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# ENANTIOSEPARATION OF THE CONSTITUENTS INVOLVED IN THE PHENYLALANINE-TYROSINE METABOLIC PATHWAY BY CAPILLARY ELECTROPHORESIS TANDEM MASS SPECTROMETRY

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Highlights:

- A CE-ESI-MS$^2$ method is developed to determine Phe-Tyr metabolic pathway enantiomers

- First simultaneous enantioseparation of the constituents of this metabolic pathway.

- ~~First MS detection of the CE enantioseparation of norepinephrine and epinephrine.~~

- Large volume sample stacking enabled to obtain LODs from 40 to 150 nM.

- Plasma samples were analyzed and L-Phe and L-Tyr were determined at µM level.

## Abstract

Catecholamines dopamine, norepinephrine, and epinephrine are well-known neurotransmitters playing different roles in the nervous and endocrine system. These compounds are biologically synthesized in the phenylalanine-tyrosine pathway which consists on the successive conversion of L-phenylalanine into L-tyrosine, L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine, norepinephrine, and epinephrine. This work describes the development of an enantioselective CE-ESI-MS$^2$ methodology enabling, for the first time, the simultaneous enantioseparation of all the constituents involved in the Phe-Tyr metabolic pathway, since all these compounds except dopamine are chiral. The developed method was based on the use of a dual CDs system formed by 180 mM of methyl-β-CD and 40 mM of 2-hydroxypropyl-β-CD dissolved in 2 M formic acid (pH 1.2) and presented the advantage of avoiding the use of any time-consuming labelling procedure. LODs ranged from 40 to 150 nM and the unequivocal identification of the compounds investigated was achieved through their MS$^2$ spectra. The applicability of this methodology to the analysis of biological samples (rat plasma) was also demonstrated.

**Abbreviations: CMT:** counter migration technique; **DA:** dopamine; **DOPA:** 3,4-dihydroxyphenylalanine; **EMO:** enantiomer migration order; **EP:** epinephrine; **HP-β-CD:** (2-hydroxypropyl)-β-cyclodextrin; **LVSS:** large volume sample stacking; **M-β-CD:** methyl-β-cyclodextrin; **NE:** norepinephrine; **PFT**: partial filling technique.

**Keywords:** capillary electrophoresis; enantiomeric multicomponent separation; phenylalanine-tyrosine metabolic pathway; plasma sample; tandem mass spectrometry;

## 1. Introduction

The determination of neurotransmitters is an important tool to increase the knowledge of neurological processes. The catecholamines dopamine (DA), norepinephrine (NE), and epinephrine (EP) are well-known neurotransmitters playing important roles in many biological processes and which can also act as hormones in stressful situations [1]. Quantification of catecholamines in biological fluids is an interesting tool in the diagnosis of several disorders such as schizophrenia, Parkinson's disease or pheochromocytoma [2,3]. These catecholamines are biologically synthesized in the Phe-Tyr metabolic pathway (**Figure 1**). A known pathology related to this metabolic pathway is phenylketonuria, an inborn error of metabolism which results in large excess of Phe in blood and urine [4], what can derive in mental retardation as a consequence of low level of Tyr and catecholamines [5]. Previous works also demonstrated the relationship between some mental disorders and the availability of precursors of catecholamines in cerebrospinal fluid of monkeys [6]. Likewise, the reduction of 3,4-dihydroxyphenylalanine (DOPA) synthesis by using the alpha-methyl-p-tyrosine inhibitor also results in a transient decrease in catecholamine synthesis [7].

All the compounds involved in the Phe-Tyr metabolic pathway (see **Figure 1**) except DA are chiral, therefore it is of high interest to study their stereochemistry, especially since only the L-enantiomers of the involved amino acids can lead to these important neuroactive molecules. Thus, D-Phe and D-Tyr were found to be present in different physiological fluids such as plasma, urine, cerebrospinal fluid, and amniotic fluid [8]. Moreover, it has been shown that D-Tyr has deleterious effects inhibiting the growth of mice [9]. On the other hand, Parkinson's disease therapy includes administration of the L-form of DOPA in order to replenish DA, whereas that D-DOPA not only cannot be converted to DA but also presents side effects [10, 11].

The study of the chirality of biological molecules acquires great importance since, although in the past it was assumed that only L-amino acids were the natural existing form [12], later studies demonstrated that the D-forms were also present in some micro-organisms [13], in amphibians and invertebrates and even in mammals, including humans [14] either as a result of spontaneous racemization of L-forms in the structure of polypeptides when ageing [15,16] or as endogenous substances [17-19]. The amino acid D-Serine is an example of this, as it is an important neuroactive molecule whose levels in mammals can even exceed the concentration of many L-amino acids [20]. Thereby, it is of high importance to increase the knowledge of the activity of enantiomers, whose determination still remains as a promising topic in the analytical sciences field.

Due to the fact that a pair of enantiomers presents the same physicochemical properties with the exception of opposite optical rotation of plane-polarized light and differential strength of the interactions with chiral compounds, their separation is a challenging task that could require the MS in order to achieve the necessary sensitivity and the unequivocal detection of the investigated compounds when these are in complex samples. Regarding the Phe-Tyr metabolic pathway, GC, LC, and CE have been the

separation techniques employed to achieve the enantiomeric separations of its individual components or partial mixtures of them using different detection systems. Thus, the enantiomeric determination of Phe and Tyr, generally in mixtures with more proteinogenic amino acids, has been reported in numerous articles but their determination in biological fluids has been less frequent. Over the last 20 years, some of these works include the use of GC-MS employing Chirasil-L-Val as stationary phase [21,22], LC-MS employing either cinchona alkaloid based chiral stationary phases [23, 24] or a chiral derivatization reagent [25-27], CE-UV with a chiral labelling agent [28], CE-LIF with a chiral derivatization reagent [29] or cyclodextrins [30,31] and CE-MS employing a chiral crown ether [32]. Note that all these methodologies required time-consuming derivatization steps except the last one [32]. Regarding the other chiral constituents of the Phe-Tyr metabolic pathway, DOPA, NE and EP, a considerable minor number of articles reporting their enantiomeric separation have been published. In the last 20 years, enantioseparation of DOPA, NE, or EP has not been reported by GC. In the case of LC, none described the enantioseparation of DOPA, while the individual or simultaneous enantioseparation of NE and EP was reported only in two works [27, 33]. Concerning CE, some articles have been published dealing with the enantiomeric separation of DOPA [34-40], as well as mixtures of DOPA with Phe and Tyr using MS detection [41], or even the enantiomeric separation although not simultaneously of DOPA, and other components of the Phe-Tyr metabolic pathway using UV detection [42]. Regarding the simultaneous enantioseparation of NE and EP by CE, some studies have been described using UV [1, 43-47] or electrochemical detection [48], but these compounds have never been enantioseparated using a CE-MS system.

In summary, to the best of our knowledge, the constituents of the Phe-Tyr metabolic pathway have never been simultaneously enantioseparated. Only an old

method published in 1996 [42] described the separation within 60 min of these compounds using sulfated-β-CD as chiral selector in CE-UV but, the simultaneous separation was not possible due to the overlapping of DA, and NE and EP enantiomers. In addition, this methodology is not compatible with MS detection under the conditions assayed. Thus, it is of special interest to develop a methodology enabling the simultaneous enantioseparation and unequivocal detection of the compounds involved in the Phe-Tyr metabolic pathway in a single run, without the need of using time-consuming derivatization steps.

