Bernardo-Bermejo, S., Marina, M.L. and Castro-Puyana, M. (2021) 'A rapid electrokinetic chromatography method using short-end injection for the enantioselective separation of tryptophan', Microchemical journal, 168, p. 106508.

A rapid electrokinetic chromatography method using short-end injection for the enantioselective separation of tryptophan

Samuel Bernardo-Bermejo[1], María Luisa Marina[1,2], María Castro-Puyana[1,2 *]

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

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

Correspondence: Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona, Km. 33.600, 28871 Alcalá de Henares, Madrid, España.

E-mail: maria.castrop@uah.es

Tel.: +34 918856430

HIGHLIGHTS

- Development of a rapid EKC method enabling the enantioseparation of tryptophan.
- First chiral analysis of tryptophan using a short-end injection.
- Enantiomeric resolution was 7.4 in 2.5 min.
- No previous derivatization of tryptophan was necessary.
- The method showed a high potential for the quality control of dietary supplements.

Abstract

A rapid enantioselective methodology for the analysis of tryptophan was developed in this work by electrokinetic chromatography using short-end injection and an anionic cyclodextrin as chiral selector. No previous derivatization of tryptophan was necessary. The influence of different experimental variables on the enantiomeric separation was investigated. The use of a 100 mM formate buffer (pH 2.2) containing 1.25% sulfated-γ-cyclodextrin with an uncoated fused-silica capillary of 50 μm inner diameter with a total length of 48.5 cm (effective length of 8.5 cm), and an injection by applying a pressure of -50 mbar (short-end injection) for 4 s, enabled the enantiomeric separation of tryptophan within 2.5 min with a resolution of 7.4. As desirable, the enantiomeric impurity, D-tryptophan, was the first-migrating enantiomer. The analytical characteristics of the developed methodology were evaluated in terms of linearity, precision, accuracy, and limits of detection and quantification, showing its good performance to be applied to the analysis of tryptophan-based dietary supplements. A relative limit of detection of 0.1% was obtained for the enantiomeric impurity, D-tryptophan, in the presence of the L-enantiomer. Results showed that the developed methodology is an interesting alternative for the enantioselective analysis of tryptophan enabling the rapid quality control of dietary supplements.

Keywords: Electrokinetic Chromatography, Enantioseparation, Short-end injection, Cyclodextrin, Amino acids, Tryptophan, Dietary supplements.

1. Introduction

Tryptophan, one of the 20 amino acids which can be found as part of proteins, is included in the group of essential amino acids. This fact implies that it must be incorporated through the diet, consuming tryptophan-containing food or dietary supplements, since it cannot be synthesized by the organism. A daily intake of 4 mg of tryptophan per kg body weight is recommended by The World Health Organisation [1]. As the other proteinogenic amino acids (except glycine), tryptophan has a chiral carbon atom, leading to two enantiomeric forms. L-tryptophan, which is the natural form, is involved in the synthesis of proteins and presents a high interest in different pharmacological and clinical areas. For instance, this enantiomer is the precursor of different bioactive compounds such as the neurotransmitter serotonin, implied in behavior and pleasure [2], or the hormone melatonin, involved in sleep regulation, among others. Besides, L-tryptophan was reported to be employed as an antidepressant agent [3] and is a potential biomarker to detect human diseases such as cell renal carcinoma [4] or to diagnose cataracts at the molecular level [5]. Conversely, D-tryptophan, which can be originated as a consequence of different racemization processes, has a low biological activity [6, 7] and is considered an enantiomeric impurity.

Tryptophan is present in a great variety of foods and its popularity has increased in the last years due to its interesting properties. Nowadays, there are a lot of commercial dietary supplements containing this amino acid. To ensure the safety of these supplements, legal regulations do not allow the presence of D-tryptophan [8]. This enantiomer may not be metabolized efficiently so that its presence decreases the nutritional value of food or dietary supplements [9]. In this context, the development of

chiral strategies for the enantiomeric determination of tryptophan in food or dietary supplements has high relevance.

Different analytical methodologies based on the use of two-dimensional liquid chromatography (LC-LC) [10, 11], nano-LC [12], gas chromatography (GC) [13] or subcritical fluid chromatography (SFC) [9] were proposed for the analysis of tryptophan enantiomers in different food samples such as cooked ham, minced meat, dry-cured Iberian ham, smoked salmon, soft cheese and dietary supplements. Regarding the analytical methodologies based on the use of nano-LC and GC techniques, previous derivatization of tryptophan with 9-fluorenylmethoxycarbonyl (FMOC) and ethylchloroformate, respectively, was necessary to carry out its detection [12, 13]. Both in LC-LC as well as nano-LC and SFC, D-tryptophan eluted after L-tryptophan which is not the desirable elution order since the majority peak could overlap the minority peak under these conditions [9-12]. Among all the above-mentioned analytical strategies, SFC allowed the fastest separation of tryptophan enantiomers (less than 7 min with a resolution value of 1.6), achieving a limit of detection (LOD) of 0.5 µg/mL for L-tryptophan [9]. However, the lowest LOD (0.2 mg/L) and the highest resolution (2.6) were obtained by LC-LC [10].

In the field of chiral analysis, there is no doubt that capillary electrophoresis (CE) is one of the most powerful tools for the enantioseparation of chiral compounds due to its simplicity, short analysis times, high-resolution power, high separation efficiency, and versatility [14]. Among the different CE modes, electrokinetic chromatography (EKC) is by far the preferred to carry out a chiral analysis. In this mode, the chiral selector is directly added to the separation medium which provides high flexibility since both the nature of the selector and its concentration can easily be changed. The wide variety of

chiral selectors that can be employed contributes not only to the high flexibility of EKC but also to reduce the economic costs derived from the use of chiral chromatographic columns [15]. Among all the chiral selectors that can be used in EKC, cyclodextrins (CDs) continue to be nowadays the most widely used [16-19].

Numerous articles published in the literature have employed tryptophan as a model compound to evaluate the discrimination power of a variety of chiral selectors in CE. Some representative examples are given here which include the use of neutral or charged CDs [20-22], antibiotics [23-25], chiral ligand ionic liquids [26], molecular imprinted silica nanoparticles [27], L-tryptophan DNA aptamer [28] or the combination of CDs with metal ion ligands [29] or chiral ionic liquids [30]. A few articles were focused on the development of CE methodologies for the enantiomeric determination of tryptophan [31-38]. Thus, micellar electrokinetic chromatography (MEKC) indirect approaches with fluorescence [36] and mass spectrometry (MS) [31] detection enabled the separation of tryptophan diastereomers (formed by derivatization of tryptophan enantiomers with an enantiopure chiral derivatization reagent ((+)-1-(9-fluorenyl) ethyl chloroformate) under achiral conditions (analysis time higher than 17 min and resolution lower than 1.8). The chiral separation of tryptophan was also achieved using a low amount of β-CD as chiral selector in an EKC-MS system that originated a partial resolution in 30 min [34]. The determination of tryptophan enantiomers in biological fluids (artificial cerebrospinal fluid samples) was perfomed using three different strategies based on the use of indirect MEKC-MS [32], and EKC-MS or MEKC-fluorescence with β-CD as chiral selector [33, 35]. In all cases, the analysis times were between 20 and 80 min and the enantiomeric resolutions obtained ranged from 0.9 to 7.1 (this last resolution value was observed in 80 min). Regarding the analysis of food samples, as far as we know, only two articles

reported the use of CE for the enantiomeric determination of tryptophan in food samples. Qi *et al.* employed a chiral ligand exchange CE (LE-CE) methodology with UV detection and a Zn(II) complex as a chiral selecting system for the analysis of rice samples [37], whereas En-Ping *et al.* developed a strategy based on the combination of poly(ethylene oxide) (PEO)-based stacking, MEKC using β-CD as chiral selector, and FMOC derivatization to determine tryptophan in beers [38]. In both cases, D-tryptophan eluted before L-tryptophan and the analysis times were higher than 20 min with enantiomeric resolutions lower than 4.0. LODs were 0.15 μg/mL and 40.7 nM for D-tryptophan and 0.15 μg/mL and 40.1 nM for L-tryptophan, respectively. However, the possibility of reaching a relative LOD (RLOD) of 0.1% for the D-enantiomer with respect to the majority enantiomer, was not reported.

