own as chiral selectors in CE [11-16]. However, most of the works reported their synergistic effect when used in dual systems of chiral selectors in CE [17]. Although there are several works aimed to study the recognition mechanisms between cyclodextrins and chiral molecules [18], mechanisms concerning ionic liquids remain unclear. In fact, only two works have reported the enantioseparation of model analytes by CE using CILs as chiral selectors and

the possible recognition mechanism between selectors and analytes by molecular modeling [19, 20]. On the one hand, Zhang et al. evaluated the combination of two spirocyclic chiral ionic liquids (1-butyl-3-methylimidazolium(T-4)-bis[(2S)-2-(hydroxy-κO)-3-methyl-butanoato-κO]borate and 1-butyl-3-methylimidazolium(T-4)-bis[(αS)-α(hydroxy-κO)-4-methyl-benzeneacetato-κO]borate) with neutral CDs for the separation of propranolol, tropicamide, duloxetine, nefopam and amlodipine [19]. Better separations, in terms of resolution and selectivity, were achieved with the dual system in comparison with the use of a CD single system. Molecular modeling experiments indicated different hydrogen bonding interactions in dual and single systems and a more stable recognition complex formed between one of the spirocyclic CILs and 2-hydroxypropyl-β-CD with the model analytes. On the other hand, Yang et al. performed the separation of nefopam, ketoconazole, econazole and voriconazole with a dual system formed by maltodextrin and two chiral ionic liquids (tetramethylammonium-$_D$-pantothenate and tetramethylammonium-$_D$-quinate) [20]. By using molecular modeling experiments, it was possible to demonstrate that the presence of the CIL in the maltodextrin system could improve the chiral recognition ability and the selectivity of the single system indicating the existence of a synergistic effect in the maltodextrin/CIL dual system.

NMR is a useful technique to obtain reliable and accurate information concerning structure and chemical mechanism of analyte-chiral selector interactions. Thus, by resorting to Overhauser effect (NOE)-based experiments, it is possible to obtain information on the spatial proximities of hydrogen atoms of both the selector and the analyte. Moreover, NMR analyses permit to mimic the solution state in which enantioseparations by CE are performed [21]. Some works reported the use of ^{19}F NMR spectroscopy of a mixture of racemic Mosher's acid sodium salt and chiral ionic liquids

104 in common NMR solvents in order to evaluate the chiral recognition properties of chiral
105 ionic liquids allowing a quantitative comparison of the strength of the recognition
106 properties between chiral ionic liquids and a racemic substrate [22-24]. However, as far
107 as we know, there are no articles reporting the combined use of CE and NMR to study
108 analyte-CILs molecular interactions.
109 Homocysteine (Hcy) is a thiol containing non-protein amino acid involved in
110 methionine´s metabolism that is considered a biomarker of cardiovascular and
111 neurodegenerative diseases [25, 26]. Few works reported the chiral separation of Hcy by
112 HPLC [27-30] and only two works employed CE for this purpose [31, 32]. The former
113 relied on the use of a high concentration of γ-CD (50 mM) to obtain the baseline
114 separation of Hcy enantiomers in long analysis times (up to 40 min) [31] whereas the
115 latter, performed by our research group, was based on the synergistic effect originated by
116 the use of a dual system of γ-CD and the CIL (R)-N,N,N-trimethyl-2-aminobutanol-
117 bis(trifluoromethanesulfon)imidate ($EtCholNTf_2$). Under these conditions, the
118 enantiomeric separation of Hcy was achieved within a short analysis time (11 min) and
119 with high resolution (8.0) [32]. Moreover, a reversal in the enantiomeric migration order
120 was observed for Hcy when using this dual system compared to that obtained when each
121 chiral selector was employed alone in the separation buffer. This fact could be attributed
122 to the existence of a new "entity" originated as a consequence of the presence of both
123 chiral selectors which modified the interactions of Hcy with them with respect to the
124 existing interactions with each chiral selector separately.

125 The purpose of the present study was to investigate by NMR the intermolecular
126 interactions taking place in the system formed by FMOC-Hcy, γ-CD and ($EtCholNTf_2$)
127 and that could justify the different behavior observed in the enantiomeric separation of

FMOC-Hcy by CE, when a dual system was employed as chiral selector with respect to the use of each chiral selector alone.

2. Materials and methods

2.1. Reagents and samples

All reagents were of analytical grade. Boric acid, sodium hydroxide and pentane were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile was obtained from Scharlau (Barcelona, Spain). The chiral selector γ-CD was provided by Fluka (Buchs, Switzerland). Water used to prepare solutions was purified through a Milli-Q system from Millipore (Bedford, MA, USA). DL-homocysteine (DL-Hcy), L-homocysteine (L-Hcy), the derivatization reagent 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl), deuterated solvents (D_2O) and sodium deuteroxide (NaOD, 40% v/v in D_2O) were supplied from Sigma-Aldrich (Madrid, Spain).

