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Enantiomeric Separation of Free L- and D-Amino Acids in Hydrolyzed Protein Fertilizers by Capillary Electrophoresis Tandem Mass Spectrometry

Laura Sánchez-Hernández,[†] Nuria Sierras Serra,[‡] María Luisa Marina,[†] and Antonio L. Crego*[,†]

[†]Department of Analytical Chemistry, Faculty of Chemistry, University of Alcalá, Carretera Madrid-Barcelona, Km 33,600, 28871 Alcalá de Henares, Madrid, Spain

[‡]Bioibérica S.A., Polígono Industrial "Mas Puigvert", Carretera Nacional II, Km 680.6, 08389 Palafolls, Barcelona, Spain

ABSTRACT: Two capillary electrophoresis−tandem mass spectrometry (CE−MS^2) methods were optimized in this work using cyclodextrins (CDs) as chiral selectors in order to determine the degree of racemization of the free amino acids contained in different hydrolyzed protein fertilizers used as plant biostimulants. The methodologies developed were characterized by the specificity of MS^2 experiments enabling the identification of all protein amino acids, except for cysteine. The enantiomeric separation of up to 14 amino acids was achieved with resolutions above 1.0 and limits of detection between 0.02 and 0.8 μM. The methods were applied to the analysis of complex samples such as hydrolyzed protein fertilizers to evaluate the presence of D-amino acids after different kinds of hydrolysis treatments. The results corroborated the absence or almost negligible presence of enantiomeric conversions of the L-amino acids into D-amino acids in the case of fertilizers obtained by enzymatic hydrolysis, as well as the high racemization rate for those obtained through a chemical hydrolysis.

KEYWORDS: *amino acids, biostimulants, chemical hydrolysis, chiral separation, cyclodextrins, enzymatic hydrolysis, fertilizers, racemization degree*

INTRODUCTION

L-Amino acids play a part in many physiological processes in plants. Among others, they have an important nutritional role during germination, during synthesis of proteins, or in the formation of phytohormones.[1] Moreover, the exogenous intake of free L-amino acids by plants, either foliar or through the roots, provides beneficial properties such as the improvement of the photosynthetic process, and helps to withstand stress conditions, such as water stress or salinity.[2] Products containing amino acids, known as biostimulants, have been reported to show growth-regulator activity and to promote plant metabolic processes.[3,4] However, while L-amino acids are biologically active, the metabolism role of D-amino acids in plants is still doubtful,[5−8] and the few existing studies on this topic indicate undesired agronomic effects such as growth inhibition or apparent toxicity.[9−11] Therefore, the presence of D-amino acids in these kinds of fertilizers may be considered as a negative indicator of the biostimulant quality.

Hydrolyzed protein fertilizers (HPFs) are obtained mainly by chemical hydrolysis, or through enzymatic hydrolysis, and they typically contain free amino acids, oligopeptides, and polypeptides. During chemical hydrolysis, strong agents (acids or alkali) and high temperatures are used which should produce the racemization of L-amino acids to their mirror image configuration leading to a loss in their biological activity.[12,13] In the search for processes that do not induce racemization, enzymatic hydrolysis has become an alternative which preserves the original enantiomeric form from the original raw material. Consequently, the challenge to develop analytical methods for the determination and control of enantiomeric amino acid purity in commercial HPFs is an interesting topic.

Chiral separation of amino acids has been accomplished by chromatographic techniques such as HPLC[14,15] and GC.[15,16] However, the electrophoretic technique capillary electrophoresis (CE) has become one of the preferred for enantiomeric separations.[17,18] Its popularity can be ascribed to its high efficiency, short analysis times, low sample consumption, and wide scope of applications avoiding expensive chiral columns as in HPLC. Moreover, its combination with mass spectrometry (MS) detection makes it a powerful technique for performing rapid, efficient, selective, and sensitive analysis.[19] Additionally, a significant increase of the signal-to-noise ratio and the ability to simultaneously measure and identify several compounds in the same sample can be obtained using MS^2 experiments.

Only four research groups have developed methods for the chiral analysis of protein amino acids by CE−MS.[20−25] Schultz et al.[20] described a CE−MS method using 30 mM (18-crown-6)-2,3,11,12-tretracarboxylic acid as chiral selector without a previous derivatization of amino acids. The separation of 10 DL-amino acids in 40 min was achieved, and the method was applied to the analysis of lysed red blood cell samples.[21] On the other hand, Simó et al.[22] developed a CE−MS methodology based on a physically polymer coated capillary and reverse polarity in order to prevent the entrance of β-cyclodextrin (β-CD) into the mass analyzer, while in a second work from the same research group, the strategy was based on a modified β-

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CD directly introduced in the MS system at a low concentration.[23] In both cases, a derivatization with fluorescein isothiocyanate (FITC) was chosen and the methods enabled the enantioseparation of seven amino acids in 50 min and five amino acids in 20 min, respectively. Both methods were applied to the analysis of food samples (soy, vinegars, and orange juices). Yuan et al.[24] developed a CE−MS method for the simultaneous enantiomeric quantification of phenylalanine, tyrosine and 3,4-dihydroxyphenylalanine (DOPA), in less than 12 min. In this work, a negatively charged CD (sulfated-β-CD) in combination with the partial filling technique (PFT) was employed. However, the developed method was applied only to the determination of the enantiomeric purity of the non-protein amino acid levodopa and to the study of DOPA metabolism in neuronal models. More recently, Zhang et al.[25] have described a capillary electrochromatography (CEC) methodology for the enantiomeric separation of dansyl protein amino acids. The use of a silica monolithic column chemically modified with L-pipecolic acid as chiral stationary phase enabled the chiral separation of three amino acids (Phe, Ser, Thr). Nevertheless, with MS detection, no baseline resolutions with analysis times of 100 min were achieved and no application was carried out.

Finally, regarding the chiral separation of non-protein amino acids, our research group has undertaken development of CE−MS methodologies. Different approaches for the sensitive and unequivocal enantiomeric determination of DL-carnitine[26−28] or DL-ornithine[29] in different matrices (foods or pharmaceutical formulations) have been developed. Thus, with two succinyl-γ-CDs of different degree of substitution and PFT strategies[26] or direct introduction of the chiral selector at low concentrations into the CE−MS system,[27,28] the enantioseparation of FMOC-carnitine was achieved. However, without the use of PFT strategy and with MS^2 experiments, an improvement in the precision and sensitivity of the method (LOD ten times lower) was achieved in 30 min.[27,28] Hence, the same strategy consisting of the direct introduction but with low concentrations of γ-CD as chiral selector was employed for the enantiomeric separation of FITC-ornithine.[29] Excellent sensitivity was obtained in an analysis time of 30 min.