This work is aimed to develop an EKC methodology to carry out the fast separation of tryptophan enantiomers using CDs as chiral selectors without any previous derivatization step. This method was subsequently applied to the analysis of tryptophan-based dietary supplements to achieve their quality control.

2. Materials and methods

2.1. Reagents and samples

All reagents employed were of analytical grade. Formic acid and sodium hydroxide were obtained from Sigma Aldrich (St. Louis, MO, USA). The chiral selectors 2-hydroxypropyl-β-CD (DS 4), β-CD, γ-CD, and sulfated-β-CD (DS 12) were purchased from Fluka (Buchs, Switzerland). Sulfated-β-CD (DS 12) was also provided by Aldrich and Sigma (Madrid, Spain). Heptakis(2,3,6-tri-O-methyl)-β-CD (DS 13), methyl-β-CD (DS 1.8), α-CD, and acetyl-γ-CD (DS 7), were from Sigma. Acetyl-β-CD (DS 7), sulfated-α-CD (DS 12), phosphated-β-CD (DS 4), sulfobutyl-β-CD (DS 6.3), and

157 sulfated-γ-CD (DS 14) were from Cyclolab (Budapest, Hungary). Water used to prepare
158 solutions was purified through a Milli-Q System from Millipore (Bedford, MA, USA).

159 Six different dietary supplements were acquired in different dietetic centers in
160 Guadalajara (Spain). DS-1 and DS-1-E containing L-tryptophan (other components:
161 vitamin B6), DS-2 and DS-2-E containing L-tryptophan (other components: $MgSO_4$,
162 vitamin B3 and B6, biotin and maltodextrin), and DS-3 and DS-3-E containing L-
163 tryptophan (other components: vitamin B1, B2, B3, B6, B9, and vitamin C). DS-1-E, and
164 DS-3-E expired in 2017 whereas DS-2-E expired in 2018.

165 *2.2. CE conditions*

166 CE experiments were performed with an Agilent 7100 CE system (Agilent
167 Technologies, Waldbronn, Germany) equipped with a DAD working at 220 nm with a
168 bandwidth of 4 nm. The instrument was controlled by HP^{3D} CE ChemStation software
169 from Agilent Technologies. Separations were carried out using 100 mM formate buffer
170 (pH 2.2) containing 1.25% (w/v) of sulfated-γ-CD as BGE and uncoated fused-silica
171 capillaries of 50 µm ID (362.8 µm OD) with a total length of 48.5 cm and an effective
172 length of 8.5 cm purchased from Polymicro Technologies (Phoenix, AZ, USA). The
173 samples were injected by applying a pressure of -50 mbar (short-end injection) for 4s, and
174 the electrophoretic separation was achieved using 30 kV and a working temperature of
175 25°C.

176 Before its first use, new capillaries were rinsed (applying 1 bar) with 1 M sodium
177 hydroxide for 30 min, followed by 5 min with Milli-Q water and conditioned with buffer
178 solution for 60 min. At the beginning of each day, the capillary was pre-washed (applying
179 1 bar) with 0.1 M sodium hydroxide for 10 min, Milli-Q water for 5 min, buffer for 30

min, and BGE for 10 min. Between injections, the capillary was conditioned with BGE for 2 min.

2.3. Preparation of solutions and samples

The separation buffer solution was prepared by diluting the appropriate volume of formic acid with Milli-Q water (100 mM, pH 2.2). The BGE was obtained by dissolving the appropriate amount of each CD in the separation buffer.

Stock standard solutions of D and L-tryptophan were prepared by dissolving the appropriate amount in Milli-Q water to obtain concentrations of 5 mM and 10 mM, respectively.

The preparation of the sample solutions of the six dietary supplements analyzed was performed weighing and mixing homogeneously the content of five capsules which corresponded to weighed amounts of each dietary supplement ranging from 1.390 g to 2.840 g. Considering the labeled amount of tryptophan in each supplement, an appropriate amount of the powder obtained was dissolved (by ultrasonication for 10 min) in Milli-Q water to obtain a standard solution of 2000 mg/L. Subsequently, the obtained solutions were centrifuged (15 min, 4000*g* at 25°C) and filtrated using 0.45 µm pore size disposable nylon filters from Scharlau (Barcelona, Spain).

2.4. Data treatment

Values for the enantiomeric resolution (calculated from the migration times of enantiomers and their peak widths at half height) and migration times were obtained using the Chemstation software from Agilent Technologies. Excel Microsoft, Statgraphics Centurion XVI, and Origin 8.0 software were used to carry out experimental data

analysis, calculation of different parameters, and composition of graphs with different electropherograms.

3. Results and discussion

3.1. Development of a rapid EKC methodology for the enantioselective separation of tryptophan

Tryptophan is an essential amino acid that possesses a positive charge at pH values lower than 2.3 (pKa(Cα-COOH)=2.38, pKa(Cα-NH_3^+) =9.39). Acidic conditions were selected to try the chiral separation of tryptophan by EKC. A 100 mM formate buffer (pH 2.2), a capillary with a total length of 40 cm, a working temperature of 25ºC and a wavelength of 220 ± 4 nm were employed to carry out a screening of 8 neutral CDs (namely α-CD, β-CD, γ-CD, 2-hydroxypropyl-β-CD, methyl-β-CD, heptakis(2,3,6-tri-O-methyl)-β-CD, acetyl-β-CD, acetyl-γ-CD) at a concentration of 10 mM, and 5 anionic CDs (sulfated-α-CD, phosphated-β-CD, sulfobutyl-β-CD, sulfated-β-CD from three different commercial suppliers but with the same DS of 12, and sulfated-γ-CD) at a concentration of 1.00% (w/v). A separation voltage of 20 kV was employed to perform the screening when neutral CDs were evaluated whereas a voltage of -20 kV was used when anionic CDs were tested as chiral selectors. **Table S1** summarizes all the CDs employed as well as the migration times obtained for each enantiomer and the enantiomeric resolution achieved. Among the neutral CDs, only acetyl-β-CD provided a partial resolution of 0.8. Contrarily, all the anionic CDs tested, except sulfated-α-CD, allowed to achieve the chiral separation of tryptophan enantiomers with resolution values ranging from 2.2 to 18.7. Among them, sulfated-γ-CD was chosen as the most appropriate selector since it gave rise to the highest enantiomeric resolution (18.7) in less than 14 min.

To develop a rapid methodology for the enantiomeric separation of tryptophan and once the chiral selector was selected, the effect of the separation voltage and the temperature was investigated. First, the separation voltage was changed from -20 kV to -30 kV and temperature values of 25ºC and 20ºC were tested. Under these conditions, the analysis time was halved when using 25ºC, obtaining D-tryptophan in 4.6 min and L-tryptophan in 6.6 min (resolution 15.1). When the temperature was 20ºC, the resolution increased to 16.1 but the analysis time was longer (5.1 and 7.5 min for D- and the L-enantiomers, respectively). This result could be expected since, as it is well known, the enantioresolution of chiral compounds can increase when decreasing the working temperature [39, 40]. Thus, -30 kV and 25ºC were chosen for further experiments.