The chiral ionic liquid (R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethane-sulfon)imidate ($EtCholNTf_2$) was synthesized by the Center for Applied Chemistry and Biotechnology (CQAB) from the University of Alcalá following a previously described method. [23,33]

The FMOC-Hcy used for NMR experiments was synthesized following the procedure described by Salvador et al. [34] with some minor modifications.

2.2. Apparatus

Electrophoretic analyses were carried out in an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) with a DAD working at 210 nm with a bandwidth

of 4 nm. The instrument was controlled by the ChemStation software (B. 04. 03 SP1) from Agilent Technologies.

A Bruker AVANCE III 700 spectrometer, operating at 700.17 MHz (^{1}H) and fitted with an inverse 5 mm triple resonance CPTCI 700 S4 cryoprobe, z-gradient unit and a variable temperature controller (Bruker Biospin, Fällanden, Switzerland) was employed for all ^{1}H NMR experiments. A Varian MERCURY 300 spectrometer, operating at 300.16 MHz (^{1}H) and 282.42 MHz (^{19}F), fitted with an inverse 5 mm two channel (^{1}H/^{19}F and ^{13}C/^{31}P) 300ASW probe, z-gradient unit and variable temperature controller (Varian Inc, Palo Alto, CA, USA) was employed for all ^{19}F monodimensional NMR experiments. A Bruker AVANCE III 300 spectrometer, operating at 300.16 MHz (^{1}H) and 282.40 MHz (^{19}F), and fitted with an inverse 5 mm double resonance BBFO 300 S1 probe, z-gradient unit and variable temperature controller was used for the ^{1}H-^{19}F HOESY experiments.

2.3. CE conditions

CE 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) from Polymicro Technologies (Phoenix, AZ, USA). The samples were injected applying a pressure of 50 mbar for 4 s. The temperature was set at 20 ºC and the voltage at 20 kV.

New capillaries were conditioned (applying 1 bar) with 1 M sodium hydroxide for 30 min, Milli-Q water for 5 min and buffer solution for 60 min. At the beginning of each day the capillary was flushed with 0.1 M sodium hydroxide during 10 min, Milli-Q water for 5 min, buffer solution for 15 min and BGE during 10 min. Between analysis, the capillary was washed with 0.1 M sodium hydroxide (2 min), Milli-Q water (1 min) and BGE (3 min). All solutions were filtered through 0.45 µm pore size disposable nylon filters provided by Scharlau (Barcelona, Spain).

The borate buffer solution (50 mM, pH 9.0) for CE separation was obtained dissolving the appropriate amount of boric acid in Milli-Q water and adjusting the pH with 0.1 M sodium hydroxide. BGE was prepared dissolving the appropriate amount of the chiral selector in the buffer solution.

The borate buffer solution (0.2 M, pH 9.0) needed for the derivatization of Hcy was prepared dissolving the amount needed of boric acid in Mili-Q water adjusting to the desired pH with sodium hydroxide. The racemic mixture of Hcy was dissolved in borate buffer and stored at 4 ºC before its derivatization with FMOC-Cl following a protocol previously reported in the literature [35, 36]. Briefly, 200 µL of Hcy standard solution (10 mM) were mixed with 200 µL of a solution of 30 mM FMOC-Cl in ACN. The reaction was kept at room temperature for 2 minutes, then the excess of FMOC-Cl was extracted with 0.5 mL of pentane. The bottom solution was diluted ten times with Milli-Q water before injection in the CE system.

2.4. NMR analyses

The concentration of FMOC-Hcy in the NMR samples was 60-fold higher than in CE experiments in order to obtain reproducible NMR spectra. The aqueous electrolyte was 50 mM sodium borate in D_2O, adjusted to an apparent pH 9 by the addition of NaOD in D_2O. Due to the presence of an unprotected mercapto functional group (that of Hcy), the aqueous electrolyte was deoxygenated by nitrogen bubbling. Racemic FMOC-Hcy (1.0 mg), γ-CD (10.0 mg) and $EtCholNTf_2$ (15 mg) were dissolved in 0.7 mL aqueous electrolyte. The sample was vortexed for 1 min and filtered through a 0.45 µm polypropylene filter prior to data acquisition.

