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**ENANTIOMERIC SEPARATION OF NON-PROTEIN AMINO ACIDS BY
ELECTROKINETIC CHROMATOGRAPHY**

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Highlights

- Eight non protein amino acids were enantiomerically separated by EKC.
- The first enantiomeric separation of Pyro, Amino adipic by CE is presented.
- L-citrulline was quantitated in food supplements and D-citrulline was not detected.
- No racemization was observed in supplements due to the effects of storage time.

Abstract

New analytical methodologies enabling the enantiomeric separation of a group of non-protein amino acids of interest in the pharmaceutical and food analysis fields were developed in this work using Electrokinetic Chromatography. The use of FMOC as derivatization reagent and the subsequent separation using acidic conditions (formate buffer at pH 2.0) and anionic cyclodextrins as chiral selectors allowed the chiral separation of eight from the ten non-protein amino acids studied. Pyroglutamic acid, norvaline, norleucine, 3,4-dihydroxyphenylalanine, 2-aminoadipic acid, and selenomethionine were enantiomerically separated using sulfated- α -CD while sulfated- γ -CD enabled the enantiomeric separation of norvaline, 3,4-dihydroxyphenylalanine, 2-aminoadipic acid, selenomethionine, citrulline, and pipercolic acid. Moreover, the potential of the developed methodologies was demonstrated in the analysis of citrulline and its enantiomeric impurity in food supplements. For that purpose, experimental and instrumental variables were optimized and the analytical characteristics of the proposed method were evaluated. LODs of 2.1×10^{-7} and 1.8×10^{-7} M for D- and L- citrulline, respectively, were obtained. D-Cit was not detectable in any of the six food supplement samples analyzed showing that the effect of storage time on the racemization of citrulline was negligible.

Abbreviations: **Aminoadipic**, 2-Aminoadipic acid; **CD**, Cyclodextrin; **Cit**, Citrulline; **DOPA**, 3,4-Dihydroxyphenylalanine; **EKC**, Electrokinetic chromatography; **Hcy**, Homocysteine; **Norleu**, Norleucine; **Norval**, Norvaline; **Orn**, Ornithine; **Pipe**, Pipercolic acid; **Pyro**, Pyroglutamic acid; **SAM**, S-adenosyl-L-methionine; **SeMet**, Selenomethionine.

Keywords: Non-protein amino acids, Enantiomeric separation, chiral CE, Electrokinetic chromatography, Citrulline, Food supplements.

1. Introduction

Non protein amino acids are a group of compounds which are not found as protein constituents. A huge amount of non-protein amino acids (more than 800) from different origin and with diverse functions have been described. Many of them are the end products of secondary metabolism, whereas many others are intermediate of metabolic pathways [1]. Although these compounds have been studied to a lesser extent than protein amino acids, they have demonstrated to be relevant in the pharmaceutical, clinical, and food fields. In fact, some of them present relevant biological activities, for instance, DOPA is applied in the treatment of Parkinson's disease [2] and norleucine is related to the inhibition of the oxidative stress associated with Alzheimer's disease (AD) [3]. Non-protein amino acids can also be present in foods as metabolic intermediates, as products formed during food processing or as additives in food to increase some nutritional and functional properties [4, 5], and they have demonstrated to be relevant markers of the food quality and safety. For example, the study of these compounds allowed to detect adulterations of olive oil with seed oils [6-8], to evaluate food processing (fermentation, storage, thermal treatment) [9-12] as well as to determine the nutritional quality and toxicity of some foods [7, 8, 13].

A relevant factor that should be considered in the analysis of non-protein amino acids is their chiral behavior. In nature, they are generally composed by their L-forms, however the racemization into the D-form may be produced through different metabolic pathways, by different processing conditions employed by the food industry to improve food characteristics [14-16], synthesized in enzymatic pathways by the action of microorganisms or they can even be present in supplemented foodstuffs due to the fraudulent addition of racemic mixtures [17, 18]. In fact, although the use of racemic mixtures of non-protein amino acids is allowed in the pharmaceutical industry [19] the use of D-enantiomers in the food industry is forbidden and the regulatory agencies prevent their use in the elaboration of foods and dietary supplements [20]. All these facts originate that nowadays the chiral analysis of non-protein amino acids is of high interest in different fields.

CE has shown to be a powerful tool to carry out chiral separations since it allows short analysis times, high separation efficiency, versatility and feasibility to incorporate a great variety of chiral selectors to obtain high resolutions. EKC is the CE mode usually employed to achieve chiral separations. From the first research works dealing with the use of EKC [21-23], this technique has experienced an enormous growth being nowadays one of the best options for analytical enantioseparations [24]. Regarding non-protein amino acids, just a few research works reported the enantiomeric determination of non-protein amino acids in foods by CE. Thus, EKC-UV methodologies were developed for the chiral separation of ornithine in fermented foods (beer, vinegar and wine) and food supplements [11, 25-27], S-adenosyl-L-methionine (SAM) in fruit juices [28] whereas the coupling EKC-MS² enabled the evaluation of fermentation processes through the enantiomeric determination of ornithine in beer samples or to identify and quantitate L-carnitine in infant formulas and dietary supplements as well as

its enantiomeric impurity (D-carnitine) [29, 30]. Also, a CEC method with UV detection has been described to the determination of citrulline in food supplements [31].

