Targeting Tryptophan and Tyrosine Metabolism by Liquid Chromatography Tandem Mass Spectrometry.

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Abstract

An imbalance in tryptophan (Trp) and tyrosine (Tyr) metabolites is associated with neurological and inflammatory disorders. The accurate and precise measurement of these compounds in biological specimens is a powerful tool to understand the biochemical state in several diseases. In this study, a rapid, accurate and sensitive method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the targeted analysis of the metabolism of Trp and Tyr has been developed and validated. The method allows for the adequate quantification of Trp, Tyr and, eight Trp metabolites, three Tyr metabolites, together with four competitive large neutral aminoacids. Serotonin, 5-hydroxyindoleacetic acid, kynurenine, kynurenic acid, dopamine, and homovanilic acid were among the targeted compounds. Sample preparation, chromatographic separation and mass spectrometric detection were optimized in human urine, human plasma and mice prefrontal cortex extracts. The method was shown to be linear ($r>0.98$) in the range of endogenous concentrations for all studied metabolites. In general, the limits of detection were suitable for the detection of the endogenous levels. Intra- and inter- assay precisions below 25% and accuracies ranging from 80 to 120% were found for most of the analytes. The use of labeled internal standards corrected the moderate matrix effect observed for some compounds. The applicability of the method was confirmed by analyzing urine samples collected from 13 healthy volunteers and comparing the results with previously established normal ranges. In addition, urine samples from two patients and a heterozygous carrier of a family with disturbed monoamine metabolism due to a loss of function mutation in the *MAOA* gene (X-linked) were analyzed and compared with samples from controls. All data together show the potential of the developed approach for targeted metabolomic studies.

Keywords: Tryptophan, tyrosine, kynurenine, metabolites, mass spectrometry, quantitative method

Highlights

- An LC-MS/MS method for the study of tryptophan and tyrosine metabolism is presented
- Concurrent quantification of 4 competitive large neutral aminoacids is offered
- Fully validated in human serum and urine, and in mice prefrontal cortex extracts
- Urinary concentrations for 18 analytes in 13 healthy individuals were calculated
- Applicability proved by analyzing samples from a family with the Brunner syndrome

1. Introduction

The aromatic and essential amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) are converted to catecholamines and serotonin (5-HT) by enzymes in adrenal, intestinal and nervous tissue [1]. Imbalances on these pathways are associated with several neurological and inflammatory disorders.

Trp is an essential amino acid necessary for protein biosynthesis and also the precursor of a large number of biologically active metabolites [2]. Trp is metabolized via several pathways, the major ones being the 5-HT and kynurenine (Kyn) pathways (Figure 1). Trp depletion or the imbalance in its metabolic products can have pathophysiological implications [3-5].

5-HT, is an important neurotransmitter that modulates numerous behavioral and physiological functions such as sleep, mood, appetite, learning, and memory [6]. Recent studies have demonstrated decreased levels of cortical 5-HT in neonatal animals with cerebral palsy induced by maternal-fetal inflammation [7] An elevated urinary concentration of its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), is used as a biochemical test for the diagnosis of a carcinoid tumor [8], as well as for the diagnosis of phenylketonuria, and migraine [9].

The kynerunine pathway is composed of two branches, leading to either the formation of kynurenic acid (KA) and xanthurenic acid (XA) or to the generation of 3-hydroxyanthranilic acid (3OH-AA) and quinolinic acid [10]. Whereas KA, an N-methyl-D-aspartate receptor antagonist, is considered to be neuroprotective [11], the metabolic products of the other branch, including 3OH-AA and quinolinic acid, are considered to be neurotoxic [12]. Imbalances in the kynurenine pathway have been related with several pathological conditions like schizophrenia [13], major depression [14], autism and epilepsy [15], or Alzheimer disease [16].