All NMR experiments were performed at 25ºC. The ^1H 90º hard pulse (700 MHz) was optimized and set to 9.00 µs (10 W). Complete ^1H signal assignment of FMOC-Hcy, γ-

198 CD and EtCholNTf$_2$ was made on the basis of COSY, edited HSQC and 1D TOCSY data,
199 when appropriate. A ^{1}H NMR spectrum of FMOC-Hcy alone in the same buffer system
200 was recorded to assist with the full signal assignment. The ^{1}H NMR resonance of residual
201 water (4.70 ppm) was used as the internal reference. A selrogp pulse sequence from the
202 Bruker library was used for all 1D ROESY experiments. The duration and the potency of
203 the shaped pulse (Gaussian) were set to 80 ms and 1.99 x 10-6 W, respectively. The
204 duration of the low power pulse for mixing was 400 ms (0.0879 W) for all 1D ROESY
205 experiments. The number of transients was 512 for each 1D ROESY experiment. For the
206 monodimensional ^{19}F NMR experiments a s2pul sequence from the Varian library was
207 used. A small amount (1% w) of KF was added to the solutions as the ^{19}F NMR internal
208 standard (-125.3 ppm). A hoesyfhgp1d pulse sequence (Bruker library) was used for the
209 ^{1}H-^{19}F HOESY experiments. The ^{1}H and ^{19}F 90º hard pulses for the HOESY experiment
210 were set to 14.00 μs (7.63 W) and 15.00 μs (6.69 W). The selective ^{19}F pulse (Gaussian)
211 at the CF_3 signal of the ionic liquid (-82 ppm) was checked with a selgpse sequence
212 (Bruker library) and set to 5.00 ms (9.4 x 10^{-3} W). The NOE mixing time was set to 1s.

213 **2.5. Data treatment**

214 Resolution values (Rs) for the enantiomers were obtained from their migration times and
215 peak widths at half height using the Chemstation software from Agilent Technologies.
216 Graphs composition with different electropherograms was carried out by using Origin 8.0
217 software. The effective electrophoretic selectivity [37, 38] (α_{eff}) was calculated following
218 the equation:

219 $$\alpha_{eff} = \mu_{ep1}/\mu_{ep2}$$

220 were μ_{ep1} and μ_{ep2} are the effective mobilities of enantiomers 1 and 2, respectively. All
221 NMR spectra were processed with the Mestre NOVA software (version 12.0.3, Mestrelab

Research, S. L., Santiago de Compostela, Spain). All spectra were manually phase adjusted and base line corrected. Exponential line broadening (0.3 Hz for the ^{1}H spectra, 1.0 Hz for 1D ROESY and 1D HOESY and 5.0 Hz for the ^{19}F experiments) was applied.

3. Results and discussion

3.1. Enantiomeric separation of FMOC-homocysteine by CE

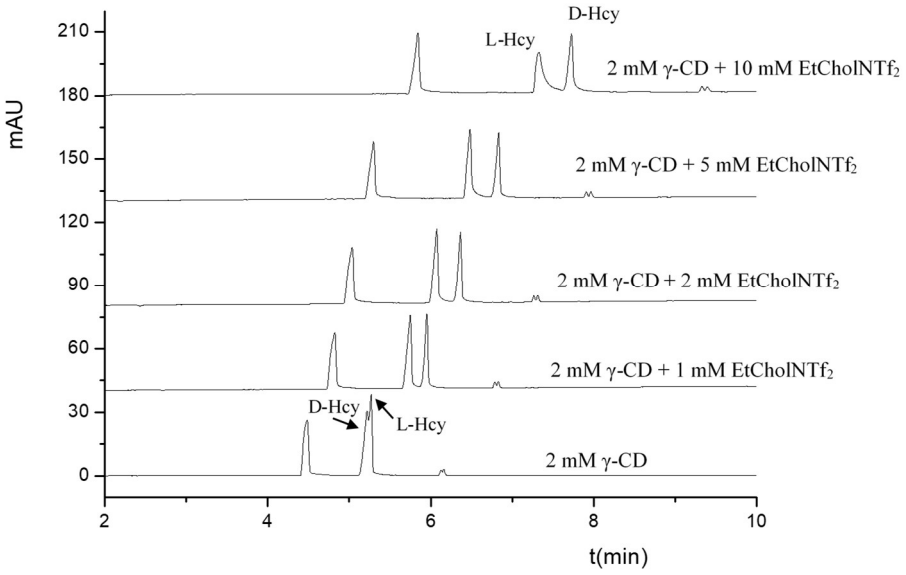
The enantioseparation of FMOC-Hcy using γ-CD and EtCholNTf$_2$ as chiral selectors was recently achieved by our research group using 50 mM phosphate buffer at pH 7.0 as running buffer [32]. Excellent results were reached using the dual system formed by γ-CD and EtCholNTf$_2$ as the chiral selectors. However, when NMR experiments were carried out in order to study the existing interactions in this system and to justify the reversal observed in the enantiomer migration order when using the dual system, the low solubility of the analyte in the employed buffer did not allow performing the NMR study. In fact, for NMR experiments, FMOC-Hcy concentration was 60-fold higher than in CE experiments where the low solubility of the analyte passed unnoticed due to the low concentration employed. In order to overcome this problem, borate buffer at pH 9.0 was chosen as the separation buffer to carry out both CE and NMR analysis and to study the interactions between the analyte and both chiral selectors.