The aim of this work was to develop novel EKC-UV methodologies based on the use of cyclodextrins as chiral selectors to carry out the chiral separation of ten non-protein amino acids (pyroglutamic acid, norvaline, norleucine, 3,4-dihydroxyphenylalanine (DOPA), selenomethionine, homocysteine, ornithine, 2-aminoadipic acid, citrulline and pipercolic acid) that have demonstrated to play an important role in some metabolic routes or to have some interesting properties and healthy benefits. L-citrulline and L-ornithine, which can be synthesized during the metabolism of L-arginine [32], have been used in dietary supplements to favour the metabolism of corporal fatty excess and reduce ammonia levels [31, 27]. D-Cit has demonstrated to have different *in vivo/in vitro* behavior compared with L-Cit [31] while D-orn has shown toxic effects at high levels and to be an inhibitor of urea synthesis by competition with the L-enantiomer [25]. L-norvaline is also related with L-arginine metabolism since it is an inhibitor of arginase activity, reducing urea production and increasing NO production [32]. L-pyroglutamic acid and selenomethionine are also of high relevance in human health since the first has a number of remarkable cognitive enhancing effects [33] (its L-enantiomer is bioactive to improve blood circulation in the brain, whereas D-pyroglutamic remains inactive [34]) and the second is considered the major source of selenium to humans which is indispensable for human health since its deficit could cause an adverse impact on immune system. While L-selenomethionine occurs naturally, D-selenomethionine is toxic at high levels [35]. Regarding pipercolic acid, it is considered to be a neuromodulator which play a role in the central inhibitory γ -aminobutyric acid system. The presence of its D enantiomer in fluids and tissues can be due to food intake or production by intestinal bacteria. [12]. Due to the scarce number

of studies devoted to the enantiomeric separation of these non-protein amino acids by CE, the development of analytical methodologies with this aim presents a high relevance.

2. Materials and methods

2.1 Reagents and samples

All reagents employed were of analytical grade. Boric acid, sodium hydroxide, ammonium hydroxide, and pentane were obtained from Sigma-Aldrich (Madrid, Spain). Formic acid, hydrochloric acid, and acetonitrile were from Scharlau (Barcelona, Spain). The chiral selectors β -CD, Heptakis (2,3,6-tri-O-methyl)- β -CD, (2-Hydroxi)propyl- β -CD (DS~3), γ -CD, carboxymethyl- β -CD were purchased from Fluka (Buchs, Switzerland), methyl- β -CD, Heptakis(2,6-di-O-methyl)- β -CD, sulfated- β -CD, succinyl- β -CD from Sigma-Aldrich (Madrid, Spain), and (2-hydroxi)propyl- γ -CD, methyl- γ -CD, acetylated- β -CD, acetylated- γ -CD, carboxyethylated- β -CD (DS~3), phosphated- β -CD, sulfated- γ -CD (DS~14), and sulfated- α -CD (DS~12) from Cyclolab (Budapest, Hungary). Water used to prepare solutions was purified through a milli-Q System from Millipore (Bedford, MA, USA).

DL-Norvaline (Norval), DL-Norleucine (Norleu), 3,4-Dihydroxy-DL-phenylalanine (DOPA), DL-Homocysteine (Hcy), DL-2-Aminoadipic acid (Aminoadipic), DL-Selenomethionine (SeMet), D-Pipecolic acid (D-pipe) and D-Ornithine (D-Orn), were supplied from Sigma-Aldrich (Madrid, Spain), whereas D-Citruline/L-Citruline (D-Cit/L-Cit), D-Pyroglutamic acid/L-Pyroglutamic acid (D-Pyro/L-Pyro), L-Pipecolic (L-Pipe) and L-Ornithine (L-Orn) were obtained from Fluka (Buchs, Switzerland).

The derivatization reagent 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) was purchased from Sigma-Aldrich (Madrid, Spain).

The six different food supplements analyzed were acquired in different Madrid's markets (Spain).

2.2 CE conditions

CE experiments were carried out with an Agilent 7100 CE system (Agilent Technologies, Waldbronn, Germany) equipped with a DAD working at 210 nm with a bandwidth of 4 nm. The instrument was controlled by HP^{3D}CE ChemStation from Agilent Technologies. Separations were performed using uncoated fused-silica capillaries of 50 μm ID (362.8 μm OD) with a total length of 58.5 cm (50 cm effective length) or with a total length of 48.5 cm (40 cm effective length) purchased from Polymicro Technologies (Phoenix, AZ, USA). The samples were injected by applying a pressure of 50 mbar from 4 to 20 s, and the electrophoretic separation was achieved using reverse-polarity mode (voltage of -20 kV) and working temperature from 15 to 25 °C.

Before its first use, new capillaries were rinsed (applying 1 bar) with 1M sodium hydroxide for 30 min, followed by 5 min with Milli-Q water and conditioned with buffer solution for 60 min. At the beginning of each day the capillary was pre-washed (applying 1 bar) with 0.1 M sodium hydroxide during 10 min, Milli-Q water for 5 min, and buffer for 40 min, and BGE during 10 min. Between injections, the capillary was conditioned with 0.1 M hydrochloric acid (2 min), Milli-Q water (1 min) and BGE (5 min).

2.3 Preparation of solutions and samples

The borate buffer solution (200 mM, pH 9.0) required to dissolve the non-protein amino acids before the derivatization step was prepared by dissolving the appropriate amount of boric acid in Milli-Q water. The separation buffer solution was prepared by diluting

the appropriate volume of formic acid with Milli-Q water and adjusting the pH to the desired value with hydrochloric acid before completing the volume with water to get the desired buffer concentration (100 mM). The BGE was obtained by dissolving the suitable amount of the appropriate chiral selector (CDs) in the separation buffer.

Stock standard solutions of each non-protein amino acid were prepared dissolving the appropriate amount in borate buffer (200 mM, pH 9.0). These solutions were stored at 4 °C until the derivatization step with FMOC.

To prepare sample solutions of the six dietary supplements analyzed, the content of four capsules was weighed, powdered and mixed homogeneously. Taking into account the labeled amount of Cit in each supplement, an appropriate amount of the powdered obtained was dissolved in Milli-Q water to obtain a standard solution of 50 mM. Dissolution was performed by ultrasonication for 10 min followed by centrifugation (15 min, 4000g at 25°C) and filtration. Before derivatization, these sample solutions were diluted in borate buffer (200 mM, pH 9.0) to obtain the appropriate concentration.