Taking into account that the concentration of the CD can affect considerably the chiral separation [41], different percentages of sulfated-γ-CD ranging from 0.25 to 2.00% (w/v) were evaluated. **Figure 1A** shows that CD percentages lower than 1.00% originated a higher migration time, a lower resolution, and a peak broadening. However, there is not a clear trend for the variation of the analysis time when the CD concentration was higher than 1.00% as can be observed in **Figure 1B.** In fact, the analysis time decreased when increasing the CD percentage from 1.0% to 1.25% but it increased when the percentage varied from 1.25 to 1.5% being the analysis time for 1.50% very similar to that obtained for 2.00% CD. Although the signals were slightly more intense using 1.50% and 2.00% sulfated-γ-CD and the resolution was slightly higher, a CD percentage of 1.25% was selected because it allowed minimizing the amount of CD employed and decreasing the capillary current.

Considering the high resolution obtained for tryptophan enantiomers, three different strategies were assayed to reduce the analysis time as much as possible: i)

applying pressure during the electrophoretic separation, ii) performing a short-end injection (8.5 cm effective length), iii) using a shorter capillary of 33.5 cm total length.

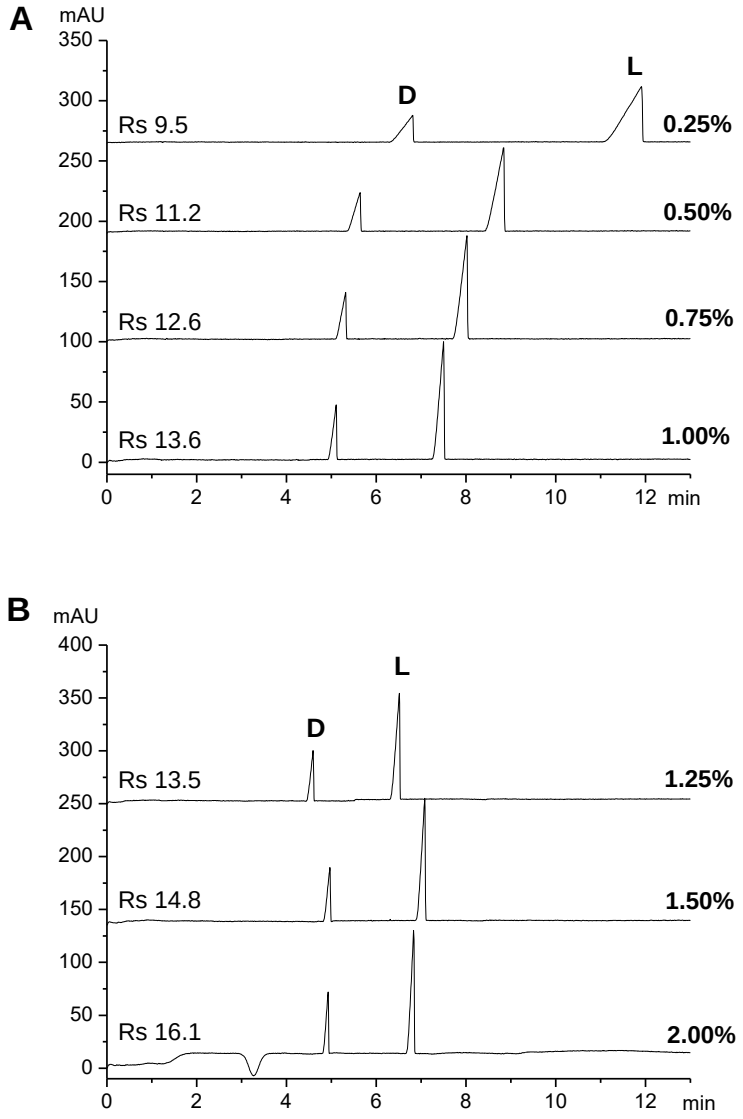

Figure 1. Electropherograms showing the effect of the percentage of sulfated-γ-CD on the chiral separation of DL-tryptophan (solution 0.5 mM in the D-enantiomer and 1mM in the L-enantiomer), (A) from 0.25% to 1.00% (w/v) and (B) from 1.25% to 2.00% (w/v). Experimental conditions: BGE, 100 mM formic acid (pH 2.2) containing different percentages of sulfated-γ-CD; uncoated fused-silica capillary, 48.5 cm (40 cm to the detector window) x 50µm ID; applied voltage, -30kV; temperature, 25 ºC; injection 50 mbar for 4 s; UV detection at 220 ± 4 nm.

In the first case, the effect of the pressure was studied in a range from 10 to 40 mbar (10, 20, 30, and 40 mbar) employing a separation voltage of -30 kV and 1.25% sulfated-γ-CD.

In agreement to that previously reported [42], a decrease in both analysis time and resolution when increasing the pressure was observed (see **Figure 2A**). The optimum pressure was considered to be 20 mbar because it was possible to have a short analysis time (around 4.5 min), a high resolution (10.0), and also a better sensitivity compared to those obtained at 30 and 40 mbar. The second strategy was based on performing a short-end injection by applying -50 mbar for 4s, and using a reverse polarity to the normal injection mode to change the direction of migration (i.e 30 kV). This type of injection introduced by Altria et al. [43] enables not only a substantial reduction in the analysis time but also a several-fold increase in sensitivity [44]. In this case, the total length of the capillary was 48.5 cm but the effective length was only 8.5 cm. As it can be seen in **Figure 2B,** the enantiomeric separation was carried out within 2.5 min (1.4 and 2.4 min for D and L-tryptophan, respectively) with a high resolution (7.4). Finally, the total length of the capillary was reduced to 33.5 cm (25.0 cm effective length). In this case, a CD percentage of 1.00% sulfated-γ-CD was employed due to the high capillary current observed under these conditions when the CD percentage was 1.25%. The separation voltage was also optimized by testing -20, -25 and -30 kV although a value of -20 kV was selected to avoid the high and unstable capillary currents observed at -25 and -30 kV. Under these optimized conditions, the enantiomeric separation of tryptophan was carried out in 5 min with a resolution of 9.5 (**Figure S1A)**. In a similar way as for the long capillary, the effect of the application of pressure during the analysis and the use of short-end injection was also studied with this capillary with a shorter length.