Taking in mind that both the nature and the pH of the separation buffer was different to those previously employed, the enantioseparation of FMOC-Hcy when using different concentrations of γ-CD (from 1 to 10 mM) was investigated. As it can be seen in **Table 1**, the highest Rs (a value of 0.9) was obtained at the highest γ-CD concentration. Also, the discrimination power of EtCholNTf$_2$ was studied, but in this case, the separation of FMOC-Hcy was not achieved when the CIL was used as the sole chiral selector in a concentration range from 20 to 60 mM. This result is different from that observed in our

247 previous work when 50 mM phosphate buffer (pH 7.0) was employed as running buffer
248 since its use enabled the separation of Hcy enantiomers at a 10 mM concentration of
249 EtCholNTf$_2$. Regarding the separation power of the dual system formed by the
250 combination of the CIL and γ-CD for Hcy, a fixed concentration of γ-CD (2 mM) and
251 different concentrations of EtCholNTf$_2$ (from 1 to 10 mM) were used. Under these
252 conditions, higher Rs values were achieved when compared to those obtained with the
253 single CD system (**Figure 1**), being the combination of 2 mM γ-CD plus 5 mM
254 EtCholNTf$_2$ that enabling to reach the highest chiral resolution (Rs 3.8). As previously
255 observed when using phosphate buffer (pH 7.0), a reversal in the enantiomeric migration
256 order was also observed when using the dual system if compared to the use of the CD
257 alone (see Table 1), being L-Hcy the first migrating enantiomer (see **Figure 1)**.
258
259 **Table 1.** Migration times, enantiomeric resolutions and electrophoretic selectivity for
260 Hcy with different chiral selectors.

Chiral selector	[CS](mM)	t_1 (min)	t_2 (min)	R_s	α (eff)	Enantiomer Migration order
γ-CD	1	4.745	-	-	-	-
	2	4.970	5.018	0.6	1.01	D - L
	5	5.153	5.224	0.7	1.01	D - L
	10	5.217	5.288	0.9	1.01	D - L
2 mM γ-CD + EtCholNTf2 dual system	1	5.709	5.910	2.3	1.04	L - D
	2	6.074	6.364	3.2	1.05	L - D
	5	6.484	6.833	3.8	1.05	L - D
	10	7.310	7.711	3.2	1.05	L - D

261



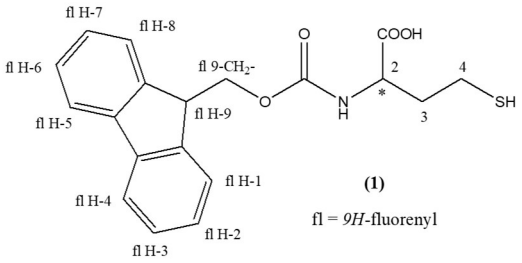
262

263 **Figure 1.** Electropherograms corresponding to the enantiomeric separation of Hcy using
264 a concentration of 2 mM γ-CD with different concentrations of $EtCholNTf_2$.
265 Experimental conditions: 50 mM borate buffer (pH 9.0); uncoated fused-silica capillary,
266 58.5 cm (50 cm to the detector window) x 50 μm ID; UV detection at 210 nm; applied
267 voltage, 20 kV; temperature 20 ºC; injection by pressure, 50 mbar for 4s.

268

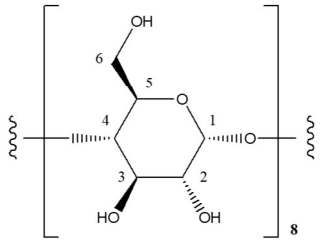
269 **3.2. NMR studies**

270 The structures and NMR chemical shifts of FMOC-Hcy (compound 1), γ-CD (compound
271 2), and $EtCholNTf_2$ (compound 3) are shown in **Figure 2**. The ^1H NMR spectrum is
272 shown in **Figure 3A.** All signals of γ-CD and $EtCholNTf_2$ looked sharp and well defined,
273 though some signal overlapping resulted, namely with H-5 (3.74 ppm) and both H-6 (3.72
274 and 3.76 ppm) of the γ-CD. The signals of FMOC-Hcy were, however, weaker and far
275 broader and some of them were overlapped with those of residual water, γ-CD and
276 $EtCholNTf_2$. Due to their broadness and comparative low intensity no selective
277 irradiations on Hcy hydrogens were attempted.