All solvents and samples were filtered prior use through 0.45 µm pore size disposable nylon filters from Scharlau (Barcelona, Spain). A pH meter (Metrohn 744, Herisau, Switzerland) was used for the pH adjustment of buffer solutions.

2.4 Derivatization

Derivatization of non-protein amino acids (except pyro which was not derivatized) was carried out following a methodology previously described in the literature with slightly modifications [36, 37]. The desired concentration of FMOC chloride in ACN was freshly prepared each day taking into account that an excess of at least three times of FMOC was necessary to obtain a complete derivatization of the non-protein amino acids.

Briefly, 300 μL of a 10 mM non-protein standard amino acid or sample solutions (diluted with 200 mM borate buffer at pH 9.0) were mixed with 300 μL of FMOC chloride dissolved in ACN. The solution was kept at room temperature for 2 min to complete reaction, and the resulting solution was extracted with 0.6 mL pentane to remove FMOC excess. Then, this solution was diluted ten times with Milli-Q water before injection in the CE system.

2.5 Data treatment

Chiral resolution values (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. Experimental data analysis, calculation of different parameters, and composition of graphs with different electropherograms were carried out using Excel Microsoft, Statgraphics Centurion XVI, and Origins 8.0 software.

3. Results and discussion

3.1 Development of enantioselective analytical methodologies for the separation of non-protein amino acids.

In order to develop chiral analytical methodologies for the separation of the enantiomers of the non-protein amino acids investigated, a pre-capillary derivatization step with FMOC chloride was performed since the lack of absorbance of most amino acids makes essential their derivatization when UV detection is employed. FMOC chloride was chosen because it is a fast labeling reagent producing stable amino acids derivatives using simple reaction conditions [38]. Besides to allow the UV detection, FMOC derivatization enables to obtain large molecules which can favor in some cases the interaction with the chiral selectors. Regarding chiral selectors, cyclodextrins were

chosen because they have demonstrated to be one of the best options in the field of chiral separations by EKC [39, 40].

3.1.1 Effect of the cyclodextrin nature and the separation buffer (nature and pH)

The buffers employed were 100 mM borate at pH 9.0 and 100 mM formate at two different pH values, pH 2.0 and pH 4.5. Under basic conditions, the discrimination power of ten different neutral cyclodextrins (β -CD, Methyl- β -CD, Heptakis(2,6-di-O-methyl)- β -CD, Heptakis(2,3,6-di-O-methyl)- β -CD, (2-Hydroxi) propyl- β -CD, (2-Hydroxi) propyl- γ -CD, Methyl- γ -CD, Acetylated- β -CD, Acetylated- γ -CD, γ -CD) at a concentration of 10 mM was investigated. These experiments were carried out using a separation voltage of 30 kV, and a working temperature of 25 °C. From the ten amino acids analyzed, only Amino adipic and SeMet were partially resolved (R_s values of 0.2 and 0.9, respectively) using Heptakis(2,6-di-O-methyl)- β -CD as chiral selector.

On the other hand, when acidic conditions were employed, the potential of different anionic cyclodextrins was evaluated. In this case, the selectors tested were sulfated- α -CD, sulfated- β -CD, sulfated- γ -CD, phosphated- β -CD, carboxymethylated- β -CD, carboxyethylated- β -CD, and succinyl- β -CD when pH 4.5 was employed, and the three sulfated CD along with the phosphated- β -CD when pH 2.0 was used. All these experiments were performed at a fixed concentration of cyclodextrin (10 mM), using a separation voltage of -20 kV, and a working temperature of 25 °C. None of the cyclodextrins employed in 100 mM formate buffer at pH 4.5 allowed the enantiomeric discrimination of the non-protein amino acids studied. However, at pH 2.0, the use of the above-mentioned four anionic cyclodextrins enabled the resolution of eight of the ten non-protein amino acids studied (Pyro, Norval, Norleu, DOPA, Amino adipic, SeMet, Cit and Pipe) (see **Table 1**). Consequently, 100 mM formate buffer at pH 2.0 was chosen as running buffer for further experiments and the effect of the temperature

was investigated for the cyclodextrins giving rise to the best enantiomeric resolutions (sulfated- α -CD, sulfated- β -CD, sulfated- γ -CD or phosphated- β -CD).

3.1.2 Effect of the temperature

In order to evaluate the influence of the temperature on the enantiomeric separation of the non-protein amino acids, the values of the chiral resolution obtained with the four cyclodextrins tested (sulfated- α -CD, sulfated- β -CD, sulfated- γ -CD, and phosphate- β -CD) at 15°C, 20°C, and 25 °C were compared. **Table 1** shows both the migration times for each enantiomer and the chiral resolution for each non-protein amino acid analyzed at different temperatures. As it can be observed, when phosphate- β -CD was used as chiral selector, the variation of temperature did not affect significantly either migration time or chiral resolution of those amino acids which could be enantioseparated. In general, both migration times and chiral resolutions are slightly lower when increasing the value of temperature. For this reason, 15 °C seemed to be the most appropriate working temperature when phosphated- β -CD was used, since it allowed a high number of enantiomeric separations (Norval, DOPA, SeMet and cit). A different behavior regarding the effect of the variation of the temperature could be observed in the case of the use of sulfated cyclodextrins as chiral selectors. In fact, when using sulfated- β -CD, the resolution of Norval was higher when increasing the temperature from 15 to 20 °C while the value of resolution obtained for Pipe was almost constant for the three temperatures tested. An important factor to take into consideration is that the migration times obtained with this cyclodextrin were too high, in most of cases more than 60 min (**Table 1**). Both sulfated- α -CD and sulfated- γ -CD showed a high discrimination power for the non-protein amino acids studied. For these two cyclodextrins, the influence of the temperature was also different. The results obtained with sulfated- α -CD were better at 15 °C whereas those obtained with sulfated- γ -CD were not affected by the

temperature. This fact can be clearly observed in **Table 1**. A decrease of the temperature to the lowest value (15°C) allowed an increment of the number of non-protein amino acids which can be enantiomerically resolved as well as a decrease in the migration times when sulfated- α -CD was employed. From the ten compounds studied, six of them (Pyro, Norval, Norleu, DOPA, Amino adipic, and SeMet) were separated with resolution values from 0.6 to 3.5. When sulfated- γ -CD was used, the best results were obtained using both 15 and 25 °C, since at 20 °C an overlapping between the peaks corresponding to Fmoc and some enantiomers was observed. In this case, an increase in the temperature gave rise to a decrease on the migration times, and slightly variations in the resolution values. Using both 15 or 25 °C, it was possible to achieve the chiral separation of Norval, DOPA, Amino adipic, SeMet, Cit, and Pipe with resolution values from 0.9 to 7.4 (see **Table 1**).