To our knowledge, only one study dealing with the analysis of D-amino acids in HPFs has been reported.[30] Thus, a MEKC-UV methodology for the study of racemization degree of DL-Ala using a polyacrylamide coated capillary and 0.1% SDS and 10 mM β-CD in the BGE was developed. In this work, only one representative amino acid (as dansyl-alanine) was considered to characterize this racemization, given the extreme difficulty of multiple chiral amino acid analysis in this type of sample.

Due to the high interest of the development of methodologies enabling the reliable determination of D-amino acids in complex matrices, such as HPFs, the aims of this work were the following: (i) to optimize chiral CE−ESI-MS^2 methodologies enabling the simultaneous enantioseparation and identification of a high number of protein amino acids; (ii) to apply the developed methodologies to the analysis of commercial HPFs; and (iii) to investigate the presence of D-amino acids in the commercial HPFs in order to evaluate the quality of the fertilizers analyzed.

MATERIALS AND METHODS

Chemicals. All reagents employed were of analytical grade. Isopropanol was supplied by Scharlau Chemie (Barcelona, Spain). Ammonium solution at 25%, sodium hydroxide, and acetone were supplied by Merck (Darmstadt, Germany). Boric acid, DL-alanine (DL-Ala), L-histidine (L-His), DL-His, L-lysine (L-Lys), DL-Lys, L-valine (L-Val), DL-Val, L-methionine (L-Met), DL-Met, L-leucine (L-Leu), D-Leu, L-tyrosine (L-Tyr), D-Tyr, DL-threonine (DL-Thr), L-arginine (L-Arg), DL-Arg, L-asparigine (L-Asn), L-serine (L-Ser), DL-Ser, L-proline (L-Pro), L-tryptophan (L-Trp), D-Trp, L-glutamic acid (L-Glu), L-phenylalanine (L-Phe), L-cysteine (L-Cys), DL-Cys, D-glutamine (D-Gln), DL-ornithine (DL-Orn), D-Orn, β-CD, γ-CD, and FITC were purchased from Fluka (Buchs, Switzerland). L-Ala, DL-Pro, DL-isoleucine (DL-Ile), DL-Phe, and L-Gln were from Aldrich (Steinheim, Germany). L-Thr, DL-Asn, D-Glu, L-aspartic acid (L-Asp), and DL-Asp were from Sigma (St. Louis, MO, USA). L-Ile and ammonium carbonate were from Sigma-Aldrich (Steinheim, Germany). Distilled water was deionized using a Milli-Q system (Millipore, Bedford, MA, USA).

HPF Samples. Four commercial HPFs were analyzed. The samples were obtained through different types of hydrolysis: HPF#1, commercial biostimulant obtained by an enzymatic hydrolysis (Terra-Sorb); HPF#2, commercial biostimulant with a mixture of enzymatic and chemical hydrolysis; HPF#3, commercial biostimulant from enzymatic hydrolysis followed by a concentration process; HPF#4, standard commercial biostimulant obtained by chemical hydrolysis from collagen. Other hydrolytic features of the samples are indicated in Table 1. These samples are complex not only because they contain free amino acids in solution but also due to the presence of peptides of different sizes, purine and pyrimidine bases, and traces of DNA and RNA. However, sample treatment was simple. Samples were diluted in Milli-Q water and their pHs were adjusted to 10.0 with 1 M NaOH. Then, they were diluted in 0.1 M borate at pH 10.0 to the desired concentration before taking the aliquot for their derivatization with FITC.

Table 1. Hydrolysis Features of the Fertilizers

	HPF#1	HPF#2	HPF#3	HPF#4
type of hydrolysis	enzymatic	enzymatic and chemical	enzymatic	chemical
hydrolysis grade (%)	67.0	21.9	65.5	27.2
free amino acids[a] (% w/w)	5.6	9.1	21.9	8.8
total amino acids[a] (% w/w)	8.9	41.6	33.5	32.4

[a]Values measured after the hydrolysis treatment by RP-HPLC with a C18 column according to AccQ-Tag method from Waters.[31]

Amino Acid Derivatization. Derivatization of amino acids with FITC was carried out under basic conditions in order to modify the amino groups of the amino acids according to the procedures reported in the literature.[32,33] Thus, a mixture of 100 μL of the amino acids standards or fertilizer solutions diluted in 0.1 M borate at pH 10.0 was reacted with 200 μL of 15 mM FITC in acetone. This solution was kept in the dark for 16 h before injection into the CE system.

Capillary Electrophoresis Tandem Mass Spectrometry Conditions. The analyses were carried out in a $HP^{3D}CE$ instrument (Agilent Technologies, Palo Alto, CA, USA) coupled through a G1607A orthogonal electrospray interface (ESI) (Agilent Technologies, Palo Alto, CA, USA) to a model 1100 series ion trap mass spectrometer (LC/MSD) (Agilent Technologies, Palo Alto, CA, USA) for MS detection. LC/MSD Trap Software 5.2 was used for MS control and data analysis. Uncoated fused-silica capillaries of 100 cm × 50 μm i.d. for MS detection were used. Before first use, the capillary was conditioned with 1 M NaOH by flushing at 1 bar for 20 min, followed by 5 min with water and 20 min with 50 mM ammonium carbonate (pH 10.0) buffer. After each run, the capillary was conditioned with acetone (1 bar, 3 min), 0.1 M NaOH (1 bar, 2 min), Milli-Q water (1 bar, 2 min), and separation buffer (1 bar, 4 min). Injections were made at the anodic end (inlet) using a pressure of 50 mbar for 15 s. The electrophoretic separation was achieved with a voltage of 20 kV and at a constant temperature of 25 °C.

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For MS operating conditions the electrical contact at the electrospray needle tip was established using a sheath liquid based on isopropanol/25 mM ammonium carbonate (50/50 v/v) and delivered at a flow rate of 0.33 mL/min by a model 1100 HPLC pump (Agilent Technologies, Palo Alto, CA, USA) and a split of 1/100 (i.e., at a flow rate of 3.3 μL/min). The nebulizer and drying gas conditions were 2 psi of N_2 and 3 L/min N_2 at 350 °C. The ion trap mass spectrometer operated with the ESI source in the positive ion mode (4.5 kV), and the m/z range scanned was from m/z 100 to 600 with a scan resolution corresponding to m/z 13,000 per second. The trap parameters were programmed in ion charge control mode to accumulate 200,000 ions with a maximum accumulation time of 300 ms, and one scan per experiment. The ion optic parameters were tuned in "smart mode" with the following assumptions: a target mass of m/z 500, a compound stability of 50%, and 100% of trap drive level. In the MS^2 experiments, the fragmentation was carried out by collision induced dissociation with the helium present in the trap for 10 ms with fragmentation amplitude of 1.0 V, and isolation width for precursor ions of m/z 4.0. MS^2 spectra of all experiments were performed using multiple reaction monitoring (MRM) mode. Finally, extracted ion electropherograms (EIEs) were obtained with extraction window of ± m/z 0.2 and using a smoothed option of the software (Gauss at 1 point).