On the other hand, Tyr is synthesized from the essential amino acid Phe by the action of the enzyme phenylalanine hydroxylase. Plasma Tyr has been proposed as a useful assessment of thyroid function [17]. The main metabolic pathway for Tyr involves its transformation in the neurotransmitter dopamine (DA). DA is subsequently metabolized into several metabolites like 3-methoxytyramine (3Me-Ty) and homovalinic acid (HVA) (Figure 1). Quantification of the major urinary product HVA and others catecholamine metabolites is routinely performed in patients suspected of having neuroblastoma or pheochromocytoma [18].

The rate of brain 5-HT and DA synthesis depends on the Trp and Tyr concentration respectively [19,20], which in turn depends on the ratio between plasma concentration of Trp/Tyr and other large neutral amino acids (LNAAs) that compete for blood-brain barrier (BBB) transport [21]. These LNAAs are Phe, leucine (Leu), isoleucine (Ile), valine (Val) and methionine (Met) [19,22]. It has been proved that, depending on the dietary intake of these amino acids, there can be substantial differences in the plasmatic ratios Trp/LNAAs and Tyr/LNAAs, with subsequent consequences on the catecholamine synthesis in the brain [23].

In summary, the accurate and precise measurement of these compounds in biological specimens is a powerful tool to understand the biochemical state in several diseases. To date, a number of methods for the analysis of Trp and its metabolites have been developed, mostly based on liquid or gas chromatography with various detection modalities, such as UV absorbance, fluorescent, and electrochemical [24-28]. However, if multiple species need to be analyzed with high sensitivity, which is very useful when a small amount of sample is available, then the use of mass spectrometers is preferred [29]. Thus, several LC-MS/MS methods have been recently developed for the detection of Trp and some of its metabolites [30-32], as well as for Tyr and its metabolites [33].

The use of LC-MS/MS instruments allows for the simultaneous quantification of targeted components of different metabolic routes, thus providing a more comprehensive picture of the biological changes in a single analysis. Moreover, these targeted strategies can easily incorporate some other analytes of interest that are not directly involved in the Trp and Tyr metabolic routes, such as the rest of LNAAs. To our knowledge, there is not any published method for the simultaneous determination of the complete panel of Trp and Tyr metabolites and LNAA competitors.

The first goal of the present study was to develop and validate a suitable method for the simultaneous quantification of Trp, Tyr, their metabolites, and their LNAA competitors in three different biological matrices; human urine, human plasma and murine prefrontal cortex (PFC) homogenates. The second aim was to demonstrate the clinical application of this targeted metabolomic approach in urine samples. For this purpose, a cohort of healthy individuals (n=13) was analyzed and the values obtained with the present method compared with the previously reported. Additionally, spot urine samples from three individuals from a family with the Brunner syndrome**,** a rare X-linked genetic disorder caused by a mutation in the Monoamineoxidase A (*MAOA*) gene that leads to an excess of monoamines in the brain [34,35], were analyzed and the results compared with spot urine samples collected from a control group.

2. Material and methods

2.1. Chemicals and reagents

Phenylalanine (Phe), valine (Val), leucine (Leu), isoleucine (Ile), methionine (Met), tryptophan (Trp), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), kynurenine (Kyn), 3-hydroxykynurenine (3OH-Kyn), anthranilic acid (AA), 3-hydroxyanthranilic acid (3OH-AA), kynurenic acid (KA), xanthurenic acid (XA), tyrosine (Tyr), dopamine (DA), 3-methoxytyramine (3Me-Ty), homovalinic acid (HVA), ammonium formate (HPLC grade), EDTA and other reagents for tissue purification were purchased from Sigma-Aldrich (St Louis, MO, USA). Dopamine-d_4 (DA-d_4), kynurenic acid-d_5 (KA-d_5), phenylalanine-d_5 (Phe-d_5) and serotonin-d_5 (5HT-d_5) were supplied by Toronto Research Chemicals (Toronto, Canada). Tyrosine-d_4 (Tyr-d_4), tryptophan-d_5 (Trp-d_5), 3-methoxytyramine-d_4 (3Me-Ty-d_4), 5-HIAA-d_4, 3OH-Kyn-$^{13}C_6$, and Kyn-$^{13}C_6$ were from Alsachim (Illkirch-Graffenstaden, France). Formic acid (LC/MS grade), acetonitrile and methanol (LC gradient grade) were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain). For structures of the analytes see Figure **1**.