Although it is well known that the enantiomeric resolution of chiral compounds can be modified with the temperature, it is not possible to establish a general behavior when this parameter varies since a decrease or an increase in the resolution can be found for different analytes. These differences can be explained by the effect that the temperature may have on the buffer viscosity, peak efficiency or selector-selectand interactions [41, 42].

From the results obtained, it can be concluded that eight out of ten non-protein amino acids investigated could be separated employing sulfated- α -CD or sulfated- γ -CD. However, the chiral separation of Pyro and Norleu was only achieved when sulfated- α -CD was used, whereas Pipe and Cit were only enantioseparated employing sulfated- γ -CD. For those amino acids for which the chiral separation could be reached by using both cyclodextrins, the best results concerning chiral resolution as well as migration times, were obtained using sulfated γ -CD. The variation of the concentration of both

cyclodextrins (sulfated- α -CD and sulfated- γ -CD) from 1 to 10 mM (1, 2, 5, and 10 mM) and of the separation voltage (-20, -25, -30 kV) did not enable to improve the results obtained so a CD concentration of 10 mM and -20kV were selected.

Figure 1 shows the electropherograms obtained for the eight FMOC-non-protein amino acids enantioseparated under the conditions giving rise to the highest chiral resolution. It can be observed in **Table 1** that the simultaneous separation of mixtures of some non-protein amino acids were also possible as shown in **Figure 2** corresponding to the separation of Pipe, Cit, and Aminoadipic using sulfated γ -CD as chiral selector, as an example.

3.2 Application of the developed methodologies to the analysis of food supplements.

The developed EKC methodology using a 100 mM formate buffer at pH 2.0 and 10 mM sulfated γ -CD as chiral selector was applied to the enantiomeric determination of Cit in food supplements. This non-protein amino acid is added to food supplements due to its capability to remove the lactic acid produced during strong training. While L-Cit is involved in the urea cycle, it is the precursor of arginine, reduces ammonia levels, and has a relevant role in NO cycle [43], D-Cit has shown a different behavior both *in vitro* and *in vivo* [44-46].

3.2.1 Optimization of the enantioseparation of Citrulline

In order to decrease the migration time for Cit enantiomers, the capillary length was shortened up to 40 cm. However, using this capillary and a working temperature of 15 °C, an overlapping between the second-migrating enantiomer (D-Cit) and FMOC was observed. To avoid that, both the buffer pH (from pH 2.0 to pH 3.0) and the temperature (from 15 °C to 25 °C) were modified. Under these conditions, the migration time obtained for Cit was around 18 min with $R_s > 2.5$, and the separation between FMOC and D-Cit was higher than 4 min (R_s 10.3) which avoided any possible overlapping

between their peaks. Finally, to obtain the best sensitivity without a loss in resolution, the influence of the variation of the enantiomeric resolution as a function of the injection time (4, 10, 15 and 20 s) using a fixed pressure of 50 mbar was investigated. The results obtained demonstrated that an injection of 50 mbar during 15 s gave rise to the highest signal with enough resolution (R_s 2.7).

3.2.2 Analytical characteristics of the developed EKC methodology for Citrulline determination

Under the optimized conditions and prior to the enantiomeric determination of Cit in food supplements, the analytical characteristics of the developed EKC methodology (linearity, precision, accuracy, limits of detection (LOD) and limits of quantification (LOQ)) were evaluated in order to demonstrate the method suitability for routine quality control.

The linearity of the method was established from five calibration levels ranging from 0.2 to 2.5×10^{-4} M of L-Cit (i.e ranging from 10 to 125% of a nominal concentration (0.2 mM) of L-Cit), and from 0.1 to 2.0×10^{-5} M of D-Cit (i.e from 0.5 to 10 % of a nominal concentration (0.2 mM) of D-cit). As it can be seen in **Table 2**, satisfactory results were obtained in terms of linearity with a correlation coefficient higher than 0.997 for both enantiomers, confidence intervals for the slopes did not include the zero value, and confidence intervals for the intercept included the zero value (in both cases for a 95 % confidence level). In addition, an ANOVA test enabled to confirm that experimental data fit properly to a linear model (p -values > 0.05 for L and D-cit).

A comparison between the confidence intervals for the slopes obtained by the external standard and the standard additions calibration method (four known amounts of DL-Cit were added to a food supplement containing a constant concentration of L-Cit) showed that there were no statistically significant differences between the slopes of each

calibration straight line (for a 95 % confidence level). Therefore, there are not matrix interferences and the external calibration method can be used to quantify the content of Cit in food supplements. Moreover, taking into account that the response relative factor (RRF, factor which enables to study if the response of a minor component is equivalent to those for the major component and it is calculated dividing the slopes of the calibration lines, $\text{slope}_{\text{minor component}}/\text{slope}_{\text{major component}}$) was between 0.8 and 1.2 such as the European Pharmacopoeia establishes [47], the response for D-Cit can be considered equal to that of L-Cit which implies that the percentage of D-Cit can be determined from the ratio between the areas of L and D-Cit.