RESULTS AND DISCUSSION

Study of the Separation and Simultaneous Identification of Amino Acids by CE−ESI-MS^2. As mentioned above, the development of methods for a reliable evaluation of the presence of protein D-amino acids in complex matrices is still an urgent demand. In this work, the study and optimization of a chiral CE−ESI-MS^2 methodology for the simultaneous separation and identification of amino acids was proposed based on a previous CE−ESI-MS^2 methodology developed by our group for the enantioseparation of the non-protein amino acid ornithine.[29] In the reported method, a sensitive and unequivocal determination of FITC-Orn enantiomers using a low concentration of the chiral selector γ-CD (0.75 mM) under basic conditions was achieved. The direct introduction of the separation buffer into the CE−MS system was possible without significantly influencing the sensitivity.

Table 2. Precursor Ions (Protonated Molecular Ions) and Product Ions from the FITC-Amino Acids Obtained by CE−ESI-MS^2

	precursor ion		product ion	
amino acid	m/z	ion	m/z	ion
Ala	479	$[FITC\text{-}AlaH]^+$	390	$[FITC\text{-}H]^+$
Arg	564	$[FITC\text{-}ArgH]^+$	390	$[FITC\text{-}H]^+$
Asn	522	$[FITC\text{-}AsnH]^+$	390	$[FITC\text{-}H]^+$
Asp	523	$[FITC\text{-}AspH]^+$	390	$[FITC\text{-}H]^+$
Cys			nd[a]	
Gln	536	$[FITC\text{-}GlnH]^+$	390	$[FITC\text{-}H]^+$
Glu	537	$[FITC\text{-}GluH]^+$	390	$[FITC\text{-}H]^+$
His	545	$[FITC\text{-}HisH]^+$	390	$[FITC\text{-}H]^+$
Ile	521	$[FITC\text{-}IleH]^+$	390	$[FITC\text{-}H]^+$
Leu	521	$[FITC\text{-}LeuH]^+$	390	$[FITC\text{-}H]^+$
Lys	463	$[(FITC_2\text{-}LysH_2]^{2+}$	390 and 536	$[FITC\text{-}H]^+$
				$[FITC\text{-}LysH]^+$
Met	539	$[FITC\text{-}MetH]^+$	390	$[FITC\text{-}H]^+$
Orn[b]	456	$[(FITC_2\text{-}OrnH_2]^{2+}$	390 and 522	$[FITC\text{-}H]^+$
				$[FITC\text{-}OrnH]^+$
Phe	555	$[FITC\text{-}PheH]^+$	390	$[FITC\text{-}H]^+$
Pro	504	$[FITC\text{-}ProH]^+$	390	$[FITC\text{-}H]^+$
Ser	495	$[FITC\text{-}SerH]^+$	390	$[FITC\text{-}H]^+$
Thr	509	$[FITC\text{-}ThrH]^+$	390	$[FITC\text{-}H]^+$
Trp	595	$[FITC\text{-}TrpH]^+$	390	$[FITC\text{-}H]^+$
Tyr	571	$[FITC\text{-}TyrH]^+$	390	$[FITC\text{-}H]^+$
Val	507	$[FITC\text{-}ValH]^+$	390	$[FITC\text{-}H]^+$

[a]Not detected. [b]Values obtained from ref 29.

In this work, the derivatizing agent FITC was also chosen for the derivatization of the amino acids. FITC is an interesting derivatizing reagent that possesses good reactivities with primary and secondary amines, having the advantage of conferring good detectability of the amino acids not only by laser induced fluorescence (LIF) and UV detectors[34] but also by MS detection.[22,23,29,35] The amino acids derivatized with FITC (FITC-AAs) provided an increase in amino acid size that led to a better sensitivity in ESI-MS because these larger molecules can be ionized with a higher yield and a lower MS background noise at values higher than m/z 150. Furthermore, the derivatization moiety gives rise to additional interactions with the CDs, improving the separation performances. Previous results showed that only for two amino acids (Phe and Tyr) a complete enantioseparation using α-CD was obtained, possibly due to the aromatic functionalizing moiety of these aromatic amino acids.

First, using a 0.75 mM γ-CD in 50 mM ammonium carbonate buffer (pH 10.0), the individual identification and the enantiomeric separation of the 19 chiral protein amino acids were investigated in MS and MS^2 modes. All FITC-AAs were detected except Cys. In this case, neither derivatized Cys nor double derivatized Cys_2 was found using MS detection. This result allowed us to hypothesize that the derivatization of Cys with FITC could be hindered due to the characteristics of the thiol (−SH) group of Cys. This nonexpected behavior could be the cause of the need for a previous thiol protection by a thiol-alkylation step reported recently,[36] prior to derivatization of Cys by the derivatizing agent 6-methoxyquinoline-4-carboxylic acid succinimide ester under similar basic conditions as for the FITC reaction.

On the other hand, mass spectra obtained for all the FITC-AAs detected using MS mode enabled us to identify the protonated molecules as $[FITC\text{-}AAH]^+$ in all cases except for Lys (amino acid with two primary amino groups) that formed a derivative with two FITC molecules resulting in a doubly protonated molecule as $[(FITC_2\text{-}LysH_2]^{2+}$ (Table 2). The derivatization (Figure 1) with two FITC molecules also was shown for Orn previously,[29] but for other amino acids with two primary amino groups (such as Arg, Asn and Gln) only derivatization with one molecule of FITC was observed. In the case of Arg, this different behavior could be due to the fact that the terminal amino group is a part of a guanidine group (NH_2−C(=NH)−NH−) whose resonance forms inactivate the hydrogen of the amine to form the derivative. In the case of Gln and Asn, the existence of an amide group (NH_2−CO−) less reactive than the amine group (NH_2−CH_2−) of Lys or Orn could justify this behavior.

Second, with the aim of increasing the molecular specificity of the methodology, the fragmentation pattern was investigated for all amino acids using MS^2 experiments. The results are also shown in Table 2. As it can be observed, only a fragment ion of m/z 390 corresponding to the $[FITC\text{-}H]^+$ ion was attained for all the amino acids except for Orn and Lys for which the $[FITC\text{-}AAH]^+$ ion corresponding to the protonated derivative with one FITC alone also appeared.