2.2. Instrumentation

We used an Acquity UPLC system, (Waters Associates) for the chromatographic separation coupled to a triple quadrupole (Quattro Premier XE) mass spectrometer provided with an orthogonal Z-spray-electrospray interface (ESI) (Waters Associates, Milford, MA, USA). The drying and nebulising gas was nitrogen. The desolvation gas flow was set to approximately 1200 L h^{-1} and the cone gas flow to 50 L h^{-1}. A capillary voltage of 3 kV was used in positive ionization mode. The nitrogen desolvation

158 temperature was set to 450ºC and the source temperature to 120ºC. Collision gas was
159 argon at a flow of 0.21 ml/min. The injection volume was 10 µl.

160 The LC separation was performed at 55 ºC using an Acquity BEH C_{18} column (100 mm,
161 2.1 mm i.d., 1.7 µm) (Waters Associates), at a flow rate of 300 µL min^{-1}. Water and
162 methanol both with formic acid (0.01% v/v) and ammonium formate (1mM) were
163 selected as mobile phase solvents. A gradient program was used for the separation of the
164 analytes; the percentage of (B) solvent was linearly changed as follows: 0 min, 1%; 0.5
165 min, 1%; 7 min, 40%; 8.5 min, 90%; 9 min, 90%; 9.5 min, 1%; 12 min, 1%. The analysis
166 was performed in a total run time of 12 min.

167 Analytes were determined by a Selected Reaction Monitoring (SRM) method by
168 acquiring two transitions for each compound (**Table 1**). The most specific transition was
169 selected for quantitative purposes. MassLynx software was used for data management.

170

171 **2.3. Biological samples**

172 *2.3.1. Murine prefrontal cortex homogenates*

173 Four male and five female CD1 outbreed mice purchased from Charles River (Barcelona,
174 Spain) and shipped to our animal facility (Ubiomex, PRBB) were used for this study.
175 Animals were 10-15 weeks old at the start of the study. Mice were housed in standard
176 cages in a temperature (21°± 1°C), humidity (55% ± 10%), and light-cycle controlled
177 room. Food and water were available *ad libitum*. Male and female mice were allowed to
178 acclimatize to the new environmental conditions for at least one week before starting the
179 experiments. To obtain the brain samples, mice were sacrificed by decapitation and brains
180 were rapidly removed. Prefrontal cortex area (PFC) was dissected using a brain tissue
181 blocker. All samples were immediately frozen in dry ice and stored at -80ºC. Every effort

was made to minimize the number of animals. . All procedures were conducted in accordance with the guidelines of the European Community, Directive 2010-63EU and BOE-2013-1337 regulating animal research and approved by the local ethical committee (CEEA-PRBB).

2.3.2. Human urine sample collection

Spot urine samples collected from six healthy volunteers were used for the validation of the method. In order to evaluate the applicability of the developed method, 24h urine samples (complete amount of urine excreted in one day) collected from 13 healthy Caucasian volunteers (8 males, 26-41 years-old and 5 females, 23-67 years-old) were analysed. Results were compared with normal ranges previously reported.

Spot urine samples from three recently described individuals (two affected brothers and the carrier mother) with documented loss of function mutations in the X-linked *MAOA* gene were obtained [36]. The older brother had a diagnosis of pervasive developmental disorder not otherwise specified while the younger fulfilled criteria for autism with moderate intellectual disability and abnormal and impulsive behavior. The mother (mutation carrier) presented with a depressive disorder and there was a history of psychiatric disease in other unavailable maternal relatives, consistent with X-linked pattern of inheritance. Results were compared with spot urines collected from 30 healthy volunteers. Additional clinical studies (performed at the clinical laboratories, Hospital del Mar) included levels of normetanephrine, serotonine and related metabolites (vanilmandelic acid, HVA and 5-HIAA) in blood and 24h urine samples. Both brothers had a documented disturbance of the catecholamine and serotonin metabolism with high

levels of 5-HT (2x), and normetanephrine (2,4x) as well as other catecholamine metabolites in blood and urine, milder in their mother.