Precision was evaluated through instrumental and methodology repeatability as well as intermediate precision. Instrumental repeatability was determined from six repeated injections of a standard solution of DL-Cit at two concentration levels (12.5×10^{-6} M and 1.5×10^{-4} M). RSD values were 1.5 % for migration times and lower than 2.9 % for corrected peak areas. Regarding method repeatability, it was evaluated with three replicates of a standard solution of DL-Cit at two concentration levels (12.5×10^{-6} M and 1.5×10^{-4} M) injected in triplicate on the same day. RSD values obtained in this case, were lower than 1.7 % and 5.8 % for migration times and corrected peak areas, respectively. Finally, intermediate precision was assessed injecting (in triplicate) three replicates of a standard solution of DL-Cit at two concentration levels (12.5×10^{-6} M and 1.5×10^{-4} M) during three consecutive days. RSD values for migration times were lower than 4.7 % whereas for peak areas were lower than 5.6 % (see **Table 2**).

Accuracy of the method was evaluated as the recovery obtained for Cit enantiomers when spiking (prior to derivatization) the six food supplements with known concentration of L-Cit standard solutions (5 and 100 % of a nominal concentration of L-Cit of 0.1 mM) or D-Cit (5 % of a nominal concentration of 0.1 mM L-Cit). **Table 2**

shows that the mean recoveries obtained for the six food supplements analyzed ranged from 85 to 111 %.

LODs and LOQs were calculated as the minimum concentration yielding an S/N ratio of 3 and 10 times. LODs were 2.1×10^{-7} M for D-Cit and 1.8×10^{-7} M for L-Cit and LOQs were 7.1×10^{-7} M and 6.1×10^{-7} M for D- and L-Cit, respectively. According to the LOD calculated for D-Cit and the nominal concentration injected for L-Cit (0.2 mM), it was possible to determine the relative limit of detection (RLOD) which allows to measure the minimum enantiomeric impurity that can be detected as a function of the amount of the main compound analyzed (calculated as (LOD for the minor enantiomer)/concentration of the major enantiomer injected) \times 100). The RLOD calculated was about 0.1 %, which implies that using the developed EKC methodology is possible to carry out the detection of impurities up to a 0.1 %. This LOD is similar to that reported previously for D-Cit using CEC [31].

3.2.3 Analysis of food supplements.

Once demonstrated the suitability of the developed EKC method for the enantioselective determination of Cit, it was applied to the analysis of six different food supplements, three of them recently acquired (FS1, FS2, and FS3) and other three submitted to a long storage time (FS4, FS5, and FS6). The results obtained for the content of L-Cit in samples FS1, FS2 and FS3 were 478.2 ± 5 , 647.8 ± 2 , and 1457.7 ± 36 mg L-Cit per capsule, respectively, what corresponded to percentages of 96, 65 and 97 % with respect to the labeled content of L-Cit. D-Cit was not detected in any of these supplements so its concentration was below the LOD of the method. In the case of FS4, FS5 and FS6 samples, the contents for L-Cit were 488.4 ± 7 , 395.8 ± 2 , and 72.5 ± 3 mg L-Cit per capsule, respectively. These values corresponded to percentages of 65, 79 and 8 % of the labeled amounts demonstrating that the content of L-Cit decreased with the

storage time since these percentages determined before the expiration of the samples were 112, 98 and 11 % [31]. However, the decrease of L-Cit content could not be attributed to a racemization from L to D-cit since the D enantiomer could not be detected in any of these samples and therefore its concentration was below 0.1 %.

Figure 3 shows the electropherograms corresponding to DL-Cit standard and to two of the food supplements analyzed (non-spiked and spiked with D-Cit). As it can be observed D-cit was not detected in any of the sample analyzed.

As it has been mentioned in the introduction, the analysis of Cit in food supplements was described previously by using a CEC methodology based on the use of cellulose tris(3-chloro-4-methylphenylcarbamate) as chiral stationary phase. Taking into account that EKC is undoubtedly a more versatile technique due to the easy use of chiral selectors in the separation buffer, and considering that the EKC methodology developed in this work can be successfully applied to the quantitative determination of L-Cit and the analysis of its enantiomeric purity, it can be considered an interesting alternative for the routine quality control of Cit in food supplements.

4. Conclusions

Different EKC methodologies based on the use of sulfated- α -cyclodextrin or sulfated- γ -cyclodextrin as chiral selectors were developed to carry out the enantiomeric separation of a group of non-protein amino acids. The use of a 100 mM formate buffer at pH 2.0 as running buffer and pre-capillary derivatization with FMOC enabled the enantiomeric separation of eight from the ten non-protein amino acids studied (pyro, norleu, norval, DOPA, aminoadipic, SeMet, pipe, and cit) and the simultaneous separation of some of them in a run. Moreover, an optimized methodology using sulfated- γ -cyclodextrin as chiral selector was applied to the enantiomeric analysis of Cit in food supplements. The

LOD obtained for D-Cit was 2.1×10^{-7} M which represents the possibility of detecting up to a 0.1 % of this enantiomer showing the high sensitivity of this methodology. L-Cit was determined in six food supplements where D-Cit was not detectable in any case. Since three of the food supplements were submitted to a long storage time, the results showed that no racemization occurred due to the effects of storage time.

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Figure captions

Figure 1. Electropherograms corresponding to the chiral separation of different FMOC-non-protein amino acids (0.2 mM) obtained under the best separation conditions. Pyro; using sulfated α -CD (S- α -CD) at 15°C, Norval; using sulfated γ -CD (S- γ -CD) at 25°C, Norleu; using sulfated α -CD (S- α -CD) at 15°C, DOPA; using sulfated γ -CD (S- γ -CD) at 25°C, Amino adipic; using sulfated γ -CD (S- γ -CD) at 15°C, SeMet; using sulfated γ -CD (S- γ -CD) at 15°C, Cit; using sulfated γ -CD (S- γ -CD) at 15°C, and Pipe; using sulfated γ -CD (S- γ -CD) at 25°C. Experimental conditions: BGE, 10 mM of the corresponding CD in 100 mM formate buffer (pH 2.0); uncoated fused-silica capillary, 58.5cm (50 cm to the detector window) x 50 μ m ID; UV detection at 210 nm (except for Pyro which was detected at 200 nm); applied voltage, -20 kV; injection by pressure in the cathodic end, 50 mbar for 4 s. * Indicates the derivatizing reagent (FMOC).