Since CE offers numerous advantages for chiral analysis such as high separation efficiency, excellent enantioselectivity and low reagents and solvents consumption (including chiral selectors), what makes it an environmental-friendly technique [49-52], this technique was employed in this work with the aim of developing an enantioselective CE-ESI-MS$^2$ methodology to carry out, for the first time, the simultaneous enantioseparation and unequivocal detection of all the Phe-Tyr metabolic pathway constituents avoiding the use of any derivatization procedure. Firstly, investigation on the enantiodiscrimination of several chiral selectors towards these compounds is presented in CE-UV. Secondly, the methodology is implemented in a MS system and, after testing its analytical characteristics, it is applied to the analysis of rat plasma. We consider that testing our enantioselective CE-ESI-MS$^2$ methodology to separate and detect all the Phe-Tyr metabolic pathway constituents in biological samples such as rat plasma could be particularly relevant due to the enormous potential of this biological sample in translational research.

## 2. Materials and methods

### 2.1. Reagents

All reagents were of analytical grade. MS-grade acetonitrile and methanol were obtained from Scharlau Chemie (Barcelona, Spain) while formic acid, perchloric acid, ammonium carbonate, ammonium hydroxide, and EDTA were from Sigma (St. Louis, MO, USA). Ammonium acetate was from Merck (Darmstadt, Germany), ascorbic acid was obtained from VWR (Radnor, PA, USA) and isoflurane from Abbott (Madrid, Spain). The employed water was MilliQ quality (Millipore, Bedford, MA, USA). β-CD, sulfated-β-CD (degree of substitution (DS): 12-14), methyl-β-CD (M-β-CD) (DS 1.7-1.9), 2-hydroxypropyl-β-CD (HP-β-CD) (DS~4.2), 2,6-di-O-methyl-β-CD (DM-β-CD), and carboxymethyl-β-CD (CM-β-CD) (DS~3) were purchased from Fluka (Switzerland). Acetyl-β-CD (A-β-CD)(DS 7), γ-CD, methyl-γ-CD (M-γ-CD) (DS~12), 2-hydroxypropyl-γ-CD (HP-γ-CD) (DS~4.5), HP-γ-CD (DS~3.2), carboxyethylated-β-CD (CE-β-CD) (DS ~3), carboxymethylated-γ-CD (CM-γ-CD) (DS ~3), and carboxyethylated-γ-CD (CE-γ-CD) (DS ~3) were purchased from Cyclolab (Hungary). DL-Phe and DL-EP were from Aldrich (St. Louis, MO, USA), L-Phe, D-Tyr, L-Tyr, and DL-NE in Fluka (Buchs, Switzerland) and DL-DOPA, L-DOPA, L-NE, L-EP, DA, and trigonelline hydrochloride in Sigma (St. Louis, MO, USA).

### 2.2. CE-UV conditions

Electrophoretic experiments were carried out on a HP³ᴰCE system from Agilent Technologies (Palo Alto, USA) with a diode array detector. The electrophoretic system, and the data collection were controlled by HP³ᴰCE ChemStation software. The BGE employed consisted of a dual CDs system based on 100 mM M-β-CD and 60 mM HP-β-CD dissolved in 2 M formic acid (pH 1.2). Separations were performed in an uncoated

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fused-silica capillary of 50 μm I.D. and a total length of 88.5 cm, purchased from Polymicro Technologies (Phoenix, USA) at +30 kV and 15 ºC. Injections were carried out applying 5000 Pa (50 mbar) for 5 s. Detector parameters were a wavelength of 200 ± 5 nm. At the beginning of the day the capillary was flushed with buffer solution for 10 min and at the end of the day with MilliQ-water for 5 min. In order to ensure the repeatability between injections, the capillary was flushed with buffer solution for 2 min and BGE for 5 min.

### 2.3. CE-ESI-MS$^2$ conditions

CE-MS analyses were performed in a HP$^{3D}$CE instrument (Agilent Technologies, Palo Alto, USA) coupled through an orthogonal coaxial sheath interface (model G1607A from Agilent Technologies, Palo Alto, USA) to the Electrospray Ionization (ESI) source of an Ion Trap mass spectrometer (model AmaZon SL from Bruker Daltonics, Germany) for MS detection. For MS control and data analysis, TrapControl Software 7.0 for AmaZon was used. BGE employed was 2 M formic acid (pH 1.2). The separation was achieved in an uncoated fused-silica capillary of 120 cm and 50 μm I.D., at +30 kV and 15 ºC. Injections were performed applying 5000 Pa (50 mbar) for 250 s. Between analysis, the capillary was flushed applying 100000 Pa (1 bar) with BGE for 4 min, and a mixture of 180 mM M-β-CD and 40 mM HP-β-CD dissolved in 2 M formic acid (pH 1.2) for 2.5 min.

MS conditions were a 50:50 (v/v) methanol/water with 0.1 % (v/v) formic acid sheath liquid at 3.3 μL/min by a 2.5 mL syringe pump (Hamilton, USA). The nebulizer and drying gas (N$_2$) were 20684 Pa (3 psi) and 5 L/min at 200 ºC. The mass spectrometer operated with the ESI source in the positive ion mode at -4.5 kV with an end plate of -500 V. The ion optical parameters were tuned in the "expert mode". In MS$^2$ experiments

the Ion Charge Control (ICC) was activated with a target up to 100,000 ions using 200 ms of accumulation time and 1 average. The *m/z* scanned range was from 100 to 210 m/z in the "Enhanced Resolution" mode (8,100 (*m/z*)/s). MS$^2$ experiments were accomplished by the fragmentation of the precursor ions of the six compounds (*m/z* 154.0, 152.0, 184.0, 198.0, 165.9 and 181.9 for DA, NE, EP, DOPA, Phe, and Tyr, respectively) and the internal standard (IS) trigonelline (*m/z* 138.0) by means of the pseudo-multiple reaction monitoring mode. The isolation width of each precursor ion was set to 2.0 *m/z* where its fragmentation was carried out by collision-induced dissociation with the helium present in the trap. Extracted Ion Electropherograms were obtained extracting the corresponding product ions with an extraction window of -0.3/+0.7 *m/z* using a smoothed option of the software (Gauss at 1 point).

### 2.4. Animal sampling

Male Wistar rats (Barcelona, Spain) weighting 300-330 g were housed at a constant temperature (20 ± 2 °C) on a 12-hour light/dark cycle (lights on at 08:00 hours), with *ad libitum* access to food and water. Animals were maintained and handled according to European Union guidelines for the care of laboratory animals (Directive 2010/63/EU) and the "Principles of laboratory animal care" were followed. All procedures were approved by the bioethics committee of the Universidad Nacional de Educación a Distancia (Madrid, Spain).

Rats were anesthetized with isoflurane, decapitated and blood was recollected in heparinized tubes, centrifuged for 10 min at 2000xg (4 ºC) and plasma was collected and stored at −70°C.

*2.5. Preparation of samples*

Rat plasma samples were treated with acetonitrile to precipitate proteins (plasma:acetonitrile, 1:2), then centrifuged at 10000xg for 15 min at 4 ºC and, the supernatant was diluted with formic acid (1:1) so that its final concentration is 0.25 M. All samples contained a final concentration of the IS trigonelline of 20 μM. All solutions were sonicated and filtered with 0.2 μm polytetrafluorethylene filters prior CE analysis.