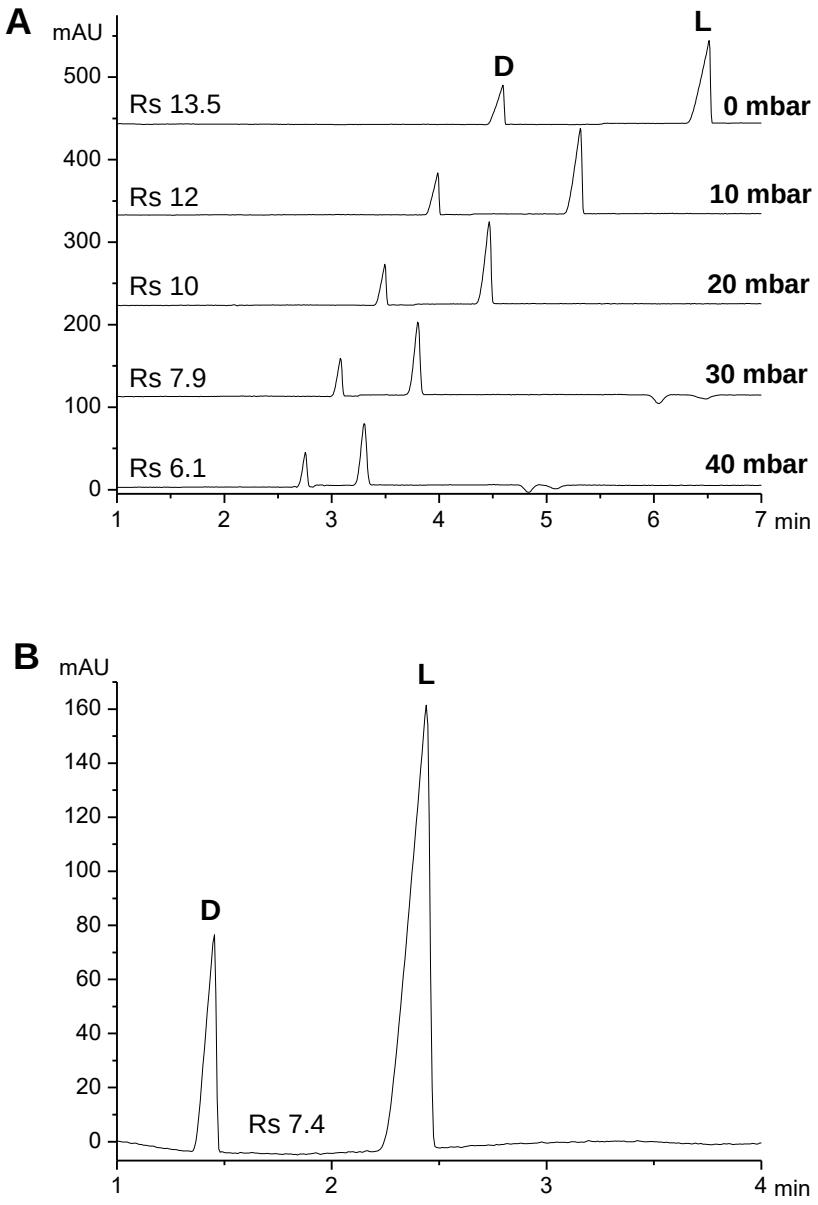

Figure 2. Electropherograms corresponding to the chiral separation of DL-tryptophan (solution 0.5 mM in the D-enantiomer and 1mM in the L-enantiomer): (A) effect of application of pressure during the analysis; (B) injection by pressure in the short-end. Experimental conditions: BGE, 100 mM formic acid (pH 2.2) containing 1.25% of sulfated-γ-CD; uncoated fused-silica capillary, 48.5 cm (40 cm (A) or 8.5 cm (B) to the detector window) x 50µm ID; applied voltage, -30kV (A) and 30 kV (B); temperature, 25 ºC; injection 50 mbar (A) or -50 mbar (B) for 4 s UV detection at 220 ± 4 nm

In the first case, 20 mbar was selected as the optimum pressure because the analysis time was short (2.2 and 2.7 min for D and L-enantiomers, respectively), the resolution was high (5.8) and peak height and width were the best among the other analysis performed by applying other pressure values (**Figure S1B**). When the injection was carried out in the short-end of the capillary (8.5 cm effective length), the migration times were 1.7 and 3.3 for D and L-enantiomer, respectively, and a resolution of 7.0 was obtained. However, there was a big peak broadening (**Figure S1C**).

Considering the results obtained when using all the strategies studied, it can be concluded that the best conditions to develop a rapid EKC method enabling the enantioselective separation of tryptophan were: 100 mM formate buffer (pH 2.2) containing 1.25% sulfated-γ-CD, uncoated fused-silica capillary of 50 μm ID with a total length of 48.5 cm (8.5 cm effective length), short-end injection by applying a pressure of -50 mbar for 4s, a separation voltage of 30 kV and a working temperature of 25°C.

3.2. Application of the developed method to the analysis of tryptophan in dietary supplements

To demonstrate the suitability of the stereoselective EKC method for the rapid analysis of tryptophan, its analytical characteristics were evaluated in terms of linearity, precision, accuracy, limits of detection (LOD), and limits of quantification (LOQ) according to the International Council on Harmonization (ICH) guidelines Q2(R1) [45]. Results obtained are grouped in **Table 1**.

Table 1. Analytical characteristics of the developed EKC method for the stereoselective determination of tryptophan in dietary supplements.

		L-tryptophan	D-tryptophan
External standard calibration method[a]			
Linear range		1-160 mg/L	1-7 mg/L
Slope ± t·sb		1.13 ± 0.02	1.36 ± 0.09
Intercept ± t·sa		2 ± 3	0.3 ± 0.4
R		0.999	0.998
p-value of ANOVA[b]		0.514	0.506
Matrix Interferences [c]	*Sample*	*Confidence interval (Slope ± t·sb)*	*Confidence interval (Slope ± t·sb)*
	DS-1	1.18 ± 0.09	1.49 ± 0.08
	DS-2	1.3 ± 0.3	1.50 ± 0.07
	DS-3	1.4 ± 0.3	1.5 ± 0.2
Precision	*Concentration level*	*RSD (%)*	*RSD (%)*
Instrumental Repeatability [d] (n = 6)	2 mg/L	t, 0.4; Ac, 1.2	t, 0.4; Ac, 1.3
	70 mg/L	t, 0.2; Ac, 0.7	t, 0.2; Ac, 2.6
Method Repeatability [e] (n = 9)	2 mg/L	t, 0.4; Ac, 1.5	t, 0.3; Ac, 1.7
	70 mg/L	t, 0.3; Ac, 1.2	t, 0.3; Ac, 2.8
Intermediate precision [f] (n = 9)	2 mg/L	t, 2.3; Ac, 5.8	t, 1.8; Ac, 4.7
	70 mg/L	t, 3.1; Ac, 7.6	t, 2.6; Ac, 9.2
Accuracy[g]		*% Mean Recovery*	*% Mean Recovery*
DS1		110 ± 7	101 ± 5
DS2		105 ± 10	105 ± 3
DS3		107 ± 9	100 ± 7
LOD [h]		0.07 mg/L	0.07 mg/L
LOQ [i]		0.25 mg/L	0.22 mg/L

[a] Nine standard solutions at different concentration levels for L-tryptophan and seven for D-tryptophan injected in triplicate for two consecutive days.
[b] p-value for ANOVA to confirm that experimental data fit properly to linear model.
[c] Comparison of the confidence intervals for the slopes corresponding to the standard addition and the external standard calibration methods.
[d] Six consecutive injections of tryptophan standard solutions at two concentration levels (2 and 70 mg/L).
[e] Three replicates of tryptophan standard solutions at two concentration levels (2 and 70 mg/L) injected in triplicate on the same day.
[f] Three replicates of tryptophan standard solutions at two concentration levels (2 and 70 mg/L) injected in triplicate during three consecutive days.
[g] Calculated as the mean recovery obtained when three dietary supplements containing 70 mg/L of L-tryptophan (nominal concentration) were spiked with know concentrations of L-tryptophan (25, 50, 75 and 100% of the nominal concentration) or D-tryptophan (3.2, 5, 7.8 and 9.2% of the nominal concentration).
[h,i] LOD and LOQ obtained for a S/N ratio = 3 or a S/N ratio = 10, respectively.