Figure 2. Electropherogram corresponding to the chiral analysis of a mixture of DL-pipe, DL-cit and DL-amino adipic (0.2 mM each non-protein amino acid). Experimental conditions: BGE, 10 mM sulfated γ -CD in 100 mM formate buffer (pH 2.0); uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) x 50 μ m ID; UV detection at 210 nm, applied voltage, -20 kV; temperature, 15 °C; injection by pressure in the cathodic end, 50 mbar for 4 s. * Indicates the derivatizing reagent (FMOC).

Figure 3. Electropherogram obtained for (A) DL-FMOC-cit standard (0.025 mM) and two different food supplements at 0.2 mM L-Cit (B) FS1 non-spiked (a) and spiked with D-cit (b) (C) FS6 non-spiked (a) and spiked with D-cit (b). Experimental conditions: BGE, 10 mM sulfated γ -CD in 100 mM formate buffer (pH 3.0); uncoated fused-silica capillary, 48.5 cm (40 cm to the detector window) x 50 μ m ID; UV detection at 210 nm,

applied voltage, -20 kV; temperature, 25 °C; injection by pressure in the cathodic end,
50 mbar for 15 s.

Figure 1.

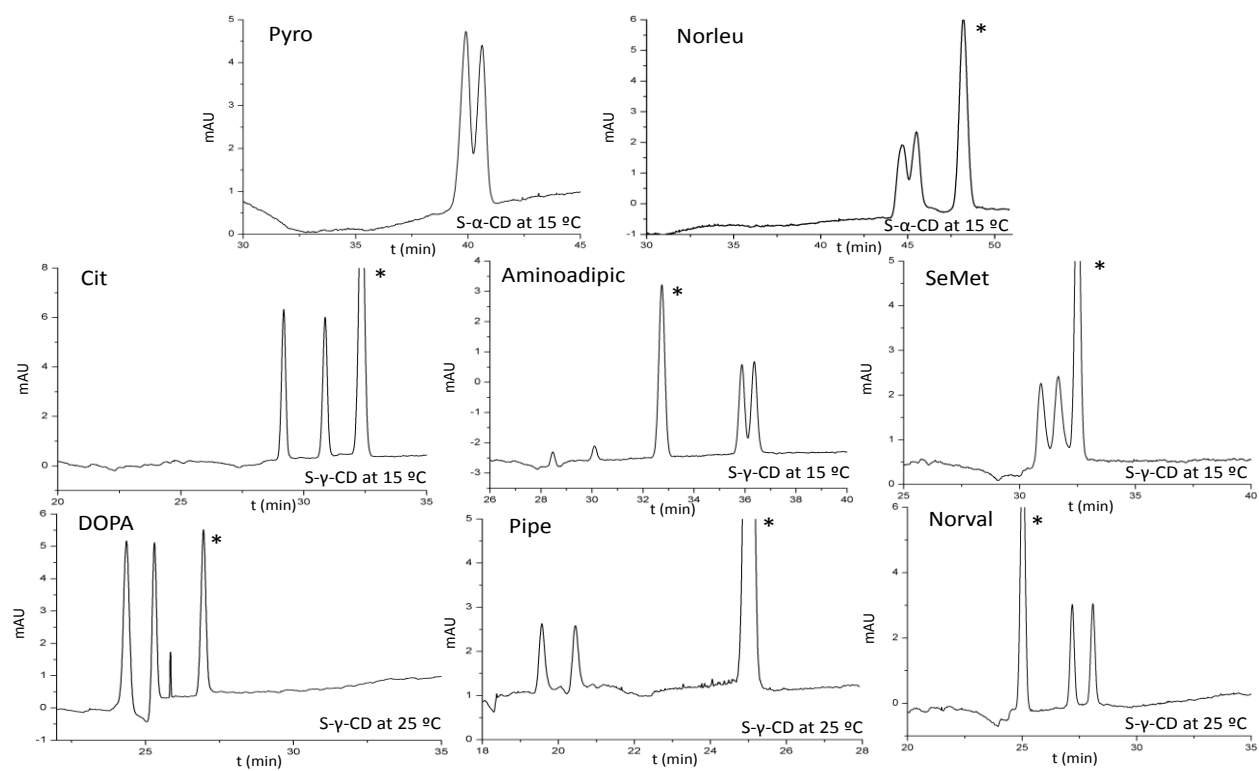


Figure 2.