Urinary concentrations of creatinine were determined by standard methods using ADVIA 2400 equipment (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA).

2.3.3 Human plasma collection

Human plasma samples used for method validation were obtained by arm venipuncture from six healthy volunteers at the hospital facilities. Plasma samples were collected in tubes containing EDTA as anticoagulant.

2.4. Sample preparation

2.4.1. Internal standard mixture

An internal standard mixture containing 1000 ng/mL of 5HIAA-d_4, 500 ng/mL of Tyr-d_4, 500 ng/mL of 3OH-Kyn-$^{13}C_6$, 500ng/mL of Trp-d_5, 500 ng/mL of Phe-d_5, 500 ng/mL of 5HT-d_5, 100 ng/mL of Kyn-$^{13}C_6$, 100 ng/mL of DA-d_4, 50 ng/mL of 3Me-Ty-d_4 and 50 ng/mL of KA-d_5 was prepared by dissolving the appropriate amounts of the stock solutions in a mixture methanol:ascorbic acid 20mM (1:1; v:v). The same internal standard mixture was used for all determinations.

2.4.2. Urine

An aliquot of 100 µL of urine was 10 fold diluted with water. After mixing, 75 µL of the internal standard mixture were added together with 75 µL of the diluted extract into the injection vial, and10 µL were injected into the system.

2.4.3. Plasma

An aliquot of 150 µL of plasma was mixed with 300 µL of acetonitrile in order to precipitate the proteins. After centrifugation, the supernatant was transferred to a clean tube and 50 µL of the internal standard mixture were added. The mixture was evaporated at room temperature under nitrogen stream (<10 psi). After reconstitution with 150 µL of water, 10 µL were injected into the system. The standards used for calibration were subjected to the sample procedure.

2.4.4. PFC homogenates

For the tissue processing, after adding 500 µl of ice-cold buffer (0.5 mN sodium metabisulfate, 0.2 N perchloric acid and 0.5 mM EDTA), the tissue (15,36 ± 0,68 mg) was homogenized using a sonicator [37]. Then, samples were centrifuged 10 min (10.000 g at 4ºC) and the supernatant was kept on ice until analysis.

Two alternative treatments were tested in the analysis of PFC homogenates. On one hand, 75 µL of the PFC extract was mixed with 75 µL of the internal standard mixture and 10 µL of the diluted extract were injected into the system. On the other hand, 75 µL of the internal standard mixture was added into 400 µL of the PFC extract. The mixture was evaporated at room temperature under nitrogen stream (<10 psi). After reconstitution with 75 µL of water, 10 µL were injected into the system. All the analytes were detected by the first method except for KYN, 3OH-KYN, AA and 3Me-Ty for which the preconcentration step was necessary. The standards used for calibration were subjected to the sample procedure.

2.5. Method validation

2.5.1. Linearity

The samples selected for validation were analyzed using the described sample treatment and their concentration estimated against a calibration curve made in water. Calibration standards at seven concentration levels were prepared in ultrapure water for each analyte. The calibration range depended on the basal concentrations expected for the analytes in the matrices tested. Calibration standards were analyzed and calibration curves were calculated by least-squares linear regression. The method was considered linear in the selected range if a correlation coefficient (r) higher than 0.98 was obtained.

2.5.2. Intra-assay accuracy and precision

Precision and accuracy of the method were checked by analyzing quality control samples (QCs). QCs (n=6) were prepared by spiking real samples at the second-to-lowest concentration level of the calibration curve. Six different samples were used for this purpose. Since selected analytes are endogenous compounds ubiquitously present in the selected matrices, QCs were always analyzed together with their unspiked extract. The spike concentration was calculated by subtracting the concentration obtained for the unspiked sample to the one of the QC.