The linearity of the method was established by plotting the corrected peak areas *versus* nine calibration levels, from 1 to 160 mg/L of L-tryptophan, and seven calibration levels, from 1 to 7 mg/L of D-tryptophan. Good correlation coefficients, 0.999 and 0.998, respectively, were obtained. For a 95% confidence level, confidence intervals for the slopes did not include the zero value, and confidence intervals for the intercept included the zero value, for both enantiomers. Moreover, to confirm that data for L- and D-tryptophan fit properly to a linear model, an ANOVA test was performed obtaining p-values higher than 0.05.

To investigate the existence of matrix interferences, a standard addition calibration method was carried out by adding four known amounts of D-tryptophan to each of the three dietary supplements analyzed (constant concentration of L-tryptophan 70 mg/L). The confidence interval for the slope was compared with the slope confidence interval obtained by the external calibration method. No matrix interferences existed since there were no statistically significant differences between the slopes at a 95% confidence level. This means that the external calibration method can be used to quantify tryptophan in dietary supplements. In addition, the response relative factor (RRF) was calculated as $slope_{D\text{-tryptophan}}$(minor component)/$slope_{L\text{-tryptophan}}$ (major component) and a value nearly of 1.2 was obtained. In this way, and considering the range established by the European Pharmacopeia (0.8-1.2) [46], the responses of D and L-tryptophan can be considered equivalent which implies that the percentage of D-tryptophan can be established from the ratio between the areas of L- and D-tryptophan.

The precision of the developed method was evaluated in terms of instrumental and methodology repeatability and intermediate precision. Instrumental repeatability was determined from six repeated injections of a standard solution of DL-tryptophan at two

concentration levels (2 and 70 mg/L). RSD values were lower than 0.4% for migration times and lower than 2.6% for corrected peak areas. The method repeatability was assessed with three replicates of a standard solution of DL-tryptophan at two concentration levels (2 and 70 mg/L) injected in triplicate on the same day. RSD values were lower than 0.4% for migration times whereas for corrected peak areas they were lower than 2.8%. Intermediate precision was evaluated by analyzing in triplicate three replicates of a standard solution of DL-tryptophan at two concentration levels (2 and 70 mg/L) during three consecutive days. RSD values were lower than 3.1% and 9.2% for migration times and peak areas, respectively.

Accuracy of the method for tryptophan enantiomers was assessed as the recovery obtained when the three dietary supplements at a concentration of 70 mg/L (nominal concentration) were spiked with L-tryptophan (from 5 to 100% of the nominal concentration) or D-tryptophan (from 3.2 to 9.2% of the nominal concentration). The recoveries obtained for both tryptophan enantiomers are included in **Table 1** which shows that they were close to 100% in all cases.

LODs and LOQs were calculated as the minimum concentration yielding an S/N ratio of 3 and 10 times, respectively. LODs of 0.17 and 0.20 mg/L were obtained for D- and L-tryptophan, respectively, while LOQs were 0.55 mg/L for D-tryptophan and 0.69 mg/L for L-tryptophan. Additionally, the RLOD was calculated as (($LOD_{D\text{-}tryptophan}$/nominal concentration of L-tryptophan (70 mg/L) x 100) obtaining a value of 0.1% which allowed to carry out the detection of the enantiomeric impurity, D-tryptophan, up to levels of 0.1% relative to the majority enantiomer.

Once the suitability of the developed method was demonstrated for the enantiomeric determination of tryptophan, it was applied to the analysis of six dietary

supplements, three of them recently acquired (DS1, DS2 and DS3) and other three stored for a long time (DS1-E, DS2-E and DS3-E). **Figure 3** represents the electropherograms corresponding to the tryptophan dietary supplement DS2 and the same sample spiked with a 0.1% of D-tryptophan.

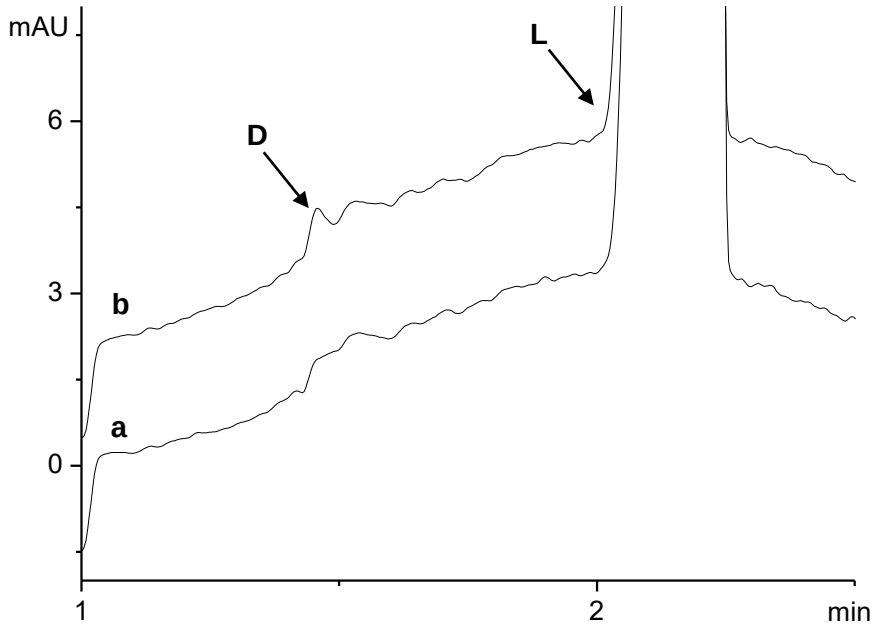

Figure 3. Electropherograms obtained for a dietary supplement at 500 mg/L L-tryptophan: (a) non-spiked dietary supplement, and (b) dietary supplement spiked with 0.1% D-tryptophan. Experimental conditions: BGE, 100 mM formic acid (pH 2.2) containing 1.25% of sulfated-γ-CD; uncoated fused-silica capillary,48.5 cm (8.5 cm to the detector window) × 50 μm ID; applied voltage, 30 kV; temperature, 25ºC; injection by pressure in the short-end, -50 mbar for 4 s; UV detection at 220 ± 4 nm.

The results obtained for the content of L-tryptophan in mg/capsule and the corresponding percentage with respect to the labeled content of L-tryptophan for the different samples are included in **Table 2**. As it can be observed, the percentages of L-tryptophan determined with respect to the labeled contents ranged from 92 ± 6 to 108 ± 2%. D-tryptophan was not detected in any of the samples analyzed at the level of the

detection limit of the developed methodology showing that the elaboration of these dietary supplements fulfils the legal normative [8] which establishes that L-tryptophan can be added to food for specific nutritional purposes (D-tryptophan is not included). On the other hand, no effect of the storage time on the amount of L-tryptophan was observed.

Table 2. Results corresponding to the enantiomeric analysis of tryptophan in dietary supplements recently acquired (DS-1, DS-2, DS-3) and submitted to a long storage time (DS-1-E, DS-2-E, DS-3-E) by the developed EKC method (n=3). D-tryptophan was not detected in any of the samples analyzed.

Dietary supplement	mg L-tryptophan/capsule	% Labeled content of L-tryptophan
DS-1	291 ± 5	108 ± 2
DS-2	479 ± 6	96 ± 1
DS-3	184 ± 11	92 ± 6
DS-1-E	277 ± 8	103 ± 3
DS-2-E	505 ± 5	101 ± 1
DS-3-E	207 ± 4	104 ± 2

Experimental conditions as in **Figure 2B.**

The results obtained in this work demonstrated that the simple developed methodology enabled to achieve the rapid chiral separation of tryptophan enantiomers (resolution 7.4 in 2.5 min) improving the results obtained with previously published methodologies in terms of analysis time and resolutions (analysis times longer than 20 min and enantiomeric resolutions lower than 4.0) [37, 38]. Besides, it has shown to be a powerful tool to carry out the fast quality control of tryptophan-based dietary supplements.