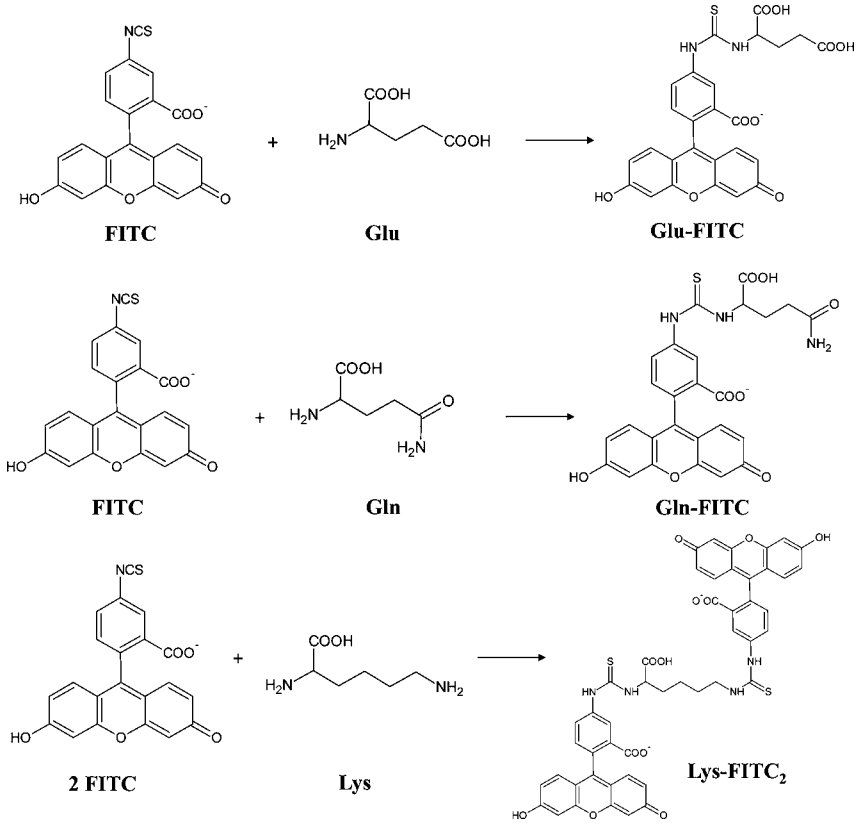

Figure 1. Scheme for the derivatization process of Glu, Gln, and Lys with FITC.

Table 3. Chiral Selector Employed in the Enantiomeric Resolution of the FITC-Amino Acids and LODs Obtained with the Optimized CE−ESI-MS^2 Methodology

amino acid	chiral selector	time (min) D-AA/L-AA	Rs[a]	LOD (μM)
Ala	γ-CD	40.0/40.6	2.0	0.2
Arg		27.4	ns[b]	0.7
Asn	γ-CD	40.2/40.8	1.5	0.2
Asp	γ-CD	58.9/60.6	2.5	0.3
Cys			nd[c]	nd
Gln	γ-CD	39.8/40.2	1.5	0.5
Glu	γ-CD	51.3/53.0	3.0	0.3
His	γ-CD	39.9/40.3	1.0	0.5
Ile		38.6	ns	0.02
Leu	γ-CD	38.5/39.2	2.0	0.1
Lys	β-CD	36.4/39.1	4.0	0.3
Met	γ-CD	39.1/39.5	1.5	0.1
Orn	β-CD	56.8/58.8	3.5	0.8
Phe	γ-CD	36.8/37.0	1.5	0.2
Pro		38.0	ns	0.1
Ser	γ-CD	40.6/41.0	1.0	0.8
Thr		35.6/35.7	ns	0.7
Trp	β-CD	31.6/31.9	1.0	0.05
Tyr	γ-CD	38.8/39.2	1.0	0.4
Val		40.5	ns	0.02

[a]Rs, resolution calculated as Rs = 1.18 $(t_2 - t_1)/(w_{1/2,1} + w_{1/2,2})$ where t_1 and t_2 are the migration times of the first- and second-migrating enantiomers, respectively, and $w_{1/2,1}$, $w_{1/2,2}$ their peak widths at half height. [b]Not separated. [c]Not detected.

Regarding the enantioseparations, the results showed that only partial resolutions were obtained for FITC-Asp and FITC-Trp under these conditions, while baseline resolution was achieved for the FITC$_2$-Lys, as was obtained for FITC$_2$-Orn in our previous work.[29,37] Therefore, we conclude that those amino acids forming derivatives with two FITC molecules have adequate resolution with 0.75 mM γ-CD but other amino acids forming derivatives with a single FITC molecule are not enantioseparated. Then, concentrations of γ-CD from 0.75 to 10 mM were investigated in order to improve the separation of amino acid enantiomers. The results showed that the enantioresolutions improved when increasing the γ-CD concentrations, obtaining the best results for a value of 5 mM. However, at higher concentrations of chiral selector, lower signal-to-noise ratios were observed for the peaks and consequently the sensitivities decreased. Accordingly, the chiral separations of Ala, Asn, Asp, Gln, Glu, Leu, Met, Phe, Ser, Tyr, and His were achieved when γ-CD concentration was increased, but incomplete separation was obtained for Lys and Orn enantiomers. These results could be attributed to the fact that a higher binding with the chiral selector is obtained when two FITC molecules are present in the derivatized amino acid. For the amino acids Trp, Thr, Arg, Ile, Pro, and Val no enantioseparation was observed.

Under the same conditions and based on the possibilities of β-CD to achieve chiral separations of FITC amino acids,[22] the individual enantiomeric separation of the 19 chiral protein amino acids and Orn was studied using 5 mM β-CD by CE−ESI-MS^2 and compared with that obtained with γ-CD in order to achieve the enantioseparation of a high number of amino acids. The results showed that the enantiomeric resolutions obtained for Ala, Asn, Asp, Glu, Ser, His, Arg, and Ile were worse when using β-CD and no separations for Gln, Leu, Met, Phe, Tyr, Pro, and Val were observed. However, the enantioseparation of Trp was obtained with resolution 1.0,