Intra-assay precision of the method is expressed as the relative standard deviation (%) of the estimated concentrations obtained for the six QCs analyzed in one assay. Intra-assay accuracy is expressed as the recovery value (%) estimated for the QCs.

2.5.3. Inter-assay accuracy and precision

Inter-assay precision is given as the relative standard deviation (%) of the estimated concentrations obtained for all replicates analyzed along the three validation assays and by two different operators (n=12). Inter-assay accuracy is obtained as described for intra-assay accuracy but considering the replicates analyzed in the three analytical batches (n=12).

2.5.4 Limit of detection (LOD)

The LOD, defined as the lowest concentrations with a value of the signal/noise ratio (S/N) of 3, was calculated by estimating the S/N from the chromatogram at the lowest concentration assayed. Establishing LODs for compounds that are endogenously present at large concentrations is of little interest. Therefore, for those compounds showing a S/N higher than 50 at the lowest calibration level, their LODs were fixed at 1/10 of the lowest calibration level.

2.5.5 Matrix effect

Due to the impossibility to obtain real blank samples, the estimation of the matrix effect was calculated by comparison between the response of the analyte in the QC (after subtraction of the response for the blank sample) and the one of a solvent standard at the same concentration. No correction of the internal standard was made for the calculation of the matrix effect.

3. Results and discussion

3.1. Method optimization

3.1.1. Sample preparation optimization

Sample procedure was optimized in order to detect the endogenous levels of the analytes performing the minimum number of treatment steps. Dilute-and-shoot based approaches are the option of choice for this purpose [38]. Thus, the use of derivatization agents, employed in other validated methods for enhancing the sensitivity, was discarded [32].

In the case of urine, a twenty fold dilution of the sample was adequate for the appropriate quantification of all analytes. This dilution also improved the chromatographic behavior of very polar compounds such as DA, which was found to be substantially altered by the presence of urinary components (Figure 2a and 2b).

Sample treatment for plasma samples involved a simple protein precipitation step, evaporation and reconstitution. The evaporation step was needed in order to eliminate the excess of acetonitrile that will disturb the chromatographic behavior of the most polar metabolites. The evaporation step was performed at room temperature under low nitrogen pressure (< 10 psi) in order to minimize the losses by evaporation. In any case, the addition of isotope-labeled internal standards corrected for any eventual evaporation of the analytes. The method for plasma samples did not involve any dilution or concentration factor. Under these conditions, analytes from all selected pathways were detected except for DA and their metabolites that are present in plasma at trace levels [39]. Some metabolites of the kynurenine pathway (XA and 3OH-AA) remained also undetectable.

In the case of PFC homogenates, a two-fold dilution of the sample allowed for the detection of the aminoacids, 5-HT, 5-HIAA and DA. In order to have information about the kynurenine pathway, a preconcentration step (five fold concentration factor) was

necessary. This concentration factor was achieved by evaporation at room temperature under nitrogen stream (<10 psi).

3.1.2. Liquid chromatographic and mass spectrometric optimization

The high polarity of the selected analytes hampers their retention in C18 columns. In the interestof maximizing this retention, methanol was preferred as organic solvent. In order to obtain suitable retention a 10 cm column was used and the chromatographic gradient started with a high percentage of aqueous solvent (99%). As explained below, the addition of ammonium in the mobile phase was found to be necessary for the proper ionization of HVA.

Most of the analytes are nitrogen-containing molecules and therefore are easily ionizable as $[M+H]^+$ (Table 1). Most of the quantitative transitions have the protonated ion as the precursor ion. The only exception was HVA. The absence of a nitrogen atom in this molecule hampered its detection in positive ionization mode. In fact, it could not be detected when formic acid was used as the only modifier in the mobile phase. However, the addition of ammonium in the mobile phase made possible the formation of the $[M+NH_4]^+$. The most specific and sensitive transitions for HVA were obtained by selecting the $[M+NH_4\text{-}NH_3]^+$ as precursor ion.