4. Conclusions

A chiral analytical methodology was developed in this work by electrokinetic chromatography enabling the rapid determination of tryptophan. After optimization of different variables, sulphated-γ-CD was employed as chiral selector at a percentage of

1.25% in a 100 mM formate buffer (pH 2.2). Among different strategies designed, the use of a short-end injection enabled the enantiomeric separation of tryptophan within 2.5 min and a resolution of 7.4. UV detection was achieved, and no previous derivatization of tryptophan was necessary. The enantiomeric impurity (D-tryptophan) was the first-migrating enantiomer as it is desirable to facilitate the detection of low amounts of this enantiomer with respect to the majority enantiomer. The method showed a good performance for the enantiomeric determination of tryptophan in dietary supplements. A relative limit of detection of 0.1 % of D-tryptophan with respect to the L-enantiomer was experimentally demonstrated. Application of the developed method to the analysis of tryptophan-based dietary supplements showed a good agreement between the content of L-tryptophan determined and the labeled one. The analysis of samples submitted to a long storage time showed that there was no effect on the L-tryptophan content. Results obtained in this work demonstrate that this method is a powerful tool for the enantioselective analysis of tryptophan enabling the rapid quality control of dietary supplements.

Acknowledgements

Authors thank the Spanish Ministry of Science and Innovation for project PID2019-104913GB-I00 and the Comunidad of Madrid and European funding from FSE and FEDER programs for project S2018/BAA-4393 (AVANSECAL-II-CM). S.B.B and M.C.P. also thank the Spanish Ministry of Economy and Competitiveness for their predoctoral (BES-2017-082458) and “Ramon y Cajal” (RYC-2013-12688) research contracts, respectively. Authors also thank J. Martínez for technical assistance.

References

[1] Food and Agriculture Organization of the United Nations, World Health Organization, United Nations University, Protein and Amino Acid Requirements in Human Nutrition: Report of a Joint WHO/FAO/UNU, Expert Consultation Technical Report Series, No 935, WHO, Geneva (Switzerland). 2007 (accessed 22 December 2020) https://apps.who.int/iris/bitstream/handle/10665/43411/WHO_TRS_935_eng.pdf?sequence=1&isAllowed=y.

[2] M. Friedman, Analysis, nutrition, and health benefits of tryptophan, In. J. Tryptophan Res. 11 (2018) 1178646918802282, https://doi.org/10.1177/1178646918802282.

[3] J. Yang, D.S. Hage, Characterization of the binding and chiral separation of D and L-tryptophan on a high-performance immobilized human serum albumin column, J. Chromatogr. A 645 (1993) 241-250, https://doi.org/10.1016/0021-9673(93)83383-4.

[4] H.O. Lee, R.G. Uzzo, D. Kister, W.D. Kruger, Combination of serum histidine and plasma tryptophan as a potential biomarker to detect clear cell renal carcinoma. J Transl Med. 15 (2017) 72, https://doi.org/10.1186/s12967-017-1178-8.

[5] A. Gakamsky, R.R. Duncan, N.M. Howarth, B. Dhillon, K.K. ButtenschN, D.J. Daly, D. Gakamsky, Tryptophan and non-tryptophan fluorescence of the eye lens proteins provides diagnostics of cataract at the molecular level. Sci Rep. 7 (2017)40375, https://doi.org/10.1038/srep40375.

[6] R.R. Langner, C.P. Berg, Metabolism of D-tryptophan in the normal human subject, J. Biol. Chem. 214 (1955) 699-707, https://doi.org/10.1016/S0021-9258(18)70919-8.

461 [7] W.C. Rose, G.F. Lambert, M.J. Coon, The amino acid requirements of man: VII.
462 General procedures; the tryptophan requirement, J. Biol. Chem. 211 (1954) 815-827,
463 https://doi.org/10.1016/S0021-9258(18)71169-1.

464 [8] Commission Decision 2001/15/EC, Off. J. Eur. Commun., L52, 2001, pp. 19-25.

465 [9] L. Sanchez-Hernandez, J.L. Bernal, M.J. del Nozal, L. Toribio, Chiral analysis of
466 aromatic amino acids in food supplements using subcritical fluid chromatography and
467 Chirobiotic T2 column, J. Supercrit. Fluids 107 (2016) 519-525,
468 https://doi.org/10.1016/j.supflu.2015.06.027.

469 [10] V. Guillén-Casla, M.E. León González, L.V. Pérez-Arribas, L.M. Polo-Díez, Direct
470 chiral determination of free amino acid enantiomers by two-dimensional liquid
471 chromatography: application to control transformations in E-beam irradated foodstuffs,
472 Anal. Bioanal. Chem. 397 (2010) 63-75, https://doi.org/10.1007/s00216-009-3376-6.

473 [11] A. Lomenova, K. Hrobonova, Application of achiral–chiral two-dimensional HPLC
474 for separation of phenylalanine and tryptophan enantiomers in dietary supplement,
475 Biomed. Chromatogr. (2020) e4972, https://doi.org/10.1002/bmc.4972.

476 [12] D. Xu, E. Sanchez-Lopez, Q. Wang, Z. Jiang, M. L. Marina, Determination of L-
477 norvaline and L-tryptophan in dietary supplements by nano-LC using O-[2-
478 (methacryloyloxy)-ethylcarbamoyl]-10,11-dihydroquinidine-silica hybrid monolithic
479 column, J. Pharm. Anal.10 (2020)70-77, https://doi.org/10.1016/j.jpha.2019.10.001.

480 [13] D. Petrova Obreshkova, D. Doncheva Tsvetkova, K. Valentinov Ivanov,
481 Simultaneous identification and determination of total content of aminoacids in food
482 supplements – Tablets by gas chromatography, Asian J. Pharm. Clin. Res. 5 (2012) 57-
483 68.

[14] E. Sánchez-López, M. Castro-Puyana, M.L. Marina, Electrophoresis | Capillary Electrophoresis: Chiral separations, in: P. Worsfold, C. Poole, A. Townshend, M. Miró (Eds.), Encyclopedia of Analytical Science, Elsevier, Netherlands, 2019, pp. 334-345.

[15] S. Bernardo-Bermejo, E. Sánchez-López, M. Castro-Puyana, M.L. Marina, Chiral capillary electrophoresis, Trends Anal. Chem. 124 (2020), 115807, https://doi.org/ 10.1016/j.trac.2020.115807.

[16] I.J. Stavrou, E.A. Agathokleous, C.P. Kapnissi-Christodoulou, Chiral selectors in CE: Recent development and applications (mid-2014 to mid-2016), Electrophoresis 38 (2017) 786-819, https://doi.org/10.1002/elps.201600322.

[17] R.B. Yu, J.P. Quirino, Chiral selectors in capillary electrophoresis: Trends during 2017-2018, Molecules 24 (2019) 1135, https://doi.org/10.3390/molecules24061135

[18] S. Fanali, B. Chankvetadze, Some thoughts about enantioseparations in capillary electrophoresis, Electrophoresis 40 (2019) 2420-2437, https://doi.org/10.1002/elps.201900144.