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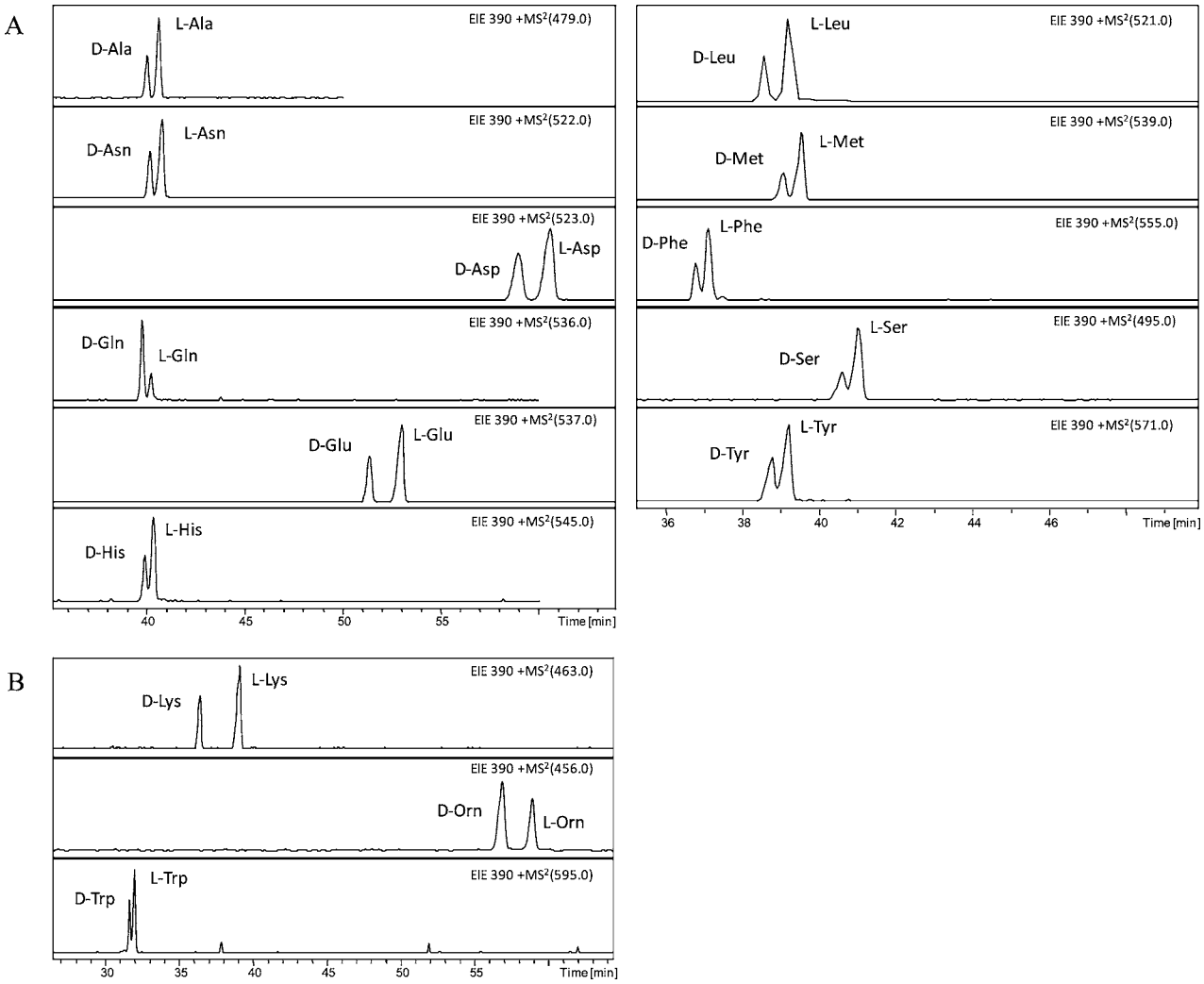

Figure 2. (A) CE−MS^2 EIEs for the 11 FITC-AAs enantioresolved using a BGE 5 mM γ-CD in 50 mM ammonium carbonate buffer (pH 10.0). (B) CE−MS^2 EIEs for the 3 FITC-AAs enantioresolved using a BGE 5 mM β-CD in 50 mM ammonium carbonate buffer (pH 10.0).

Table 4. Enantiomeric Impurity (ei %)[a] and Enantiomeric Excess (ee %)[b] Percentages Obtained in Four HPFs Analyzed

	HPF#1		HPF#2		HPF#3		HPF#4	
amino acid	ei %	ee %	ei %	ee %	ei %	ee %	ei %	ee %
Ala	0.9	98.1	8.6	82.9	2.8	94.4	44.4	11.3
Asp	1.0	98.0	19.1	61.8	1.8	96.4	42.1	15.8
Glu	0.4	99.1	8.7	82.5	1.3	97.4	31.8	36.3
Leu	nd[c]	100	nd	100	1.0	98.1	14.9	70.0
Lys	nd	100	7.1	85.9	nd	100	40.3	19.3
Orn	1.0	98.0	15.5	68.9	0.5	99.0	51.5	−2.9

[a]ei % was calculated from the equation ei % = $[(A_D)/(A_D + A_L)] \times 100$, where A_D is the corrected peak area (peak area divided by migration time) of the D-enantiomer and A_L is the corrected peak area of the L-enantiomer. [b]ee % was calculated from the equation ee % = $[(A_L - A_D)/(A_D + A_L)] \times 100$. [c]Not detected.

and moreover, better enantioresolutions were achieved for Orn and Lys than those obtained with 5 mM γ-CD. In summary, as it can be seen in Table 3, using 5 mM γ-CD or β-CD as chiral selector, enantioseparations of up to 13 protein amino acids and Orn were achieved with resolutions between 1.0 and 4.0 (Figure 2).

Since both methods only differ in the type of selector, it is possible to say that the different behavior of both CDs can be due to the differences in their cavity size (the two CDs are strictly homologous, differing only in the number of glucopyranose units present in the macrocycle, seven in the β-CD and eight in the γ-CD, respectively). Thus, for most FITC-AAs (11 out of the 14 enantioresolved), γ-CD showed a better chiral separation ability than β-CD probably because its wider cavity gives rise to more effective interactions with the functionalized moiety in FITC-AAs. However, for the biggest FITC-AA separated (FITC-Trp) and when two FITC molecules are present in the derivatized amino acid ($FITC_2$-Lys and $FITC_2$−Orn), the best results were obtained with β-CD. These results suggest that these derivatized amino acids are too big to enter into the γ-CD cavity, and in the case of the FITC-Trp the larger size of the side chain of the amino acid gives rise to more effective interaction with the β-CD, while in the $FITC_2$-Lys and $FITC_2$-Orn the molecular recognition process is likely to consist only of a partial interaction between the OH groups present on the outer surface of the β-CD.

Finally, MRM mode for the MS^2 experiments was also studied in order to achieve the separation and identification of