Two transitions were optimized for each analyte (Table 1). The most sensitive and specific one was used for quantification, the second one for qualitative purposes. All the potential precursor ions of each analyte were fragmented at several collision energies (from 5eV to 50 eV) and the resulting transitions were tested in terms of specificity and sensitivity. In general, the most abundant transitions were specific enough for the detection of the analytes at the endogenous levels. The main exception was found in the

detection of Leu and Ile. Both analytes shared the transition 132 →86 (coming from the neutral loss of HCOOH), this fact together with their similar retention times made the quantification of both analytes difficult (Figure 2c). The selection of specific transitions (132 →43 for Leu and 132 → 69 for Ile) helped in the proper quantification of both analytes (Figure 2d). Regarding the second transition, in most of the cases the precursor ion for this second transition was the same selected for the first one. However, for some analytes such as 5-HT, 3OH-AA, DA and 3Me-Ty more sensitive results were obtained when $[M+H-NH_3]^+$ or $[M+H-H_2O]^+$ were selected as precursor ions (Table 1).

3.2. Method validation

Due to the fact that electrospray ionisation is particularly prone to matrix effects, and that this effect may notably vary between different types of specimens, the present method has been independently validated in three different biological matrices; human urine, human plasma and homogenates from mouse brain. The use of adequate internal standards is one of the most suitable approaches to compensate this matrix effects. For this reason, the concentration of each analyte was calculated by using the internal standard closely eluted (Table 2).

Since all the analytes are endogenous, they are present in all matrices studied, making impossible obtaining an analyte-free matrix for conducting the validation experiments. The use of artificial matrices for the validation was considered as alternative but the idea was rejected, as these artificial matrices do not contain most of the interferences present in the real ones. For this reason, the calibrators were prepared in ultrapure water, and precision and accuracy were evaluated in real samples by comparison between the concentrations before and after the addition of known amount of the analytes.

368 The linearity range to be tested was established for each analyte depending on their
369 expected concentration in each matrix. For this purpose, five samples of each matrix were
370 analyzed by the optimized method and the concentration for each analyte was estimated.
371 Linearity range and QC concentrations for the accuracy and precision experiments were
372 selected on the basis of these ranges (see experimental section). The linear range obtained
373 for each analyte is depicted in Table 2. Satisfactory correlation coefficients ($r > 0.99$) were
374 obtained for all analytes in the selected range (Table 2). The only exception was 3OH-
375 Kyn in both human urine and plasma where correlation coefficients of $r=0.983$ and
376 $r=0.982$, respectively were calculated.

377 The LOD is not an issue for the determination of compounds present in high amounts. In
378 those cases where the S/N at the lowest calibration level was higher than 50, the LOD
379 was arbitrarily fixed at 1/10 of the lowest calibration level assayed. That was the case of
380 aminoacids and Kyn in plasma and Trp, Tyr, Phe, 5-HIAA and KA in urine. For the rest
381 of analytes, the LODs in the three matrices assayed are summarized in Table 2. They
382 range from 0.1 ng mL^{-1} for Kyn, KA and 3Me-Ty in mice PFC homogenates to 1 µg/mL
383 for HVA in urine.
384 Low to moderate matrix effects produced by the components of the different matrices
385 were found. The observed concentration due to either ion suppression or ion enhancement
386 ranged typically from 51% to 131% of the spiked value (Table 3). The most suppressed
387 compounds (50-60% for Val, DA and 5HT) showed short retention times being the co-
388 eluting interferences at these retention times the main potential reason for the
389 suppression. Three main exceptions were found in mice PFC homogenates, where Kyn,
390 3OH-Kyn and KA showed higher ion enhancements, causing an over quantification.
391 Thus, for these three analytes the use of labelled ISTDs, like the ones employed in the
392 present method, is mandatory in order to overcome this problem.