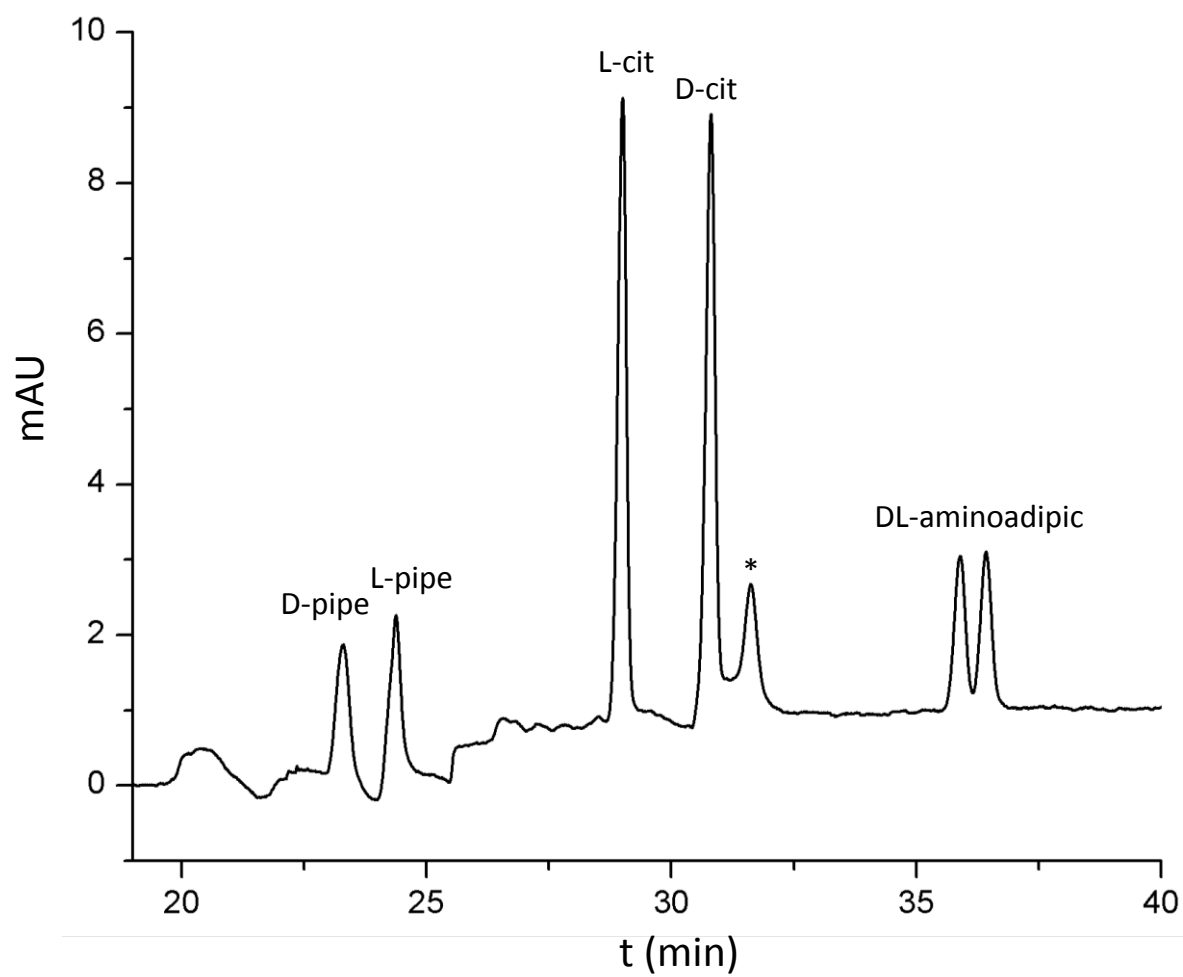


Figure 3.

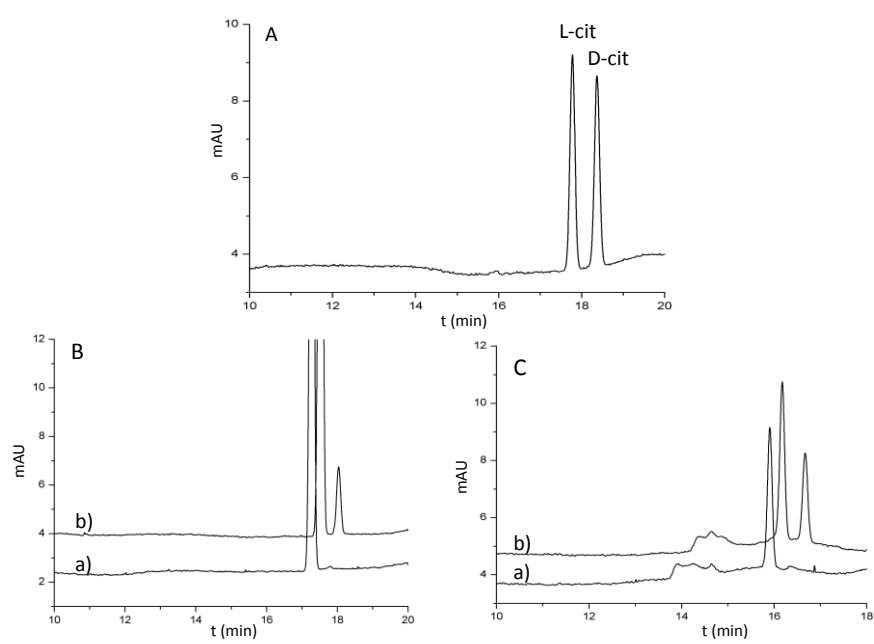


Table 1. Migration time and chiral resolution for NPAA studied by EKC using four different CDs at different temperatures.

		15 °C			20 °C			25 °C		
		t ₁	t ₂	R _s	t ₁	t ₂	R _s	t ₁	t ₂	R _s
Phosphate β-CD	Pyro*	21.3	-	-	19.6	-	-	22.6	-	-
	Norval	17.1	17.2	1.0	16.1	16.2	0.7	16.3	16.4	0.7
	Norleu	16.9	-	-	15.9	-	-	15.5	-	-
	DOPA	15.5	15.7	1.0	14.8	15.0	1.0	16.1	16.3	1.0
	aminoadipic	18.4	-	-	16.9	-	-	16.8	-	-
	SeMet	16.2	16.4	0.6	15.2	15.3	0.5	14.9	15.0	0.6
	Hcy	18.1	-	-	17.0	-	-	17.5	-	-
	Orn	18.4	-	-	17.0	-	-	16.5	-	-
	Cit	17.7	18.1	2.6	16.6	16.8	0.9	16.3	-	-
	Pipe	>40			>40			>40		
Sulfated β-CD	Pyro*	40.9	-	-	38.8	-	-	36.2	-	-
	Norval	49.2	50.0	0.9	54.5	58.5	3.4	>60		
	Norleu	50.6	-	-	>60			52.3		
	DOPA	>60			>60			44.9	-	-
	aminoadipic	>60			>60			51.4	-	-
	SeMet	>60			>60			50.0	-	-
	Hcy	>60			>60			51.3	-	-
	Orn	53.4	-	-	52.5	-	-	51.7	-	-
	Cit	50.3	-	-	50.5	-	-	49.0	-	-
	Pipe	25.0	25.8	1.4	25.4	26.2	1.5	26.1	27.0	1.5