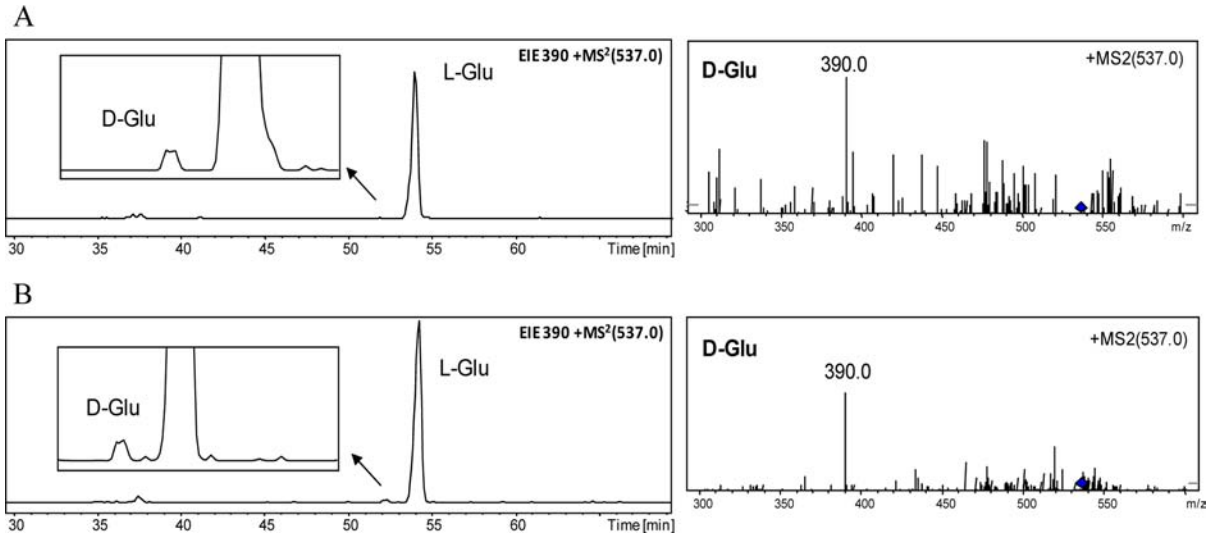

Figure 3. CE−MS^2 EIEs of FITC-Glu detected with its enantiomeric impurity (D-Glu) in HPF# 1 (A) and HPF#3 (B), and MS^2 spectra obtained for each D-Glu. CE conditions as in Figure 2.

the maximum possible number of amino acids in the same run and to reduce the number of experiments per sample. In this work, three analyses by MRM mode, one for the precursor ions of the amino acids separated using β-CD and two for the precursor ions of the amino acids separated with γ-CD, were studied. With a maximum accumulation time of 300 ms, a fragmentation time of 10 ms, and one scan for each MS^2 spectrum, the results showed that at least 12 points per peak were achieved.

Under these conditions, the LODs obtained by dilution of each standard amino acid previous to derivatization were calculated as the minimum analyte concentration yielding an S/N ratio equal to 3. Values in the 0.02-0.8 μM range per each amino acid enantiomer were achieved (Table 3).

Comparing these results with those obtained by LC−MS for the chiral separation of protein amino acid mixtures, it is possible to conclude that the results are in correlation with the values achieved in three of the reported methodologies in terms of resolution (up to 4.0) and sensitivity, with LODs in the μM range.[35,38,39] However, fewer than 14 amino acids were enantioseparated in the reported methods. Thus, the CE−ESI-MS methodologies developed in this work improve the number of chiral amino acids resolved. On the other hand, in a recent chiral LC−MS methodology,[36] the separation of 18 protein amino acids with resolutions up to 9 has been described. However, only the LOD for Ala was given (0.015 μM).

Enantiomeric Amino Acid Analysis in Hydrolyzed Protein Fertilizers. For the first time, a CE−MS method is proposed in this work for the analysis of HPFs. The enantiomeric separation and identification of a series of six amino acids (Ala, Asp, Glu, Leu, Lys, and Orn), those with an enantiomeric separation at baseline or better (Rs > 1.5) and whose degradation during the hydrolysis is not as important compared to Asn, Gln, Ser, and Trp,[40] was carried out in these samples by the optimized CE−ESI-MS^2 methodologies to study the possible presence of D-amino acids after hydrolysis treatments.

Table 4 shows the results obtained for the HPFs analyzed regarding the percentages of D-amino acid as enantiomeric impurity (ei %) and enantiomeric excess (ee %) of L-amino acid with respect to D-amino acid. The identification of the peaks was accomplished by the addition of D- or L-amino acid standard solutions. Thus, for HPF#1, obtained through an enzymatic hydrolysis, percentages of ei $\leq$ 1.0% and ee $\geq$ 98.0% were obtained, therefore, the analyzed amino acids were present almost exclusively in L-form. Similar results were obtained for HPF#3 (ei $\leq$ 2.8% and ee $\geq$ 94.4%) also prepared with enzymatic hydrolysis. These results demonstrate the quality of fertilizers #1 and #3. Figure 3 shows the CE−MS^2 EIEs of the FITC-Glu detected in both fertilizers with their enantiomeric impurities (D-Glu) and MS^2 spectra obtained for each D-amino acid. As it can be observed, although the percentages of D-Glu were very low, the product ion m/z 390 was observed in each MS^2 spectrum.

Percentage values of ei up to 19.l% and ee of only 61.8% were obtained for the amino acid Asp in the HPF#2 produced with mixed hydrolysis. Thus, the same D-Asp was detected but with higher values than in fertilizer #1 and #3 when a chemical hydrolysis is carried out after the enzymatic process (Figure 4A). Finally, the presence of the highest D-amino acid contents in the HPF#4 produced with chemical hydrolysis was corroborated. Thus, for Ala, Asp, and Lys, values near to racemic contents (ei around 40%, Figure 4B) were reached. Although minor enantiomeric impurities were generated for Leu (only 14.9%) and Glu (31.8%), note that for Orn the majority enantiomer was the D-form presenting an ei = 51.5% and, as a consequence, a negative value of ee (−2.9%) was obtained due to its higher content in the sample (Figure 4C). These results show that although similar hydrolysis features for fertilizer #2 and #4 are presented in Table 1, the enzymatic hydrolysis prior to a chemical hydrolysis avoided substantially the conversion to D-amino acids. All of this corroborates the high potential of the developed method in the control of the quality of hydrolyzed protein fertilizers.

In summary, in this study, two CE−ESI-MS^2 methodologies for the identification of amino acid enantiomers were developed for enantiomeric purity control of hydrolyzed protein fertilizers. Using a BGE of 50 ammonium carbonate (pH 10.0) containing 5 mM β-CD or 5 mM γ-CD, the chiral separation and identification of 13 protein amino acids and ornithine was achieved. The sensitivity obtained allowed for