The influence of the different samples on matrix effect in the three selected matrices was evaluated by means of the RSD of the effect observed in six different samples for each type of matrix. RSDs lower than 25% were typically obtained indicating low variation between samples. Met and 5-HT in mice PFC were the only exceptions showing RSD of 30% and 35%, respectively. Once again, the use of labelled ISTDs allowed for the correction of this variation. The low sample volume used for the determination is probably the main reason of the low variation observed, despite the large variability of the matrix components.

Intra-day accuracies ranged from 86 to 117% for all validated compound in the three types of biological specimens. Inter-day accuracies were between 80 and 120%. That confirmed the satisfactory quantitative figures of the developed method.

Regarding precision, the validation results indicate that for the majority of the quantifications the RSDs were below 15%, the only exceptions being Met in PFC, and 3OH-Kyn in urine that showed RSD of 22% and 18% respectively. As expected, the estimation of the inter-day precisions gave RSD values a bit higher, but below the 25% mark for all cases with the only exception of Met in PFC samples which showed a RSD of 35%.

3.3 Method application

3.3.1. Urine samples from healthy individuals

The applicability of the method was tested by the analysis of 24 h urine samples collected from 13 healthy volunteers (8 males and 5 females). Typical chromatograms for a male urine sample are shown in Figure 3. The validated method was able to determine the 18 targeted metabolites and LNAAs in all urine samples (Table 4).

To overcome the possible different diuresis among individuals, clinical urinary determinations are typically normalized either by the urinary concentration of creatinine or by the total volume of urine excreted in a 24 hours period. In order to make our results comparable to the ranges presented by other studies, the results found in this work are presented in both formats (Table 4).

In general, and despite the large variability of published concentrations, results were in agreement with the reported reference values confirming the suitability of the method for the quantification of Trp, Tyr, their metabolites and other LNAAs in human urine samples. It is important to emphasize that these concentrations have been found in healthy adults not taking any type of medications. The urinary concentrations of some Kyn and Tyr metabolites are increased during pregnancy and by contraceptive and corticosteroid hormones [40,41].

3.3.2. Urine samples from MAO-A deficient individuals

The majority of 5-HT is metabolized to 5-HIAA being the flavoprotein MAO (EC 1.4.3.4) the responsible of this transformation. This enzyme is mainly located in mitochondria and catalyses oxidative deamination of several monoamines to their corresponding aldehydes, ammonia and hydrogen peroxide. Two forms of MAO, are known; MAO-A and MAO-B, MAO-A having the highest affinity for 5-HT [42].

Individuals carrying a loss of function mutation in the *MAOA* gene and deficient MAO-A activity do not appropriately metabolize 5-HT, thus leading to abnormal urinary concentration of 5-HT. This build up in the 5-HT production can be easily detected by monitoring two different metabolic precursor/product ratios. As illustrated in Figure 4, in one hand the accumulation of 5-HT leads to an abnormally high ratio when compared to its precursor (Trp). On the other hand, the inability to convert 5-HT to 5-HIAA causes an

abnormal 5-HT/5-HIAA ratio. There was a good correlation of the metabolic abnormality with the genotype and also with the neurobehavioral phenotype, with more severe disturbance in the hemizygous males and milder alteration in the heterozygous female (the mother).

4. Conclusions

An LC-MS/MS method for the simultaneous quantitation of Tyr, Trp, their metabolites and competitive LNAA has been developed and validated. The present method has been independently validated in three different biological matrices; human urine, human plasma and homogenates from murine PFC. These are the specimens commonly available when conducting investigations related to the Trp and Tyr metabolism.

The adequacy of the method for its intended purpose in terms of sensitivity, precision, accuracy and linearity has been proven through the application of a validation protocol.

Regarding human samples, the ranges for a set of healthy individuals have been determined by the new method. The figures obtained for those 18 target compounds are in agreement with previously published data.