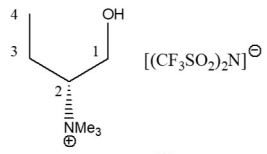


(1)

fl = *9H*-fluorenyl



(2)



(3)

1		2		3	
position	**δ ^{1}H (ppm)**	**position**	**δ ^{1}H (ppm)**	**position**	**δ ^{1}H (ppm)**
H-2 (α)	3.80a	H-1	4.97	H-1	3.82, 4.02
H-3 (β)	1.68, 1.75	H-2	3.52	H-2	3.10
H-4 (γ)	2.12, 2.21	H-3	3.80	H-3	1.69, 1.84
		H-4	3.47	H-4	0.95
9H-fluorenyl 9-C$\underline{H}_2$	4.36, 4.70b	H-5	3.72	2-NMe_3^+	3.02
9H-fluorenyl H-9	4.12a	H-6	3.72, 3.76		
9H-fluorenyl H-1,8	7.55				**δ ^{19}F (ppm)**
9H-fluorenyl H-2,7	7.30				
9H-fluorenyl H-3,6	7.37			$[(CF_3SO_2)_2N]^-$	-82.05
9H-fluorenyl H-4,5	7.73				

a - doubtful
b - underneath HDO signal

Figure 2. Structure, atom numbering, and 1H NMR chemical shifts of FMOC-Hcy (1), γ-CD (2) and (R)-N,N,N-trimethyl-2-aminobutanol-bis(trifluoromethanesulfon)imidate (3).

Irradiation at γ-CD's internal H-3 (3.80 ppm, **Figure 3A**) resulted in intermolecular NOE with the aromatic hydrogens of the FMOC protecting group of Hcy (7.30-7.73 ppm) and, in minor extent, with those at 4.36 and 4.70 ppm (9H-fluorenyl 9-CH_2O of Hcy). These result suggested insertion of the FMOC group into the cavity of γ-CD. It must keep in mind that the signal at 4.70 ppm falls right underneath the signal of residual HDO, thus

NOE enhancements involving this signal should be taken with some caution. Selective irradiation at 3.72 ppm (γ-CD's H-5 and H-6) also resulted in NOE with FMOC's aromatic and CH_2 hydrogens, further supporting the insertion of the 9H-fluorenyl residue of the studied analyte through the γ-CD's major rim. Yet intermolecular NOEs with the H-3 and H-4 protons of EtCholNTf$_2$ (respectively 1.69 and 0.95 ppm) were also observed. This would point at that this portion of the ionic liquid would be also inserted into γ-CD's cavity.

Irradiation at 3.02 ppm (trimethyl ammonium group of IL) gave some NOEs with γ-CD's internal H-3 and H-5 and also with the hydrogens at 4.36 and 4.70 ppm of Hcy (not with the aromatic ones). This would suggest that the trimethyl ammonium group of EtCholNTf$_2$ could be partially inserted into the CD cavity, though located closer to the major rim. Irradiation at the terminal methyl group (H-4) of the IL gave some small NOE interaction with γ-CD's H-3 and H-5, this further corroborating our findings.

In another set of experiments, the tentative inclusion of the ionic liquid into the CD cavity was monitored by ^{19}F NMR. The ^{19}F chemical shift of the trifluoromethyl groups of the CIL was recorded in the absence and presence of γ-CD (**Figure 3B**). A small deshielding effect was observed upon addition of γ-CD. This behaviour could be attributed to insertion of the IL into γ-CD's cavity. No substantial variation of the ^{19}F chemical shift of EtCholNTf$_2$ resulted when Hcy was also added. The inclusion of the IL into γ-CD's cavity was further corroborated by a ^{1}H-^{19}F HOESY experiment of a sample of ionic liquid and γ-CD (**Figure 3C**). Heteronuclear NOE was found between the CF_3 group of EtCholNTf$_2$ and γ-CD's H-3 and perhaps with γ-CD's H-5 and H-6. Some HOESY interaction with the trimethylammonium group of EtCholNTf$_2$ was also observed, all these in agreement with the inclusion of the CIL through γ-CD's major rim. The same HOESY interactions were observed, albeit with minor intensity, in a sample also

containing FMOC-Hcy, although no HOESY interactions involving Hcy hydrogens were observed. Interestingly, heteronuclear NOE between CF_3 group and water molecules was also seen with both samples. This could point at a strong solvation effect, with water molecules residing near the anionic moiety of the IL for comparatively long times.