[19] I. Fejos, E. Kalydi, M. Malanga, G. Benkovics, S. Beni, Single isomer cyclodextrins as chiral selectors in capillary electrophoresis, J. Chromatogr. A (2020) 1617, https://doi.org/ 10.1016/j.chroma.2020.461375.

[20] J. Chen, X.Q. Wang, M. Ghulam, H.X. Chen, F. Qu. Predefine resolution of enantiomers in partial filling capillary electrophoresis and two discontinuous function plugs coupling in-capillary, Electrophoresis 39 (2018), 2391–2397, https://doi.org/10.1002/elps.201800154.

505 [21] M. Havlikova, Z. Bosakova, G. Benkovics, J. Jindrich, M. Popr, P. Coufal, Use of
506 6-O-mono-substituted derivatives of β-cyclodextrin-bearing substituent with two
507 permanent positive charges in capillary electrophoresis, Chem. Pap. 70 (2016) 1144–
508 1154, https://doi.org/10.1515/chempap- 2016- 0053.

509 [22] J. Wang, L. Wang, J.L. Bai, Separation of different enantiomeric amino acids by
510 capillary array electrophoresis, Anal. Lett. 39 (2006) 1429-1437, https://doi.org/
511 10.1080/00032710600669002.

512 [23] A.P. Kumar, J.H. Park, Azithromycin as a new chiral selector in capillary
513 electrophoresis, J. Chromatogr. A 1218 (2011) 1314–1317,
514 https://doi.org/10.1016/j.chroma.2010. 12.106.

515 [24] L. Sánchez-Hernández, E. Domínguez-Vega, C. Montealegre, M. Castro-Puyana,
516 M.L. Marina, A.L. Crego, Potential of vancomycin for the enantiomeric resolution of
517 FMOC-amino acids by capillary electrophoresis-ion-trap-mass spectrometry,
518 Electrophoresis35(2014)1244–1250, https://doi.org/10.1002/elps.201300489.

519 [25] B. Chen, Y. Du, P. Li, Investigation of enantiomeric separation of basic drugs by
520 capillary electrophoresis using clindamycin phosphate as a novel chiral selector,
521 Electrophoresis. 20 (2009) 2747–2754, http://doi.org/10.1002/elps.200800452.

522 [26] R.J. Liu , Y.X. Du, J.Q. Chen, Q. Zhang, S.J. Du , Z.J. Feng, Investigation of the
523 enantioselectivity of tetramethylammonium l-hydroxyproline ionic liquid as a novel
524 chiral ligand in ligand-exchange CE and ligand-exchange MEKC, Chirality 27 (2015)
525 58–63, http://doi.org/10.1002/chir.22388.

526 [27] C.Y. Yue, G.S. Ding, F.J. Liu, A.N. Tang, Water-compatible surface molecularly
527 imprinted silica nanoparticles as pseudostationary phase in electrokinetic

chromatography for the enantioseparation of tryptophan. J Chromatogr A 1311 (2013) 176–182. https://doi.org/10.1016/j.chroma.2013.08.086.

[28] R. Huang, W.M. Xiong, D.F. Wang, L.H. Guo, Z.Y. Lin, L.S. Yu, K.D. Chu, B. Qui, G.N. Chen, Label-free aptamer-based partial filling technique for enantioseparation and determination of DL-tryptophan with micellar electrokinetic chromatography, Electrophoresis 34 (2013) 254–259, https://doi.org/10.10 02/elps.20120 0464.

[29] Z.F. Xu, J. Guan, H.L. Shao, S.T. Fan, X.Y. Li, S. Shi, F. Yan, Combined use of Cu(II)-L-Histidine complex and beta-cyclodextrin for the enantioseparation of three amino acids by CE and a study of the synergistic effect, J. Chromatogr. Sci. 1626 (2020) 461383, https://doi.org/10.1016/j.chroma.2020.461383.

[30] Y. Wu , G. Wang , W. Zhao , H. Zhang, H. Jing , A. Chen , Chiral separation of phenylalanine and tryptophan by capillary electrophoresis using a mixture of β-CD and chiral ionic liquid ([TBA] [L-ASP]) as selectors, Biomed. Chromatogr. 28 (2014) 610–614, https://doi.org/10.1002/bmc.3078.

[31] R.C. Moldovan, E. Bodoki, T. Kacso, A.C. Servais, J. Crommen, R. Oprean, M. Fillet, A micellar electrokinetic chromatography-mass spectrometry approach using in-capillary diastereomeric derivatization for fully automatized chiral analysis of amino acids, J. Chromatogr. A 1467 (2016) 400-408, https://doi.org/10.1016/j.aca.2016.08.040.

[32] A. Prior, R.C. Moldovan, J. Crommen, A.C. Servais, M. Fillet, G.J. de Jong,G.W. Somsen, Enantioselective capillary electrophoresis-mass spectrometry of amino acids in cerebrospinal fluid using a chiral derivatizing agent and volatile surfactant, Anal. Chim. Acta 940 (2016) 150-158, https://doi.org/10.1016/j.aca.2016.08.040.

[33] A. Prior, L. Sánchez-Hernández, J. Sastre-Torano, M.L. Marina, G.J. de Jong, G.W. Somsen, Enantioselective analysis of proteinogenic amino acids in cerebrospinal fluid by capillary electrophoresis-mass spectrometry, Electrophoresis 37 (2016) 2410-2419, https://doi.org/10.1002/elps.201600015.

[34] L. Sánchez-Hernández, N.S. Serra, M.L. Marina, A.L. Crego, Enantiomeric separation of free L- and D-amino acids in hydrolyzed protein fertilizers by capillary electrophoresis tandem mass spectrometry. J. Agric. Food Chem.61(2013) 5022–5030, https://doi.org/10.1021/jf401334.

[35] A. Prior, G. Coliva, G.J. de Jong, G.W. Somsen, Chiral capillary electrophoresis with UV-excited fluorescence detection for the enantioselective analysis of 9-fluorenylmethoxycarbonyl-derivatized amino acids, Anal. Bioanal. Chem. 410 (2018) 4979-4990, https://doi.org/10.10 07/s0 0216- 018- 1148- x.

[36] A. Prior, E. van de Nieuwenhuijzen, G.J. de Jong, G.W. Somsen, Enantioselec- tive micellar electrokinetic chromatography of DL-amino acids using (+)-1- (9-fluorenyl)-ethyl chloroformate derivatization and UV-induced fluorescence detection, J. Sep. Sci. 41 (2018) 2983–2992, https://doi.org/10.10 02/jssc.20180 0204.

[37] L. Qi, M.R. Liu, Z.P. Guo, M.Y. Xie, C.G. Qiu, Y. Chen, Assay of aromatic amino acid enantiomers in rice-brewed suspensions by chiral ligand-exchange CE, Electrophoresis, 28 (2007) 4150-4155, https://doi.org/10.1002/elps.200700281.

[38] E.P. Lin, K.C. Lin, C.W. Chang, M.M. Hsieh, On-Line sample preconcentration by sweeping and poly(ethylene oxide)-mediated stacking for simultaneous analysis of nine pairs of amino acid enantiomers in capillary electrophoresis, Talanta 114(2013)297-303, https://doi.org/10.1016/j.talanta.2013.05.039.

573 [39] B. Chankvetadze, Capillary electrophoresis in chiral analysis, J. Wiley & Sons,
574 Chichester, UK, 1997, pp. 572, https://doi.org/10.1002/elps.1150191237.