## 3. Results and discussion

*3.1. Investigation on the chiral separation of the constituents of the Phe-Tyr metabolic pathway by CE*

In order to develop a chiral methodology enabling the enantioseparation of all the constituents involved in the Phe-Tyr metabolic pathway, the enantiodiscrimination power of different chiral selectors towards these compounds was investigated using CE-UV. First, counter migration technique (CMT) was evaluated aiming to avoid the undesired presence of the chiral selector in the detection system making the future MS-coupling easier. Thus, negative sulfated-β-CD was employed due to its contrary migration to that of the studied analytes. Enantioselectivity of sulfated-β-CD was tested at concentrations ranging from 0.2 to 0.6 mM in 200 mM ammonium formate buffer (pH 3.0) and the separation was performed in 88.5 cm fused-silica capillary of 50 μm I.D., at 30 kV and 15 ºC. In the tested CD concentration range, enantioseparation for amino acids Phe, Tyr and DOPA was accomplished, however, catecholamines DA, NE and EP could not be detected, due to their extremely high interaction with the anionic CD which migrates away from the detector in normal polarity. In order to detect the catecholamines, the EOF was raised increasing the pH to 5.0 and to 7.0, but although the enantioseparation of NE

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and EP was achieved under the new tested conditions, amino acids Phe, Tyr and DOPA could not be enantioresolved.

Since it was not possible to enantioseparate all the studied compounds with the CMT approach under the same conditions, the next strategy was to carry out a screening with several neutral CDs dissolved in an extremely acid media (2 M formic acid (pH 1.2)). With this BGE all studied compounds are under the same ionic net charge ($pK_a$ values shown in **Figure 1**) and the EOF is totally suppressed so that these positively charged analytes can migrate faster than the CD. These are ideal conditions for developing a partial filling technique (PFT), strategy required when using the method in MS.

Eight β-CDs (β-CD, A-β-CD, DM-β-CD, M-β-CD, HP-β-CD, CE-β-CD, CM-β-CD, succ-β-CD) and five γ-CDs (γ-CD, M-γ-CD, HP-γ-CD, CE-γ-CD, and CM-γ-CD) were tested at concentrations ranging from 5 to 80 mM except β-CD which was studied from 1 to 12 mM due to its low aqueous-solubility. Concerning γ-CD derivatives only 80 mM of M-γ-CD allowed enantiodiscrimination of Tyr and DOPA (Rs 1.5 and 2.6, respectively) but did not offer enantioselectivity for Phe, NE, or EP. Regarding β-CD derivatives, all of them except the native β-CD allowed baseline enantioseparation for NE and EP, however, just succ-β-CD, M-β-CD, and HP-β-CD also enabled enantiodiscrimination for some of the studied amino acids where Rs increased as the concentration increased. Thereby, 80 mM of succ-β-CD could not discriminate Tyr enantiomers but offered partial enantioseparation for Phe (Rs 0.6) and DOPA (Rs 1.3). Additionally, DOPA enantiomer migration order (EMO) enabled the minor enantiomer to be the last one to migrate making it hard to be detected when large amounts of L-enantiomer are present. Unlike succ-β-CD, 80 mM of M-β-CD offered enantioseparation for Tyr (Rs 1.5) besides partial enantioseparation for Phe (Rs 1.0) and peak broadening

for DOPA. M-β-CD allowed the D-amino acids to migrate in first place whereas that in the case of the catecholamines L-enantiomers migrated first, however since Rs was high enough it was possible to detect D-enantiomers in presence of high amounts of L-enantiomers. The difference in the EMO might be justified by the different position of the chiral center (in the carbon-β for amino acids and in the carbon-α for catecholamines), thus modifying their enantiorecognition. On the other hand, 80 mM of HP-β-CD offered enantiodiscrimination for Phe and DOPA but peak broadening was observed for Tyr, and the EMO was the same as with M-β-CD.

Therefore, M-β-CD could be a potent chiral selector to carry out the enantioseparation of the studied compounds. In order to enhance the Rs its concentration was studied in a range from 80 to 180 mM where 160 mM originated the best Rs and allowed the individual enantioseparation of all the compounds including DOPA (Rs ranging from 1.5 to 6.0), although simultaneous enantioseparation could not be achieved due to the co-migration of DOPA enantiomers with Phe enantiomers (**Figure 2**). On the other hand, the optimum concentration for HP-β-CD, the CD which also offered good Rs and presented the same EMO as M-β-CD, was investigated being 160 mM also the optimum concentration. As can be seen in **Figure 2**, 160 mM of HP-β-CD avoided the co-migration of enantiomers of Phe and DOPA but the Rs obtained with this CD was worse than the one obtained with M-β-CD. It seems that M-β-CD is the CD which most influences the Rs of each analyte whereas that HP-β-CD affects the simultaneous separation of the analytes of interest thus, the use of a dual CDs system consisting of a mixture of M-β-CD and HP-β-CD was evaluated as they showed to offer combined effects. Different combinations were tested being 100 mM of M-β-CD with 60 mM of HP-β-CD the mixture which presented the best Rs for the simultaneous enantioseparation in the shortest analysis time. Finally, the effect of the separation temperature was

evaluated from 15 to 35 ºC, being 15 ºC the optimum in terms of Rs. ~~It is important to highlight that, not only the enantioseparation of these compounds, but also their individual separation was a very challenging task since the chemical structure of the studied compounds is very similar, as they belong to the same metabolic pathway (they are just differentiated by gains or losses of small molecules such as OH, CH₂ or HCO₂).~~ The enantioseparation of all components of the Phe-Tyr metabolic pathway under the optimal conditions is shown in **Figure 3.**

*3.2. Optimization of the CE-ESI-MS² methodology*

Once the chiral methodology was developed in an UV detection system, the next step consisted on transferring the method to a MS system in order to increase the sensitivity and selectivity. To avoid the undesired entry of the chiral selector in the detection system and to enhance the sensitivity, the possibility of using a PFT was studied since, as described above, it was not possible to develop a CMT. Thus, the employed capillary (88.5 cm length) was 70 % filled with the CD mixture by applying 100000 Pa (1 bar) for 1.6 min. Under these conditions, analytes migrated faster than in CE-UV as a consequence of the suction effect produced in the MS system and also as a result of the higher apparent mobility of analytes due to the reduced chiral selector band in the capillary, detecting the studied analytes with analysis times around 30 min with a remarkable loss in the Rs. In order to obtain a good enantioresolution in the shortest possible analysis time, the capillary length and the time to fill the chiral selector band were optimized. 120 cm and 2.5 min (i.e. a 83 % of the capillary was filled with the CDs mixture) were the best in terms of Rs, analytes migration times, and time distance between the last analyte (L-Tyr) and the band of CDs. Since the enantioresolution was not good enough, a re-optimization of the concentration of CDs mixture employed in the PFT was

necessary, and several combinations of the mixture of CDs were tested. Although none of the tested combinations offered baseline Rs for DOPA enantiomers, with 180 mM for M-β-CD and 40 mM for HP-β-CD, complete individual and simultaneous enantioseparation were obtained for the rest of compounds. It is important to highlight that the sensitivity increased up to 3 times when using PFT over complete filling of the capillary with the mixture of CDs as it generated ion suppression caused by its entry in the MS system.

After re-optimizing the CE separation conditions, MS parameters were optimized. It was observed that high amounts of methanol (80 %) in the sheath liquid (SL), required to obtain a stable formation of ions in the ESI interface between CE and MS, generated instability in the ionic current of the MS system, so a mixture composed of methanol:water (50:50 (v/v)) with formic acid 0.1 % (v/v) was chosen. Among all studied flow rates of SL, 3.3 µL/min offered good sensitivity and a stable ionic current in the MS system. Likewise, different ESI parameters were adjusted aiming to obtain the best sensitivity in terms of S/N. Thus, the optimum applied voltage was 4500 V as it offered the best S/N and a stable ionic current. Optimal values for nebulizer pressure and drying gas flow were 20684 Pa (3 psi) and 5 L/min, respectively. Finally, 200 ºC for drying gas temperature was the optimum value to achieve the best S/N.