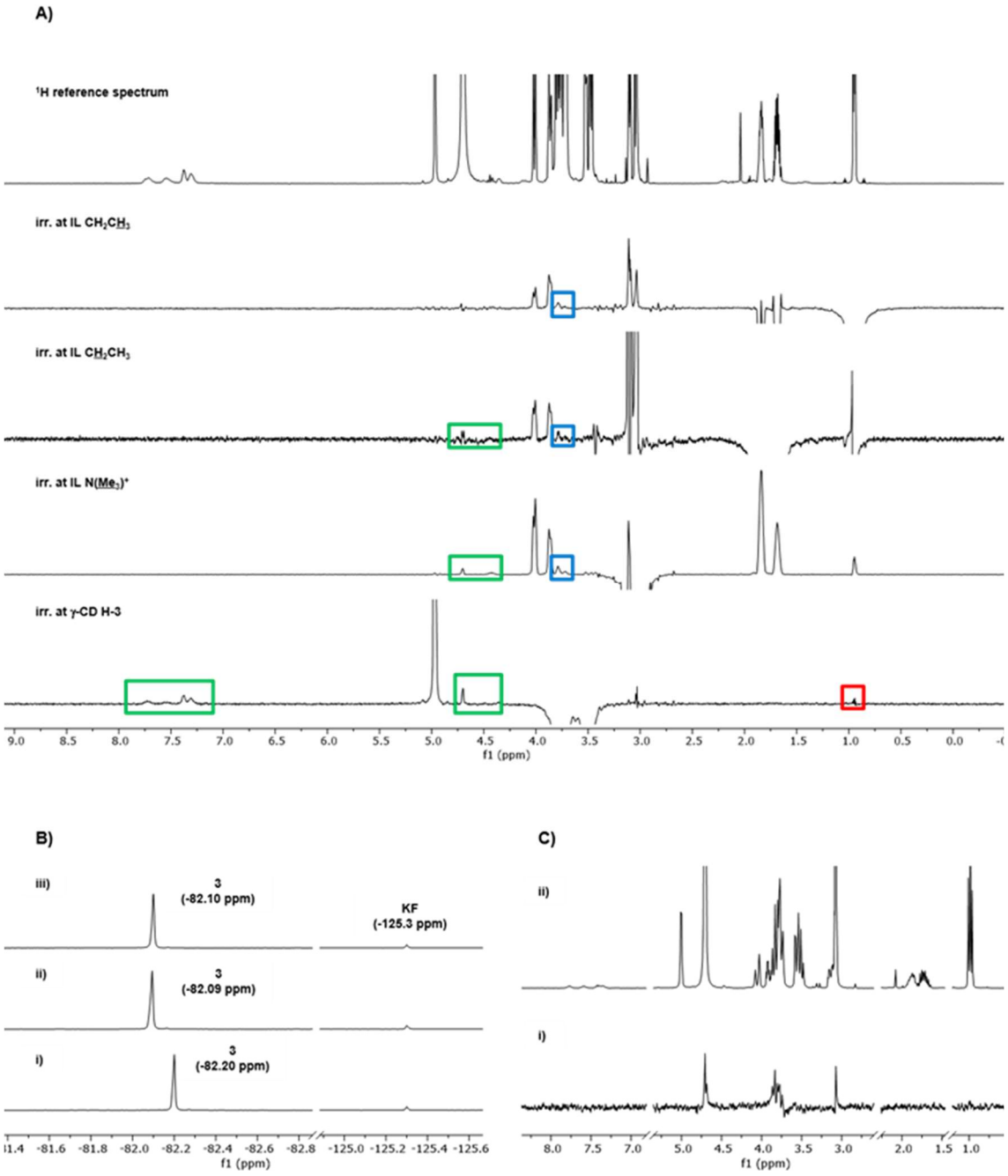


Figure 3. A) 1H NMR spectrum (top) and 1D ROESY experiments (700 MHz, D2O, 25ºC) of FMOC-Hcy (1.0 mg), γ-CD (10.0 mg) and EtCholNTf2 (15.0 mg) in 0.7 mL of

50 mM borate buffer (pH 9) in D2O. Observed intermolecular NOEs are framed in green (involving FMOC-Hcy), blue (involving γ-CD) or red (involving EtCholNTf2); **B)** 19F NMR spectrum (282 MHz, D2O, 25ºC) of EtCholNTf2 (15.0 mg in 0.7 mL of pH 9 50 mM borate buffer (i); 19F NMR spectrum (same conditions) after adding γ-CD (10.0 mg) (ii); 19F NMR spectrum (same conditions) after adding γ-CD and FMOC-Hcy (10.0 and 1.0 mg, respectively) (iii). Dissolved KF was used as the internal reference in all cases; **C)** 1H-19F 1D HOESY (300 MHz, D2O, 25ºC, irradiating at □ 19F -82.05 ppm (i) and 1H NMR spectrum (300 MHz, D2O, 25ºC (ii) of FMOC-Hcy (1.0 mg), γ-CD (10.0 mg) and EtCholNTf2 (15.0 mg) in 0.7 mL of 50 mM borate buffer (pH 9) in D2O.

As a tentative and overall conclusion, the formation of a 1:1:1 inclusion complex was envisaged (**Figure 4**). Both the 9H-fluorenyl part of FMOC-Hcy and the cationic and ionic moieties of EtCholNTf$_2$ would enter the cavity of γ-CD through the major rim. The hydrophilic groups of FMOC-Hcy and EtCholNTf$_2$ would thus remain mostly outside and exposed to the solvent.