Table 1. (Continued)

		15 °C			20 °C			25 °C		
		t ₁	t ₂	R _s	t ₁	t ₂	R _s	t ₁	t ₂	R _s
Sulfated α-CD	Pyro*	32.4	32.8	0.9	37.5	38.1	0.9	40.2	41.0	0.9
	Norval	47.3	48.4	1.6	>60			>60		
	Norleu	45.6	46.4	0.7	33.7	34.1	0.5	31.8	32.2	0.5
	DOPA	49.6	52.7	3.5	>60			>60		
	aminoadipic	47.2	48.0	1.2	49.1	-	-	57.3	58.2	0.8
	SeMet	44.7	45.3	0.6	33.8	-	-	30.4	-	-
	Hcy	50.9	-	-	>60			>60		
	Orn	49.3	-	-	>60			>60	-	-
	Cit	52.0	-	-	>60			>60		
	Pipe	41.4	-	-	>60			>60		
Sulfated γ-CD	Pyro*	30.1	-	-	26.7	-	-	26.5	-	-
	Norval	34.6	35.9	2.6	30.5	31.5	2.4	27.3	28.2	2.6
	Norleu	35.3	-	-	31.5	-	-	27.9	-	-
	DOPA	30.4	32.9	6.9	30.2	-	-	24.5	27.1	7.4
	aminoadipic	35.8	36.3	1.1	32.1	32.5	1.0	28.9	29.2	0.9
	SeMet	30.9	31.7	1.2	28.1	-	-	25.1	25.6	1.1
	Hcy	32.9	-	-	28.5	-	-	25.0	-	-
	Orn	31.9	-	-	27.9	-	-	24.5	-	-
	Cit	29.2	30.9	5.1	28.0	28.3	4.3	24.2	25.3	4.2
	Pipe	24.0	25.0	2.0	21.4	22.4	2.0	19.5	20.4	2.9

Experimental conditions: BGE, 10 mM of CD in 100 mM formate buffer (pH 2.0); uncoated fused-silica capillary, 58.5 cm (50 cm to the detector window) × 50 μm ID; UV detection at 210 nm; applied voltage, -20 kV; injection by pressure, 50 mbar for 4 s of sample. *Pyroglutamic acid without derivatization (UV detection at 200 nm). t₁, time of the first-migrating enantiomer; t₂, time of the second-migrating enantiomer.

Table 2: Analytical characteristics of the developed EKC method for the determination of cit in food supplements.

	L-Cit		D-Cit
Linearity			
Linear range ^a	0.2-2.5 x 10 ⁻⁴ M		0.1-2.0 x 10 ⁻⁵ M
Slope ± t·s _b	213.9 ± 12.4		183.4 ± 25.7
Intercept ± t·s _a	0.26 ± 1.87		-0.06 ± 0.31
R ²	0.9995		0.9972
p-value of ANOVA ^b	0.209		0.075
Matrix Interferences ^c	<i>Sample</i>	<i>Confidence interval (Slope ± t·s_b)</i>	
	Standard	213.9 ± 12.4	183.4 ± 25.7
	Food supplement	191.9 ± 10.2	186.5 ± 11.6
Precision	<i>Concentration level</i>	RSD (%)	RSD (%)
Instrumental Repeatability ^d (n = 3)	12.5 x 10 ⁻⁶ M	t, 1.5; Ac, 2.5	t, 1.5; Ac, 2.9
	1.5 x 10 ⁻⁴ M	t, 1.5; Ac, 1.9	t, 1.5; Ac, 1.9
Method Repeatability ^e (n = 9)	12.5 x 10 ⁻⁶ M	t, 0.9; Ac, 4.3	t, 0.8; Ac, 4.0
	1.5 x 10 ⁻⁴ M	t, 1.7; Ac, 5.6	t, 1.5; Ac, 5.8
Intermediate precision ^f (n = 9)	12.5 x 10 ⁻⁶ M	t, 4.2; Ac, 4.2	t, 4.7; Ac, 4.6
	1.5 x 10 ⁻⁴ M	t, 2.6; Ac, 5.4	t, 2.7; Ac, 5.6
Accuracy ^g	<i>% Mean Recovery</i>		<i>% Mean Recovery</i>
FS1 / FS2	97 ± 8 / 97 ± 11		93 ± 3 / 85 ± 1
FS3 / FS4	104 ± 7 / 99 ± 10		95 ± 4 / 92 ± 4
FS5 / FS6	100 ± 2 / 106 ± 3		108 ± 1 / 111 ± 1
LOD ^h	1.8 x 10 ⁻⁷ M		2.1 x 10 ⁻⁷ M
LOQ ⁱ	6.1 x 10 ⁻⁷ M		7.1 x 10 ⁻⁷ M

^a Five standard solutions at different concentration levels were injected in triplicate for 3 consecutive days.

^b p-value for ANOVA to confirm that experimental data fit properly to linear models.

^c Comparison of slopes corresponding to the standard addition and the external standard calibration methods.

^d Instrumental repeatability was determined from six consecutive injections of citrulline standard solutions at two concentration levels.

^e Method repeatability was calculated by using the value obtained for three replicates of citrulline standards solutions at two concentration levels injected in triplicate on the same day.

^f Intermediate precision was calculated by using the mean value obtained each day for three replicates (injected in triplicate during three consecutive days) of citrulline standard solutions at two concentration levels.

^g Accuracy was evaluated as the mean recovery obtained when six different samples solutions were spiked with known concentrations of L-Cit standard (5 % and 100 % of a nominal concentration of 0.1 mM L-Cit) or D-Cit (5 % of a nominal concentration of 0.1 mM L-Cit).

^h LOD calculated as the concentration yielding an S/N ratio of 3 ⁱ LOQ calculated as the concentration yielding an S/N ratio of 10