Before optimizing the trap parameters, the first optical parameter to be optimized was the capillary exit voltage present in the first vacuum stage (zone of collision induced fragmentation of the formed in the ESI before going into the analyzer) to avoid fragmentation of quasi-molecular ions ([M+H]$^+$). 60 V was selected to obtain the protonated molecular ions of each compound (except NE) as the base peak of the MS spectra, so they were chosen as precursor ions for MS$^2$ experiment. In the case of NE two ions were majority, being the ion at *m/z* 152.0 (resulting from the loss of a water molecule

[M-H$_2$O+H]$^+$) its base peak, more abundant than the ion [M+H]$^+$, so it was selected as the precursor ion for NE in the MS$^2$ experiment. **Table 1** shows the values for other optimized MS$^2$ parameters and **Figure 4A** shows the separation obtained.

Finally, in order to increase the method sensitivity, sample pre-concentration by means of an injection with large volume sample stacking (LVSS) was considered. Different injection times were tested being 250 s the one which presented the best sensitivity without harming the Rs and the peak shape (see **Figure 4B**). This preconcentration strategy enabled to improve sensitivity about 50 times when compared to the 5 s standard injection.

*3.3. Analytical characteristics of the CE-ESI-MS$^2$ methodology and application to the analysis of rat plasma samples*

With the aim of applying the developed CE-ESI-MS$^2$ methodology to the analysis of plasma samples, the analytical characteristics of the method were evaluated in terms of selectivity, linearity, accuracy, precision, and sensitivity for DA, L-NE, L-EP, L-DOPA, L-Phe, and L-Tyr in rat plasma.

The fact that the plasma samples were treated with acetonitrile to carry out the protein precipitation affected negatively the separation due to a decrease in the conductivity of the medium. This led to peak broadening and longer analysis times. To overcome this issue a sample dilution was considered, establishing the need to perform a 1:1 dilution of the treated sample with formic acid 0.25 M prior to injection into the CE system.

**Figure 5** demonstrates the good selectivity of the developed methodology; on the one hand there is complete resolution within the individual compounds including their enantiomers (baseline for all of them except for DOPA), on the other hand all analytes

have different *m/z* values and could be identified by their $MS^2$ spectra. Initially, it could be thought that the complete resolution within the individual compounds is not even required as they can be distinguished by MS. However, the differences between the compounds of interest in some cases are very small (only two units of *m/z*, which could involve errors in the isolated precursor ions due to the presence of isotope ions of other compounds) and other times these may differ with regard to the functional groups ~~differences may be due to very likely lost as are the groups~~ OH, $H_2O$, or COOH ~~$HCO_2$~~. Also, something important to take into account is the ion suppression, which might be caused if several analytes co-elute. Therefore, on certain occasions it is desirable to have individual separation of the studied compounds, especially when the chemical structure of the compounds is very similar. Likewise, an adequate selectivity was achieved as no interfering peaks were observed when a plasma sample was analyzed (see **Figure 6A**). This is of extreme importance since in biological samples it is common to find molecules with same monoisotopic mass, and therefore can interfere in the proper determination and/or quantification of a certain compound.

The results of the rest of analytical characteristics are shown in **Table 2**. Quantification of the analytes of interest was carried out by means of the internal standard calibration method. The IS selected was trigonelline since its chemical structure was similar to the studied compounds, it was not present in the matrix sample, and it migrated at 60 min, near the analytes without interfering (**Figure 6**). Good linearity was achieved for all compounds ($R^2 > 99$ %), when plotting the corrected peak areas of each analyte divided by the corrected peak area of the IS versus the concentration of each analyte divided by concentration of the IS. Confidence interval for the slope did not include the zero value and confidence interval for the intercept included the zero value in all cases (confidence level of 95 %). It was found that there were not matrix interferences since

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there were not significant differences between the slopes obtained by the internal standard and the standard additions calibration methods (P-values > 0.05, for a confidence level of 95 %). On the other hand, good recovery values were obtained, ranging from 87 to 105 % with precision values from 7 to 15 %. Instrumental and method repeatability, and intermediate precision were adequate, as RSD % values ranged from 1.8 to 3.3 % in the case of migration times and from 9.2 to 16.9 % for corrected peak areas. Regarding the method sensitivity, the LODs ranged from 40 to 150 nM and the LOQs from 133 to 500 nM. Although the migration times increased and Rs decreased when compared to the CE-UV method, the CE-ESI-MS$^2$ methodology enabled to improve the LODs from 200 (in the case of NE) to 3500 times (for DA).

Figure 6 shows the electropherograms corresponding to a rat plasma sample and the same sample spiked with the analytes of interest. The compounds found in the analyzed samples were L-Phe and L-Tyr, being the contents of the other compounds below their respective LODs. ~~The fact that~~ Catecholamines and DOPA could not be detected in our plasma samples since their physiological levels in healthy subjects, 0.5, 1.6, and 8.5 nM for EP, NE, and DOPA [53], respectively, are much lower than the herein reported LOD (40, 150, and 54 nM for EP, NE, and DOPA, respectively). ~~is due to could be because we analyzed healthy subjects, and their concentration were below the LOD of the proposed methodology.~~ Slightly higher levels of these compounds have been found in diseased samples (1.0, 3.7, and 10.9 nM for EP, NE, and DOPA [53], respectively), although these values are still below the LOD of our method. Even ~~It has been seen that~~ higher levels of ~~catecholamines and~~ DOPA ~~are found in diseased samples when compared to healthy subjects, these concentrations being in the nM level for catecholamines [53] and~~ have been also reported in diseased samples, in the µM range ~~for DOPA~~ [54]. Regarding D-Phe and D-Tyr, they have been reported to be present in a percentage of 0.8

and 0.6 %, for D-Phe and D-Tyr, respectively, referred to their corresponding L-forms [8]. The fact that the percentage that our method allows to detect is 2.3 and 2.2 % for D-Phe and D-Tyr, respectively, could be the reason why they could not be detected. Further improvements in sensitivity using the *sheathless* interface [55] or preconcentration strategies [56] could enable the detection of these analytes.

In spite of this issue, as can be seen in **Figure 6B** the studied analytes could be determined in spiked plasma samples analyzed by the developed CE-ESI-MS$^2$ method. The concentrations of the determined amino acids, quantified by the internal standard calibration method and considering the sample treatment were $51 \pm 3$ µM for L-Phe, and $55 \pm 3$ µM for L-Tyr. These values are in agreement with those reported in the literature [57], thus demonstrating the validity of the proposed methodology.

## 4. Conclusions

An enantioselective CE-ESI-MS$^2$ methodology has been developed enabling, for the first time, the simultaneous enantioseparation of all the constituents of the Phe-Tyr metabolic pathway. A thorough study on the chiral recognition obtained for these compounds towards different CDs has been carried out showing that the best enantioselectivity was obtained with a mixture 180 mM in M-β-CD and 40 mM in HP-β-CD in an acidic medium (2 M formic acid at pH 1.2).