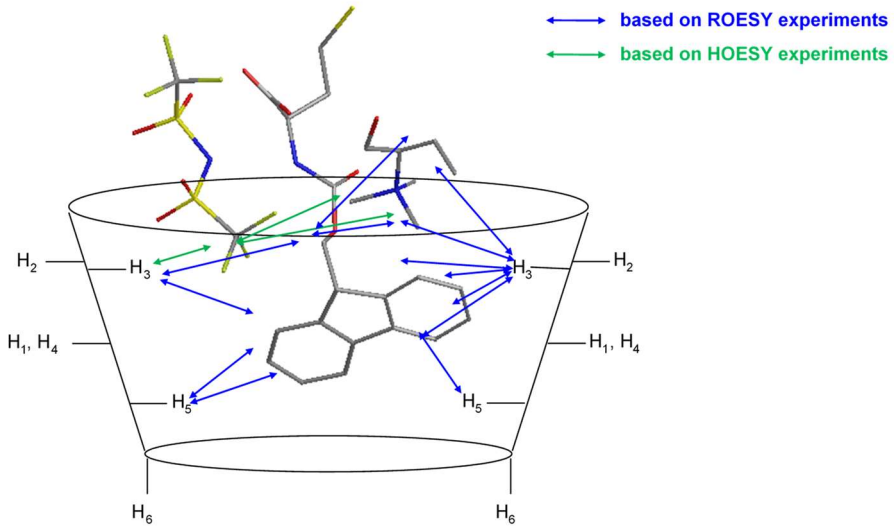


Figure 4. Tentative structure of the complex formed by FMOC-Hcy, γ-CD and EtCholNTf2. Key intermolecular interactions based upon ROESY (blue) and HOESY results (green) are represented by double arrows.

4. Conclusions

Different concentrations of γ-CD and EtCholNTf2 were tested to achieve the enantiomeric separation of FMOC-Hcy by CE in borate buffer at pH 9.0. The separation of Hcy enantiomers was observed using γ-CD obtaining Rs values lower than 0.9. However, no separation was observed with $EtCholNTf_2$ as sole chiral selector. The combined use of both chiral selectors was investigated to improve the enantiomeric separation of Hcy. The best results were obtained when 2 mM of γ-CD were combined with 5 mM of $EtCholNTf_2$ reaching a Rs value of 3.8 in a short analysis time (~7 min). A reversal in the enantiomeric migration order was also observed in the dual system, D-Hcy being the first migrating enantiomer, compared to the CD single system. NMR experiments showed intermolecular interactions between the internal hydrogens of γ-CD and the aromatic group of FMOC-Hcy, confirming the formation of an inclusion complex with the CD. Interactions of the γ-CD with the ionic liquid were also observed indicating that $EtCholNTf_2$ was also inserted into the CD cavity.

Acknowledgements

Authors thank financial support from the Spanish Ministry of Economy and Competitiveness for project CTQ2016-76368-P. M.C.P. also thanks this Ministry for her "Ramón y Cajal" research contract (RYC-2013-12688). M.G. thanks the University of Alcalá for her pre-doctoral contract and the Spanish Ministry of Science, Innovation and Universities for her pre-doctoral contract FPU17/01635. Authors also thank the Center for Applied Chemistry and Biotechnology (CQAB) from the University of Alcalá for the synthesis of the CIL, and D. Molero-Vílchez and E. Sáez-Barajas from CAI-RMN, Universidad Complutense de Madrid for technical assistance in ROESY and HOESY experiments.

363

References

[1] Ho, T.D., Zhang, C., Hantao, L.W., Anderson, J.L., *Anal. Chem.* 2014, *86*, 262-285.

[2] Tan, Z-Q., Liu, J-F., Pang, L., *TrAC Trends Anal. Chem*. 2012, *39*, 218-227.

[3] Berthod, A., Ruiz-Ángel, M.J., Carda-Broch, S., *J. Chromatogr. A* 2018, *1559*, 2-16.

[4] Nawala, J., Dawidziuk, B., Dziedzic, D., Gordon, D., Popiel, S., *TrAC Trends Anal. Chem*. 2018, *105*, 18-36.

[5] Nan, H., Anderson, J. L., *TrAC Trends Anal. Chem*. 2018, *105*, 367-379.

[6] Kartsova, L.A., Bessonova, E.A., Kolobova, E.A., *J. Anal. Chem*. 2016, *71*, 141-152.

[7] Ali, I., Suhail, M., Sanagi, M.M., Aboul-Enein, H.Y., *Crit. Rev, Anal. Chem*. 2017, *47*, 332-339.

[8] Kapnissi-Christodoulou, C.P., Stavrou, I.J., Mavroudi, M.C., *J. Chromatogr. A* 2014, *1363*, 2-10.

[9] Zhang, Q., *TrAC Trends Anal. Chem.* 2018, *100*, 145-154.

[10] Greño, M., Marina, M.L., Castro-Puyana, M., *Crit. Rev. Anal. Chem*. 2018, *48*, 429-446.