575 [40] Y. Martin-Biosca, C. García-Ruíz, M.L. Marina, Enantiomeric separation of the
576 chiral phenoxy acid herbicides by electrokinetic chromatography. Application to the
577 determination of analyte-selector apparent binding constants for enantiomers,
578 Electrophoresis 22 (2001) 3216-3225, https://doi.org/10.1002/1522-
579 2683(200109)22:15<3216::AID-ELPS3216>3.0.CO;2-X.

580 [41] J.L. Beckers, P. Bocek, The preparation of background electrolytes capillary zone
581 electrophoresis: Golden rules and pitfalls, Electrophoresis, 24 (2003) 518–535,
582 https://doi.org/10.1002/elps.200390060.

583 [42] H. Wan, A. Holmén, M. Någård, W. Lindberg, Rapid screening of pKa values of
584 pharmaceuticals by pressure-assisted capillary electrophoresis combined with short-end
585 injection. J. Chromatogr. A. 979 (2002) 369-377, https://doi.org/ 10.1016/s0021-
586 9673(02)01262-1.

587 [43] K. D. Altria, M.A. Kelly, B.J. Clark, The use of a short-end injection procedure to
588 achieve improved performance in capillary electrophoresis, Chromatographia, 43 (1996),
589 153-158, https://doi.org/10.1007/BF02292944.

590 [44] Z. Glatz, Application of short-end injection procedure in CE, Electrophoresis 34
591 (2013) 631–642, https://doi.org/10.1002/elps.201200506.

592 [45] ICH validation of analytical procedures: text and methodology Q2 (R1), In:
593 International Conference on Harmonization, 2005.

[46] European Pharmacopoeia, 4th edition, The European Pharmacopoeia Convention Inc., 2004, pp. 3843-3849 (supplement 4.6).

Supporting information

A rapid electrokinetic chromatography method using short-end injection for the enantioselective separation of tryptophan

Samuel Bernardo-Bermejo[1], María Luisa Marina[1,2], María Castro-Puyana[1,2 *]

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

Correspondence: Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona, Km. 33.600, 28871 Alcalá de Henares, Madrid, España.

E-mail: maria.castrop@uah.es

Tel.: +34 918856430

Table S1. Cyclodextrins employed as chiral selectors to achieve the enantioselective separation of tryptophan by EKC at pH 2.2.

	Cyclodextrin	Time (min)		Resolution
		D-Tryptophan	L-Tryptophan	
NEUTRAL	α-CD	8.9	-	-
	β-CD	8.4	-	-
	γ-CD	8.3	-	-
	2-hydroxypropyl-β-CD (DS 4)	9.5	-	-
	methyl-β-CD (DS 1.8)	8.6	-	-
	heptakis(2,3,6-tri-O-methyl)-β-CD (DS 13)	9.3	-	-
	acetyl-β-CD (DS 7)	10.2	10.3	0.8
	acetyl-γ-CD (DS 7)	10.2	-	-
ANIONIC	sulfated-α-CD (DS 12)	13.5	-	-
	phosphated-β-CD (DS 4)	18.8	20.2	2.4
	sulfobutyl-β-CD (DS 6.3)	17.8	19.1	2.2
	sulfated-β-CD (DS 12) (Sigma)*	14.5	15.1	2.1
	sulfated-β-CD (DS 12) (Aldrich)*	12.5	13.8	4.9
	sulfated-β-CD (DS 12) (Fluka)*	11.0	12.6	5.7
	sulfated-γ-CD (DS 14))	8.9	13.6	18.7

Experimental conditions: BGE, 10 mM CD in 100 mM formic acid (pH 2.2); uncoated fused-silica capillary, 48.5 cm (40 cm to the detector window) x 50µm ID; UV detection at 220 ± 4 nm applied voltage, 20 kV; temperature, 25 ºC.

*Commercial suppliers.

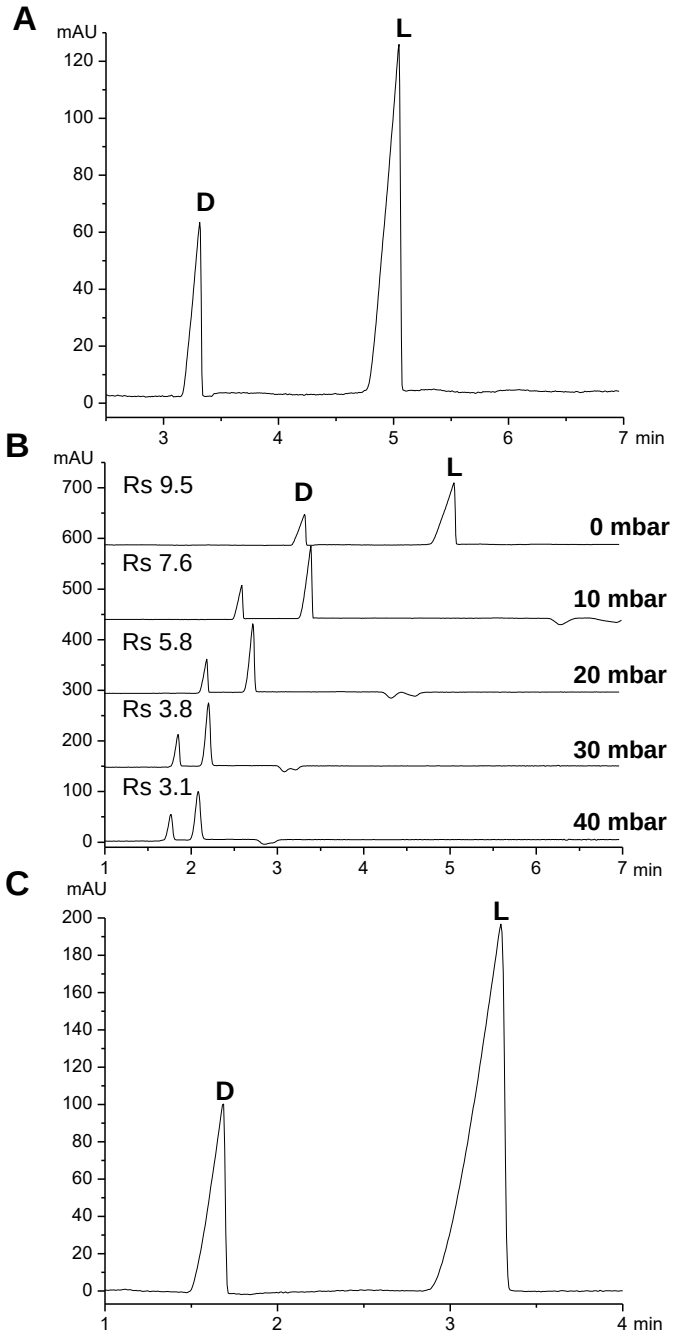

Figure S1. Electropherograms corresponding to the chiral separation of DL-tryptophan (solution 0.5 mM in the D-enantiomer and 1 mM in the L-enantiomer): (A) injection by pressure in the cathodic end; (B) effect of the pressure during the analysis; (C) injection by pressure in the short-end Experimental conditions for A and B: BGE, 100 mM formic acid (pH 2.2) containing 1.00% sulfated-γ-CD; uncoated fused-silica capillary, 33.5 cm (25 cm to the detector window) x 50μm ID; applied voltage, -20kV; temperature, 25 ºC; injection 50 mbar for 4 s; UV detection at 220 ± 4 nm. Experimental conditions in C: uncoated fused-silica capillary, 33.5 cm (8.5 cm to the detector window) x 50μm ID; applied voltage, 20 kV; injection -50 mbar for 4 s. Other conditions as in A and B.