The CE-ESI-MS$^2$ methodology developed in this work enabled to obtain LODs values ranging from 40 to 150 nM for the constituents of the Phe-Tyr metabolic pathway in addition to their simultaneous analysis in a unique run. Moreover, no derivatization steps were necessary. This is also the first time that NE and EP enantiomers are separated by CE-MS.

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The developed method was applied ~~demonstrated its applicability~~ to the analysis of biological fluids showing that L-Phe and L-Tyr were present in the analyzed rat plasma samples ~~analyzed~~ at the μM level.

**Conflicts of interest**

*Authors declare that they have no conflict of interest*

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**Figure captions**

**Figure 1.** Scheme of the Phe-Tyr metabolic pathway depicting the structures of its constituents along with their theoretical $pK_a$ values in brackets (determined by Marvin software: http://www.chemaxon.com/marvin/sketch/index.php). Chiral center is denoted by *.

**Figure 2.** Electropherograms displaying the chiral separation of the Phe-Tyr metabolic pathway constituents by CE-UV employing 160 mM M-β-CD or 160 mM HP-β-CD as chiral selector. Other experimental conditions, as in **section 2.2**. Peak identification: dopamine (DA), norepinephrine (NE), epinephrine (EP), 3,4-dihydroxyphenylalanine (DOPA), phenylalanine (Phe), and tyrosine (Tyr). Sample: standards in a proportion of 1:1 (L:D-enantiomers), being L-enantiomers and DA in a concentration of 1 mM except L-Phe which was 5 mM.

**Figure 3.** Electropherogram showing the chiral separation of the Phe-Tyr metabolic pathway constituents by the developed CE-UV methodology. Peak identification: dopamine (DA), norepinephrine (NE), epinephrine (EP), 3,4-dihydroxyphenylalanine (DOPA), phenylalanine (Phe), and tyrosine (Tyr). Experimental conditions, as in **section 2.2**. Sample: standards in a proportion of 20:1 (L:D-enantiomers), being L-enantiomers and DA in a concentration of 1 mM except L-Phe which was 5 mM.

**Figure 4.** Extracted Ion Electropherograms obtained by CE-ESI-MS$^2$ for the chiral separation of the Phe-Tyr metabolic pathway constituents with injection time of 5 s (normal injection) in (A) and 250 s (injection with LVSS) in (B). Peak identification: dopamine (DA), norepinephrine (NE), epinephrine (EP), 3,4-dihydroxyphenylalanine (DOPA), phenylalanine (Phe), and tyrosine (Tyr). Experimental conditions, as in **section 2.3**.

**Figure 5.** Extracted Ion Electropherograms obtained by CE-ESI-MS$^2$ for the chiral separation of the Phe-Tyr metabolic pathway constituents along with their MS$^2$ spectra. Peak identification: dopamine (DA), norepinephrine (NE), epinephrine (EP), 3,4-dihydroxyphenylalanine (DOPA), phenylalanine (Phe), and tyrosine (Tyr). Experimental conditions, as in **section 2.3**. Standard solutions were diluted in a 1:2 (v/v) proportion with acetonitrile and then the resulting mixture was diluted 1:1 (v/v) with formic acid 0.25 M to mimic the treatment of plasma samples. Final concentration of standards: 600 nM for L-enantiomers, including DA, and 200 nM for D-enantiomers.

**Figure 6.** Extracted Ion Electropherograms obtained by CE-ESI-MS$^2$ method of (A) a rat plasma sample (the inserts show the MS$^2$ spectra of L-Phe and L-Tyr peaks), and (B) a spiked rat plasma sample (spiked with 200 nM of D-NE, D-EP, D-DOPA, D-Phe and D-Tyr and 600 nM of DA, L-NE, L-EP, and L-DOPA). IS stands for the internal standard trigonelline. Experimental conditions, as in **section 2.3**.
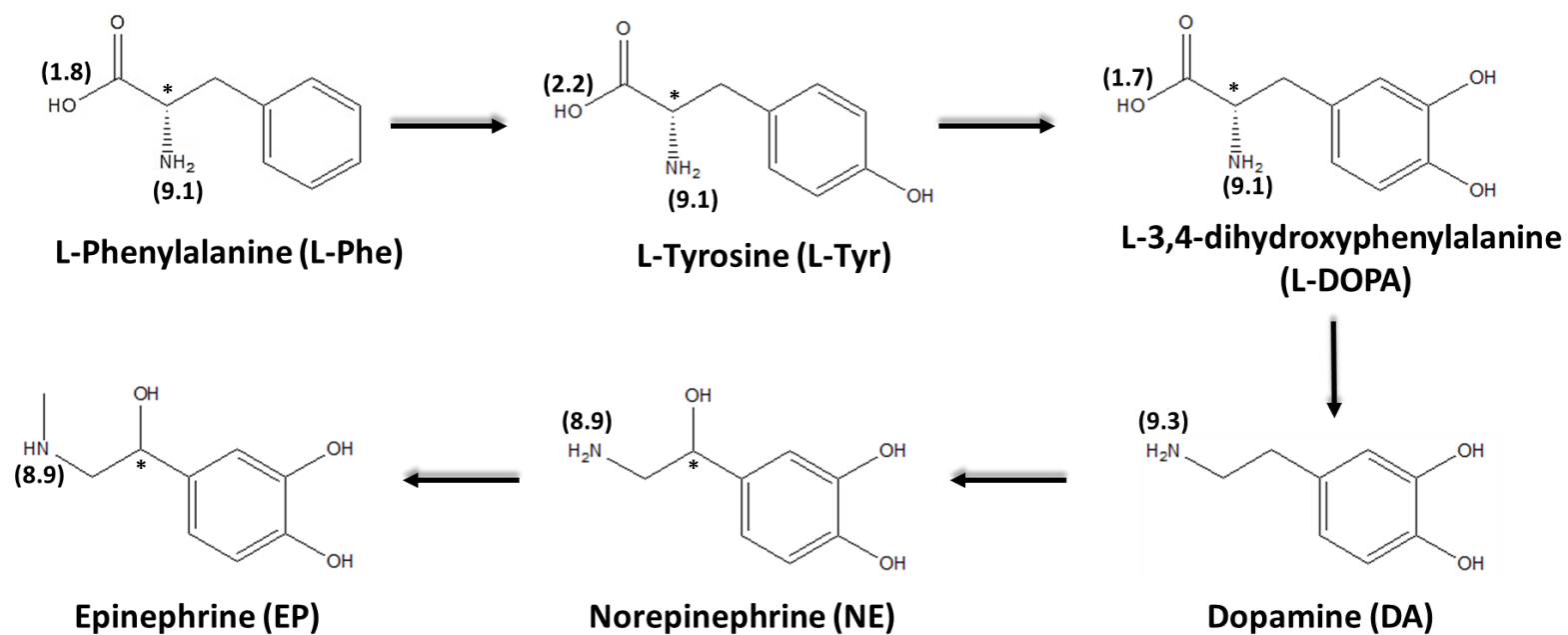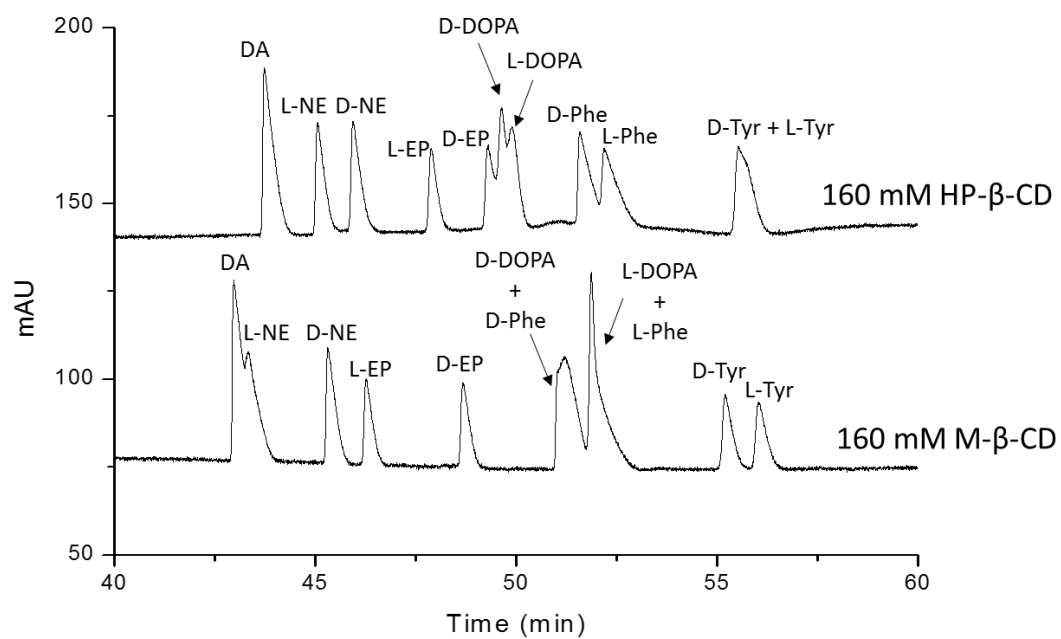
Figure_1