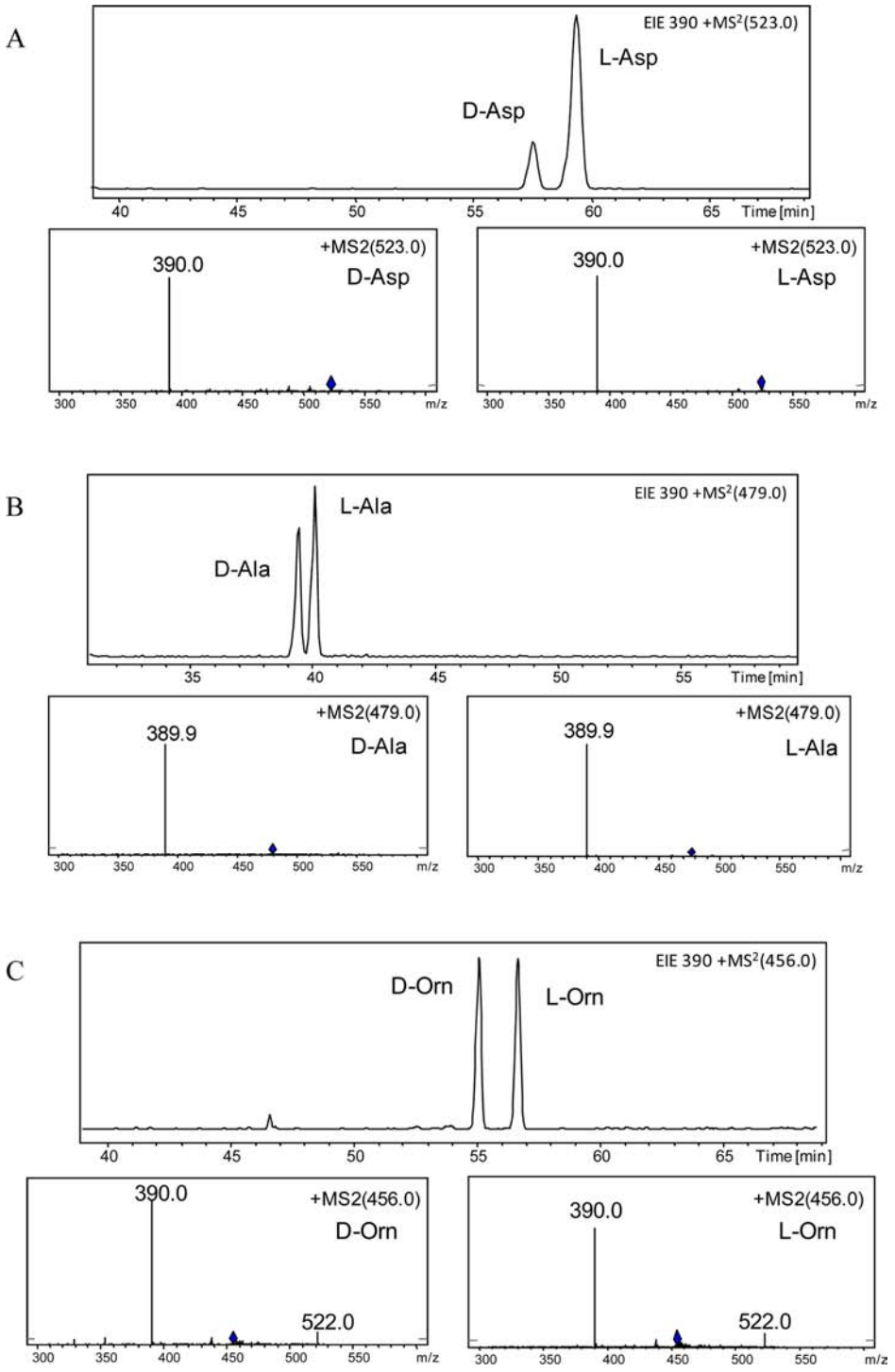

Figure 4. CE−MS^2 EIEs of (A) FITC-Asp in HPF#2, (B) FITC-Ala in HPF#4, and (C) $FITC_2$-Orn in HPF#4, all of them with MS^2 spectra obtained for each D-amino acid. CE conditions as in Figure 2.

LODs in the 0.02−0.8 μM range, which is enough to detect enantiomeric impurities less than 1%. Results showed that, depending on the type of hydrolysis of the analyzed sample, different percentages of enantiomeric impurities were produced. The enzymatic hydrolysis was the one with no or almost negligible presence of D-amino acids. This fact highlights the necessity of analytical methods such as that developed in this work to achieve the quality control of chiral amino acids in hydrolyzed protein fertilizers with the aim to ensure the beneficial properties of this kind of biostimulant for plants.

AUTHOR INFORMATION

Corresponding Author

*Phone: +34-91-8856390. Fax: +34-91-8854971. E-mail antonio.crego@uah.es.

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Notes

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■ REFERENCES

(1) Barneix, A. J.; Causin, H. F. The central role of amino acids on nitrogen utilization and plant growth. *J. Plant Physiol.* **1996**, *149*, 358−362.

(2) Rai, V. K. Role of amino acids in plant responses to stresses. *Biol. Plant.* **2002**, *45*, 481−487.

(3) Kauffman, G. L., III; Kneivel, D. P.; Watschke, T. L. Effects of a biostimulant on the heat tolerance associated with photosynthetic capacity, membrane thermostability, and polyphenol production of perennial ryegrass. *Crop Sci.* **2007**, *47*, 261−267.

(4) Kolomaznik, K.; Pecha, J.; Friebrova, V.; Janacova, D.; Vasek, V. Diffusion of biostimulators into plant tissues. *Heat Mass Transfer* **2012**, *48*, 1505−1512.

(5) Gördes, D.; Kolukisaoglu, U.; Thurow, K. Uptake and conversion of D-amino acids in *Arabidopsis thaliana*. *Amino Acids* **2011**, *40*, 553−563.

(6) Brückner, H.; Westhauser, T. Chromatographic determination of L- and D-amino acids in plants. *Amino Acids* **2003**, *24*, 43−55.

(7) Forsum, O.; Svennerstam, H.; Ganeteg, U.; Näsholm, T. Capacities and constraints of amino acid utilization in Arabidopsis. *New Phytol.* **2008**, *179*, 1058−1069.

(8) Vranova, V.; Zahradnickova, H.; Janous, D.; Skene, K. R.; Matharu, A. S.; Rejsek, K.; Formanek, P. The significance of D-amino acids in soil, fate and utilization by microbes and plants: review and identification of knowledge gaps. *Plant Soil* **2012**, *354*, 21−39.

(9) Erikson, O.; Hertzberg, M.; Näsholm, T. A conditional marker gene allowing both positive and negative selection in plants. *Nat. Biotechnol.* **2004**, *22*, 455−458.

(10) Erikson, O.; Hertzberg, M.; Näsholm, T. The *dsdA* gene from *Escherichia coli* provides a novel selectable marker for plant transformation. *Plant Mol. Biol.* **2005**, *57*, 425−433.

(11) Näsholm, T.; Kielland, K.; Ganeteg, U. Uptake of organic nitrogen by plants. *New Phytol.* **2009**, *182*, 31−48.

(12) Liardon, R.; Jost, R. Racemization of free and protein-bound amino acids in strong mineral acid. *Int. J. Pept. Protein Res.* **1981**, *18*, 500−505.

(13) Liardon, R.; Hurrel, R. Amino acid racemization in heated and alkali-treated proteins. *J. Agric. Food Chem.* **1983**, *31*, 432−437.

(14) Ilisz, I.; Berkecz, R.; Peter, A. HPLC separation of amino acid enantiomers and small peptides on macrocyclic antibiotic-based chiral stationary phases: A review. *J. Sep. Sci.* **2006**, *29*, 1305−1321.

(15) León, J.; Reubsaet, E.; Beijnen, J. H.; Bult, A.; van Maanen, R. J.; Danielle Marchal, J. A.; Underberg, W. J. M. Analytical techniques used to study the degradation of proteins and peptides: chemical instability. *J. Pharm. Biomed. Anal.* **1998**, *17*, 955−978.