[11] Risvi, S.A., Shamsi, S.A., *Anal. Chem*. 2006, *78*, 7061-7069.

[12] Yuan, L.M., Zhou, Y., Meng, X., Li, Z.Y., Zi, M., Chang, Y.X., *Anal. Lett*. 2006, *39*, 1439-1449.

[13] Ma, Z., Zhang, L., Lin, L., Ji, P., Guo, X., *Biomed. Chromatogr*. 2010, *24*, 1332-1337.

384 [14] Yu, J., Zuo, L., Liu, H., Zhang, L., Guo, X., *Biomed. Chromatogr*. 2013, *27*, 1027-
385 1033.

386 [15] Stavrou, I.J., Kapnissi-Christodoulou, C.P., *Electrophoresis* 2013, *34*, 524-530.

387 [16] Zhang, Q., Du, Y., Zhang, J., Feng, Z., Zhang, Y., Li, X., *Electrophoresis* 2015, *36*,
388 1216-1223.

389 [17] Boon, Y.H., Raoov, M., Zain, N.N.M., Mohamad, S., Osman, H., *Crit. Rev. Anal.*
390 *Chem*. 2017, *47*, 332-339.

391 [18] Chankvetadze, B., *J. Chromatogr. A* 2018, *1567*, 2-25.

392 [19] Zhang, Y., Du, S., Feng, Z., Du, Y., Yan, Z., *Anal. Bioanal. Chem.* 2016, *408*, 2543-
393 2555.

394 [20] Yang, X., Du, Y., Feng, Z., Liu, Z., Li, J., *J. Chromatogr. A* 2018, *1559*, 170-177.

395 [21] Salgado, A., Chankvetadze, B., *J. Chromatogr. A* 2016, *1467*, 95-144.

396 [22] Vasiloiu, M. Bica, K., in: Rodriguez, H. (Ed.), Ionic liquids for better separation
397 processes, Springer-Verlag, Berlin Heidelberg 2016, pp. 167-192.

398 [23] Wasserscheid, P., Bösmann, A., Bolm, C., *Chem. Comm*. 2002, 200-201.

399 [24] Jayachandra, R., Reddy, S.R., *RSC Adv*. 2016, *6*, 39758-39761.

400 [25] Li, T., Yu, B., Liu, Z., Li, J., Ma, M., Wang, Y., Zhu, M., Yin, H., Wang, X., Fu, Y.,
401 Yu, F., Wang, X., Fang, X., Sun, J., Kong, W., *Nat. Commun.* 2018, *9*, 11.

402 [26] Roostaei, T., Felsky, D., Nazeri, A., De Jager, P.L., Scheneider, J.A., Bennett, D.A.,
403 Voineskos, A.N., *Neurobiol. Aging* 2018, *62*, 243.

404 [27] Deáková, Z. Duracková, D. W. Armstrong, J. Lehotay, *J. Chromatogr. A* 2015, *1408*,
405 118-124.

406 [28] Bystrická, Z., Bystrický, R., Lehotay, J., *J. Liq. Chrom. Rel. Technol.* 2016, *39*, 775-
407 781.

408 [29] Kullman, J. P., Yu, T., Chen, X., Neal, R., Ercal, N., Armstrong, D. W., *J. Liq.*
409 *Chrom. Rel. Technol*. 2000, *23*, 1941-1952.

410 [30] Bhusban, R., Dubey, R., *J. Chromatogr. A* 2011, *1218*, 3648-3653.

411 [31] Kim, I-J., Park, S-J., Kim, H-J., *J. Chromatogr. A* 2000, *877*, 217-223.

412 [32] Greño, M., Marina, M.L., Castro-Puyana, M., *J. Chromatogr. A* 2018, *1568*, 222-
413 228.

414 [33] Bwambok, D. K., Marwani, H. M., Fernand, V. E., Fakayode, S. O., Lowry, M.,
415 Negulescu, I., Strongin, R. M., Warner, I. M., *Chirality* 2008, *20*, 151-158.

416 [34] Salvador, L.A., Elofsson, M., Kihlberg, J., *Tetrahedron* 1995, *51*, 5643-5656.

417 [35] Wang, H., Andersson, P.E., Engström, A., Blomberg, L.G., *J. Chromatogr. A* 1995,
418 *704*, 179-193.

419 [36] Sánchez-Hernández, L., Domínguez-Vega, E., Montealegre, C., Castro-Puyana, M.,
420 Marina, M. L., Crego, A. L., *Electrophoresis* 2014, *35*, 1244-1250.

421 [37] François, Y., Varenne, A. Juillerat, E., Villemin, D., Gareil, P., *J. Chromatogr. A*
422 2007, *1155*, 134-141.

423 [38] Lelièvre, F., Gareil, P., Jardy, A., *Anal. Chem.* 1997, *69*, 385-392.