**Figures**

**Figure 1.**

Figure_2

**Figure 2.**

Figure_3

**Figure 3.**

**Figure_4**

**Figure 4.**



**A) Normal injection**

*Concentration of standards: 100 µM for L-enantiomers (including DA) and 20 µM for D-enantiomers.*

**B) Large volume sample stacking**

*Concentration of standards: 2 µM for L-enantiomers (including DA) and 0.4 µM for D-enantiomers.*

50 times sensitivity improvement
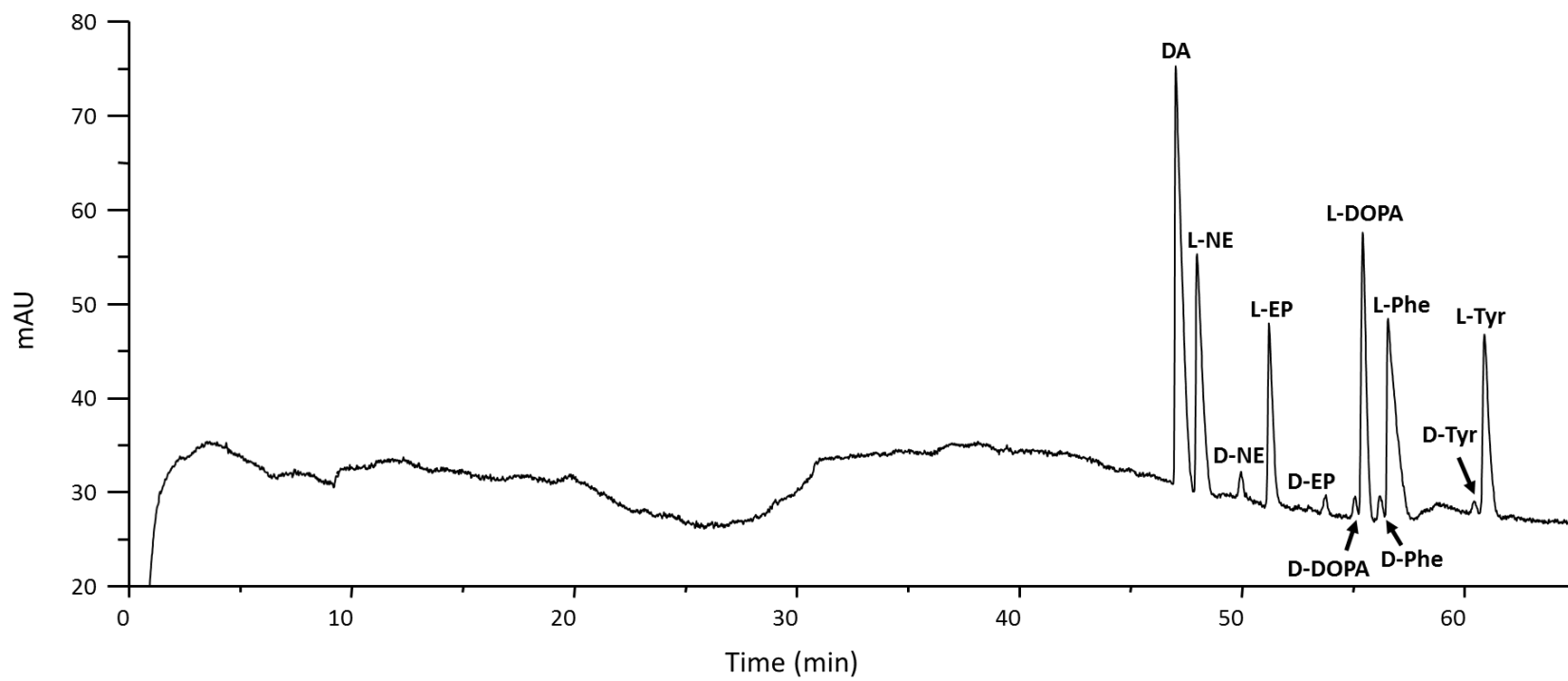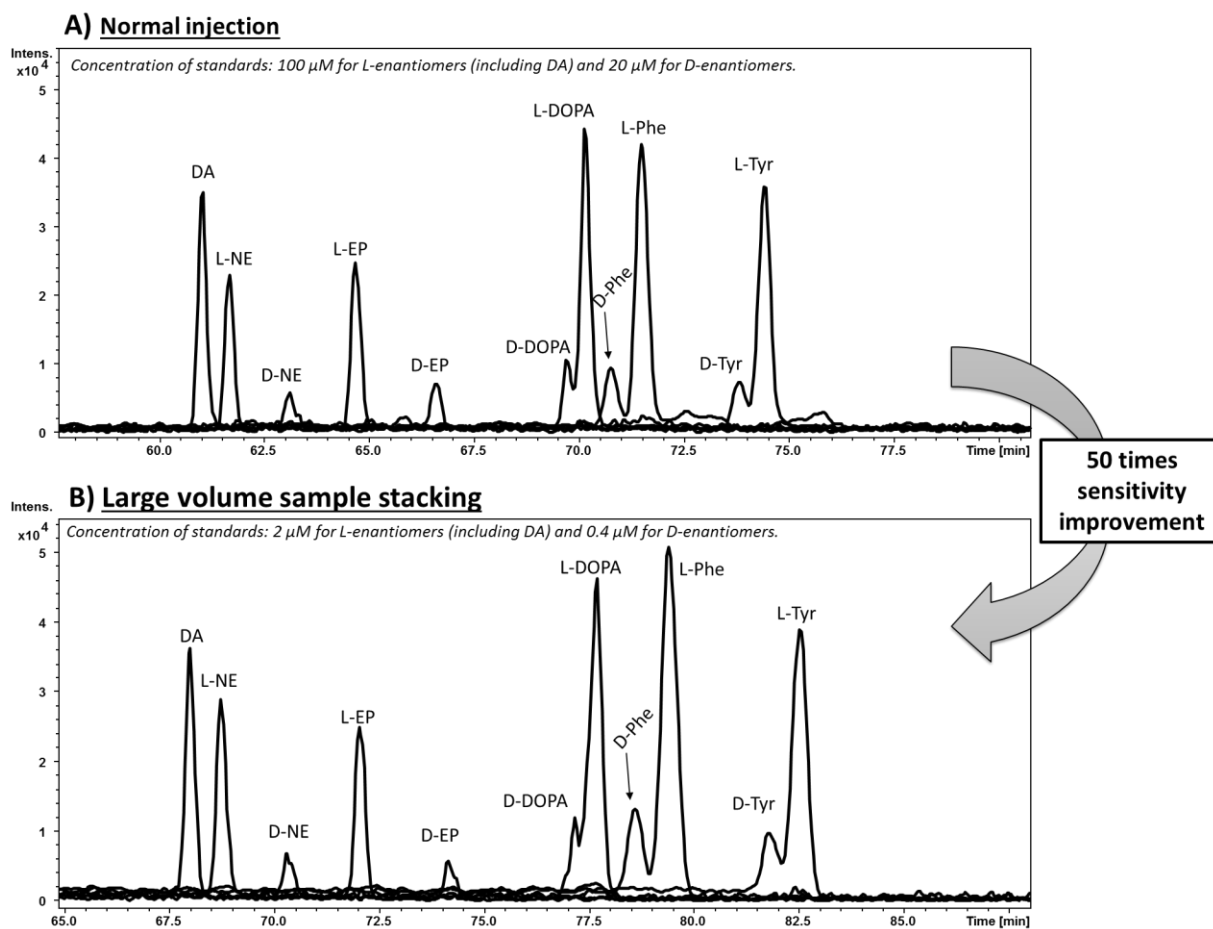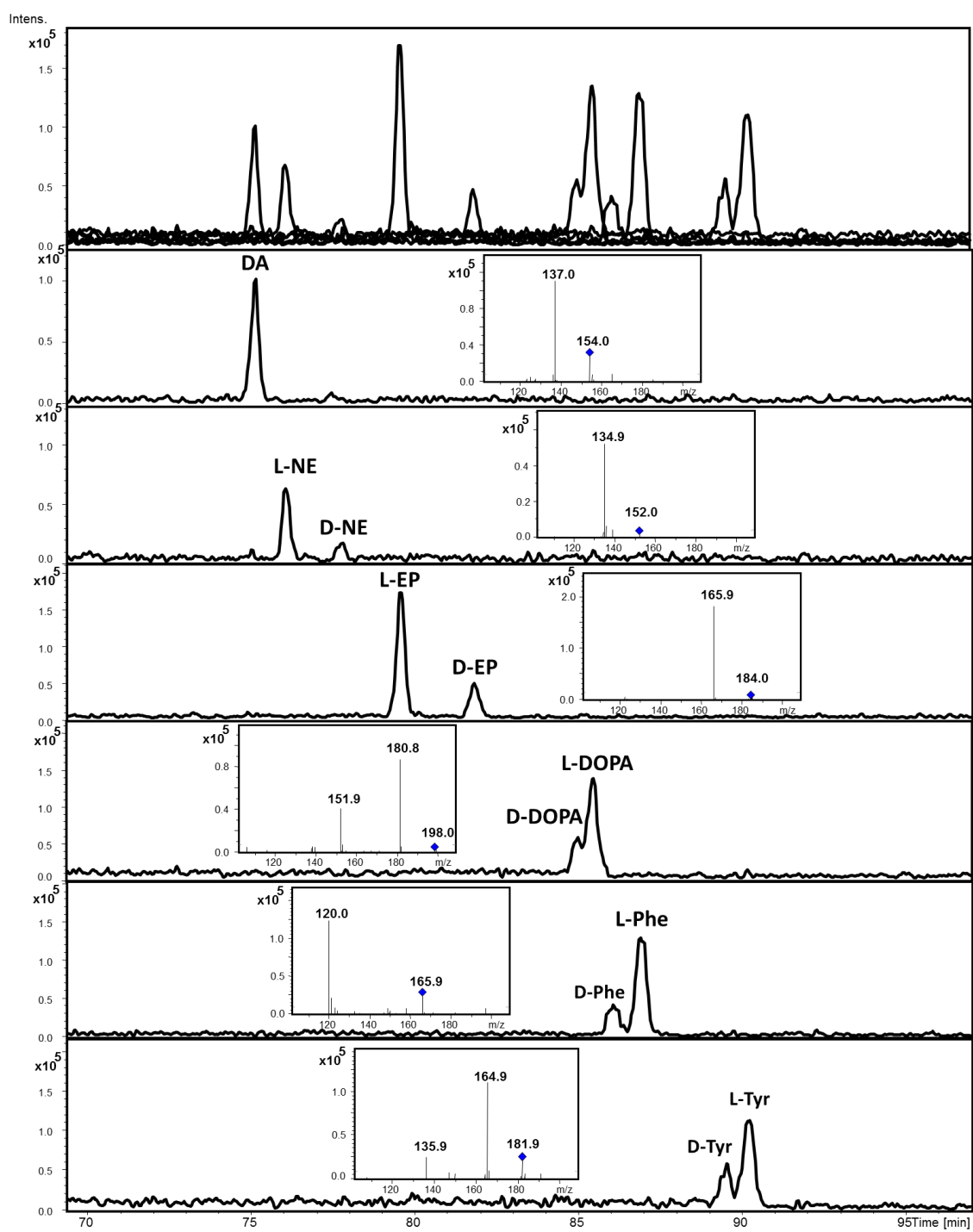
Figure_5
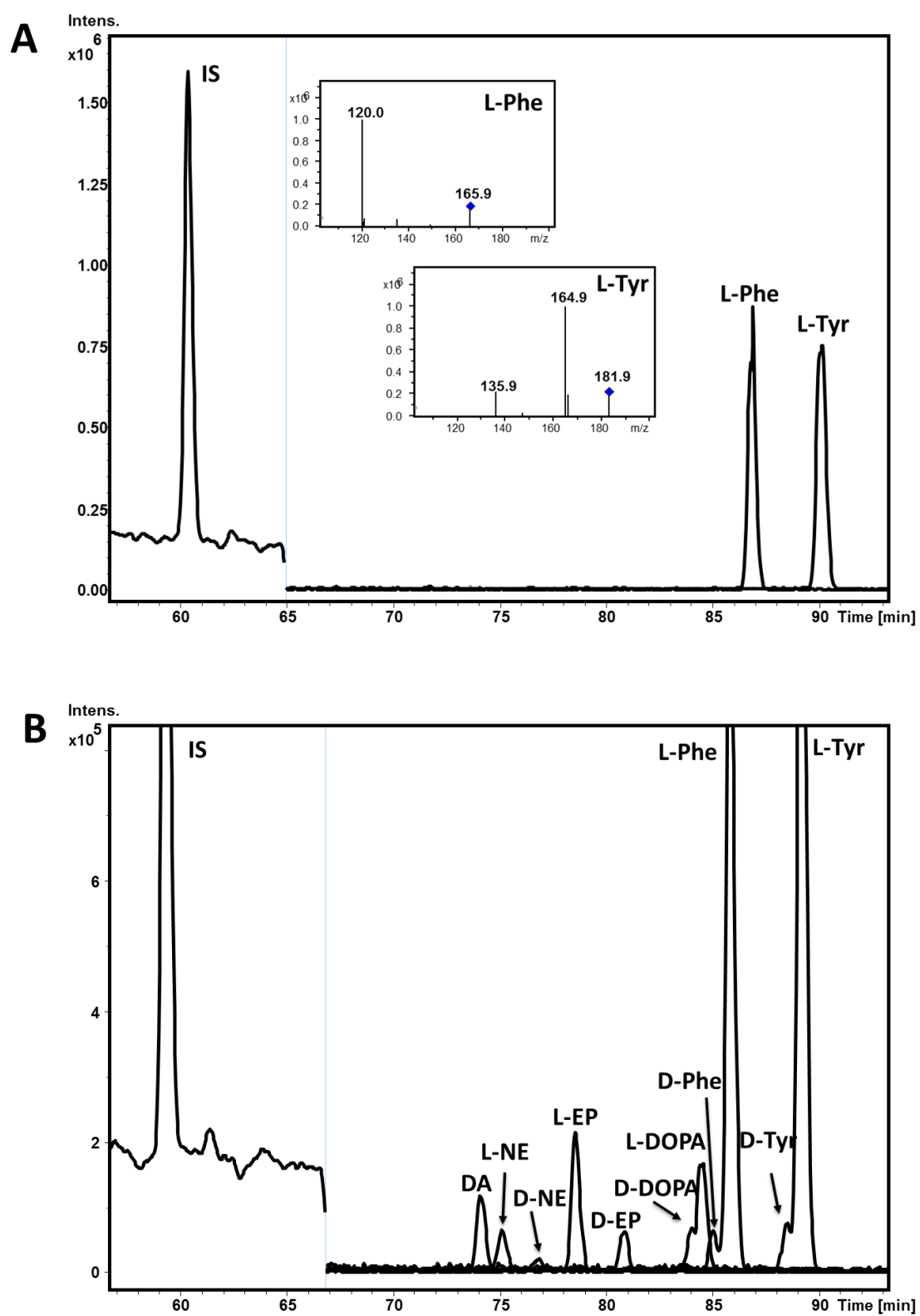
**Figure 5.**