(16) Schurig, V. Gas chromatographic enantioseparation of derivatized alpha-amino acids on chiral stationary phases-Past and present. *J. Chromatogr., B* **2011**, *879*, 3122−3140.

(17) Sánchez-Hernández, L.; Castro-Puyana, M.; Marina, M. L.; Crego, A. L. Recent approaches in sensitive enantioseparations by CE. *Electrophoresis* **2012**, *33*, 228−242.

(18) Poinsot, V.; Carpene, M. A.; Bouajila, J.; Gavard, P.; Feurer, B.; Couderc, F. Recent advances in amino acid analysis by capillary electrophoresis. *Electrophoresis* **2012**, *33*, 14−35.

(19) Desiderio, C.; Iavarone, F.; Rossetti, D. V.; Messana, I.; Castagnola, M. Capillary electrophoresis-mass spectrometry for the analysis of amino acids. *J. Sep. Sci.* **2010**, *33*, 2385−2393.

(20) Schultz, C. L.; Moini, M. Analysis of underivatized amino acids and their D/L-enantiomers by sheathless capillary electrophoresis/electrospray ionization-mass spectrometry. *Anal. Chem.* **2003**, *75*, 1508−1513.

(21) Moini, M.; Schultz, C. L.; Mahmood, H. CE/electrospray ionization-MS analysis of underivatized D/L-amino acids and several small neurotransmitters at attomole levels through the use of 18-crown-6-tetracarboxylic acid as a complexation reagent/background electrolyte. *Anal. Chem.* **2003**, *75*, 6282−6287.

(22) Simó, C.; Rizzi, A.; Barbas, C.; Cifuentes, A. Chiral capillary electrophoresis-mass spectrometry of amino acids in foods. *Electrophoresis* **2005**, *26*, 1432−1441.

(23) Giuffrida, A.; León, C.; García-Cañas, V.; Cucinotta, V.; Cifuentes, A. Modified cyclodextrins for fast and sensitive chiral-capillary electrophoresis-mass spectrometry. *Electrophoresis* **2009**, *30*, 1734−1742.

(24) Yuan, B.; Wu, H.; Sanders, T.; McCullum, C.; Zheng, Y.; Tchounwou, P. B.; Liu, Y.-M. Chiral capillary eliectrophoresis-mass spectrometry of 3,4-dihydroxyphenylalanine: Evidence for its enantioselective metabolism in PC-12 nerve cells. *Anal. Biochem.* **2011**, *416*, 191−195.

(25) Zhang, Y.; Huang, L.; Chen, Q.; Chen, Z. A silica monolithic column with chemically bonded L-pipecolic acid as chiral stationary phase for enantiomeric separation of dansyl amino acids by CEC-MS. *Chromatographia* **2012**, *75*, 289−296.

(26) Castro-Puyana, M.; García-Ruiz, C.; Crego, A. L.; Marina, M. L. Development of a CE−MS^2 method for the enantiomeric separation of L/D-carnitine: Application to the analysis of infant formulas. *Electrophoresis* **2009**, *30*, 337−348.

(27) Sánchez-Hernández, L.; Castro-Puyana, M.; García-Ruiz, C.; Crego, A. L.; Marina, M. L. Determination of L- and D-carnitine in dietary food supplements using capillary electrophoresis−tandem mass spectrometry. *Food Chem.* **2010**, *120*, 921−928.

(28) Sánchez-Hernández, L.; García-Ruiz, C.; Crego, A. L.; Marina, M. L. Sensitive determination of D-carnitine as enantiomeric impurity of levo-carnitine in pharmaceutical formulations by capillary electrophoresis−tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2010**, *53*, 1217−1223.

(29) Domínguez-Vega, E.; Sánchez-Hernández, L.; García-Ruiz, C.; Crego, A. L.; Marina, M. L. Development of a CE-ESI-ITMS method for the enantiomeric determination of the non-protein amino acid ornithine. *Electrophoresis* **2009**, *30*, 1724−1733.

(30) Cavani, L.; Ciavatta, C.; Gessa, C. Determination of free L and D alanine in hydrolysed protein fertilisers by capillary electrophoresis. *J. Chromatogr., A* **2003**, *985*, 463−469.

(31) Cohen, S. A.; Michaud, D. P. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. *Anal. Biochem.* **1993**, *211*, 279−287.

(32) Li, H.; Wang, H.; Chen, J.; Wang, L. H.; Zhang, H. S.; Fan, Y. Determination of amino acid neurotransmitters in cerebral cortex of rats administered with baicalin prior to cerebral ischemia by capillary electrophoresis-laser-induced fluorescence detection. *J. Chromatogr., B* **2003**, *788*, 93−101.

(33) Pobozy, E.; Czardowska, W.; Trojanowicz, M. Determination of amino acids in saliva using capillary electrophoresis with fluorimetric detection. *J. Biochem. Biophys. Methods* **2006**, *67*, 37−47.

(34) Underberg, W. J. M.; Waterval, J. C. M. Derivatization trends in capillary electrophoresis: An update. *Electrophoresis* **2002**, *23*, 3922−3933.

(35) D'Orazio, G.; Cifuentes, A.; Fanali, S. Chiral nano-liquid chromatography−mass spectrometry applied to amino acids analysis for orange juice profiling. *Food Chem.* **2008**, *108*, 1114−1121.

(36) Reischl, R. J.; Lindner, W. Methoxyquinoline labeling-A new strategy for the enantioseparation of all chiral proteinogenic amino

acids in 1-dimensional liquid chromatography using fluorescence and tandem mass spectrometric detection. *J. Chromatogr., A* **2012**, *1269*, 262−269.
(37) Domínguez-Vega, E.; Martínez-Girón, A. B.; García-Ruiz, C.; Crego, A. L.; Marina, M. L. Fast derivatization of the non-protein amino acid ornithine with FITC using an ultrasound probe prior to enantiomeric determination in food supplements by EKC. *Electrophoresis* **2009**, *30*, 1037−1045.
(38) Petritis, K.; Valleix, A.; Elfakir, C.; Dreux, M. Simultaneous analysis of underivatized chiral amino acids by liquid chromatography-ionspray tandem mass spectrometry using a teicoplanin chiral stationary phase. *J. Chromatogr., A* **2001**, *913*, 331−340.
(39) Song, Y.; Shenwu, M.; Zhao, S.; Hou, D.; Liu, Y.-M. Enantiomeric separation of amino acids derivatized with 7-fluoro-4-nitrobenzoxadiazole by capillary liquid chromatography/tandem mass spectrometry. *J. Chromatogr., A* **2005**, *1091*, 102−109.
(40) Hunt, S. The non-protein amino acids. In *Chemistry and biotechnology of the amino acids*; Chapman & Hall LTD: 1985; pp 55−137.