**Figure_6**

**Figure 6.**

**Table 1.** Optimal values for the ion trap parameters in the developed CE-ESI-MS$^2$ methodology.

| Parameters optimized individually for each studied compound | | | | |
|---|---|---|---|---|
| **Analytes** | **Precursor ions (m/z)** | **Product ions (m/z)** | **Cut-Off values (m/z)** | **Fragmentation voltage (V)** |
| DA | 154.0 [**DA**+H]$^+$ | 137.0 | 50 | 0.10 |
| D/L-NE | 152.0 [**NE**-H$_2$O+H]$^+$ | 134.9 | 60 | 0.20 |
| D/L-EP | 184.0 [**EP**+H]$^+$ | 165.9 | 100 | 0.15 |
| D/L-DOPA | 198.0 [**DOPA**+H]$^+$ | 151.9 / 180.8 | 47 | 0.10 |
| D/L-Phe | 165.9 [**Phe**+H]$^+$ | 120.0 | 60 | 0.12 |
| D/L-Tyr | 181.9 [**Tyr**+H]$^+$ | 135.9 / 164.9 | 42 | 0.09 |
| | | | | |
| Parameters common for all studied compounds | | | | |
| Fragmentation time | 80 ms | | | |
| Scan mode | Enhanced resolution | | | |
| Maximum accumulation time | 200 ms | | | |
| Average number of scans | 1 | | | |
| Ion charge control target value | 100.000 | | | |

**Table 2.** Analytical characteristics of the developed CE-ESI-MS$^2$ methodology.

| | DA | L-NE | L-EP | L-DOPA | L-Phe | L-Tyr |
|---|---|---|---|---|---|---|
| **Internal standard method [a]** | | | | | | |
| *Intercept $\pm t \; x \; s_{intercept}$* | $(6 \pm 50)\cdot10^{-3}$ | $(10 \pm 75)\cdot10^{-3}$ | $(9 \pm 58)\cdot10^{-3}$ | $(7 \pm 61)\cdot10^{-3}$ | $(15 \pm 100)\cdot10^{-3}$ | $(7 \pm 74)\cdot10^{-3}$ |
| *Slope $\pm t \; x \; s_{slope}$* | $1.0 \pm 0.2$ | $1.0 \pm 0.2$ | $1.3 \pm 0.2$ | $1.7 \pm 0.2$ | $1.8 \pm 0.3$ | $1.3 \pm 0.2$ |
| *$R^2$ (%)* | 99.4 | 99.6 | 99.5 | 99.6 | 99.2 | 99.2 |
| | | | | | | |
| **Standard addition method [b]** | | | | | | |
| *Slope $\pm t \; x \; s_{slope}$* | $1.03 \pm 0.03$ | $0.95 \pm 0.02$ | $1.4 \pm 0.2$ | $1.65 \pm 0.09$ | $1.7 \pm 0.2$ | $1.3 \pm 0.1$ |
| *$R^2$ (%)* | 99.96 | 99.994 | 99.1 | 99.8 | 99.7 | 99.7 |
| *Accuracy* | | | | | | |
| **Recovery [c]** | $89 \pm 9$ % | $95 \pm 12$ % | $90 \pm 9$ % | $87 \pm 9$ % | $105 \pm 15$ % | $92 \pm 7$ % |
| *Precision* | | | | | | |
| Instrumental repeatability [d] (n=6) | | | | | | |
| t, RSD (%) | 0.9 | 0.9 | 0.9 | 0.8 | 0.8 | 0.8 |
| $A_c$, RSD (%) | 7.6 | 7.3 | 6.7 | 4.9 | 7.0 | 3.7 |
| Method repeatability [e] (n=6) | | | | | | |
| t,RSD (%) | 1.2 | 1.1 | 1.1 | 1.1 | 0.9 | 1.0 |
| $A_c$, RSD (%) | 10.2 | 8.6 | 13.1 | 12.1 | 9.5 | 3.8 |
| Intermediate precision [f] (n=9) | | | | | | |
| t,RSD (%) | 1.8 | 2.0 | 2.2 | 3.3 | 3.0 | 3.2 |
| $A_c$, RSD (%) | 10.8 | 9.2 | 16.9 | 14.7 | 10.5 | 10.1 |
| *Sensitivity* | | | | | | |
| ***LOD [g] (nM)*** | 77 | 150 | 40 | 54 | 67 | 67 |
| ***LOQ [h] (nM)*** | 257 | 500 | 133 | 180 | 223 | 223 |

a) Five standard solutions at different concentration levels (from 0.3 to 30 µM).

b) Addition of five known (from 0 to 20 µM) amounts of standards to a rat plasma sample.

c) Recovery (average ± standard deviation) obtained from three independent rat plasma sample solutions spiked with known amounts of standards (1.5 µM for DA, L-NE, L-EP and L-DOPA and 5 µM for L-Phe and L-Tyr).

d) Six consecutive injections (n=6) of a rat plasma sample solution spiked with 1.5 µM of DA, L-NE, L-EP and L-DOPA and 5 µM of L-Phe and L-Tyr.

e) Six plasma samples solutions (n=6) from a rat spiked with 1.5 µM of DA, L-NE, L-EP and L-DOPA and 5 µM of L-Phe and L-Tyr, injected in triplicate.

f) Three plasma samples solutions from a rat spiked with 1.5 µM of DA, L-NE, L-EP and L-DOPA and 5 µM of L-Phe and L-Tyr, injected in triplicate in 3 different days (n=9).

g) LOD obtained for a S/N=3.

h) LOQ obtained for a S/N=10.