The wide coverage of Trp and Tyr metabolism, together with short analysis time, low sample volume, simple sample preparation and satisfactory quantitative results make this method useful for targeted metabolomic studies. From a clinical point of view, a change in the relative ratios of these metabolites can provide important insights in predicting the presence and progression of neuroinflammation in disorders such as cerebral palsy, autism, multiple sclerosis, Alzheimer disease, major depression and schizophrenia.

In addition of being a clinical tool for humans, the adequate results obtained for mice PFC homogenates indicates that the validated methodology can also be used in targeted metabolomic studies with animal models dealing with the biosynthesis and metabolism of Trp and Tyr in the brain [43].

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FIGURE CAPTIONS

Figure 1. Structures of studied analytes involved in the tryptophan and tyrosine pathways. LNAA: Large neutral aminoacids, NTs: Neurotransmitters, Val: valine, Leu: leucine, Ile: isoleucine, Met: methionine, Trp: tryptophan, Phe: phenylalanine, Tyr: tyrosine, KYN: kynurenine, 3OH-KYN: 3-hydroxykynurenine, KA: kynureninc acid, AA: anthranilic acid, 3OH-AA: 3-hydroxyanthranilic acid, XA: xanthuneric acid, 5HT: serotonin, DA: dopamine, 5HIAA: 5-hydroxyindoleacetic acid, 3Me-Ty: 3-methoxytyramine, HVA: homovanillic acid.

Figure 2. LC-MS/MS chromatograms obtained for (a) 5HT and 5HT-d_4 both after 2 fold dilution of the urine, (b) 5HT and 5HT-d_4 both after 10 fold dilution of the urine and (c) Leu and Ile using the most sensitive transition (132→ 86) and the most specific ones (132 → 69 for Ile and 132 → 43 for Leu).

Figure 3. LC-MS/MS chromatograms for a urine sample containing 681 ng/mL of Val, 634 ng/mL of Leu, 843 ng//mL of Ile, 1517 ng/mL of Met, 11447 ng/mL of Trp, 451 ng/mL of 5HT, 14582 ng/mL of 5HIAA, 982 ng/mL of Kyn, 5361 ng/mL of KA, 85 ng/mL of 3OH-Kyn, 604 ng/mL of XA, 10 ng/mL of AA, 346 ng/mL of 3OH-AA, 4516 ng/mL of Phe, 1062 ng/mL of Tyr, 166 ng/mL of DA, 26 ng/mL of 3Me-Ty and 2180 ng/mL of HVA.

Figure 4. Urinary 5HT/5HIAA and 5HT/Trp ratios from a control group (n=30) and three persons carrying mutations in the *MAOA* gene, the two male probands (1 and 2) and their heterozygous mother (3).

654 **Tables**

655 **Table 1**. Molecular weight (MW) and SRM conditions for the selected analytes
656

Compound	MW	Cone voltage (V)	Precursor ion (*m/z*)	Species	Collision energy (eV)	Product ion (*m/z*)
Val	117	15	118	$[M+H]^+$	10	72*
					25	55
Leu	131	15	132	$[M+H]^+$	20	43*
					10	86
Ile	131	15	132	$[M+H]^+$	20	69*
					10	86
Met	149	15	150	$[M+H]^+$	10	104*
					20	56
Trp	204	15	205	$[M+H]^+$	10	188*
					20	146
5HT	176	10	177	$[M+H]^+$	5	160*
		25	160	$[M+H-NH_3]^+$	20	132
5HIAA	191	25	192	$[M+H]^+$	20	146*
					30	91
Kyn	208	15	209	$[M+H]^+$	20	146*
					10	192
3OH-Kyn	224	15	225	$[M+H]^+$	10	208*
					20	162
KA	189	20	190	$[M+H]^+$	20	144*
					10	172
XA	205	20	206	$[M+H]^+$	20	160*
					10	188
AA	119	25	120	$[M+H]^+$	15	92*
					20	65
3OH-AA	153	10	154	$[M+H]^+$	10	136*
		25	136	$[M+H-H_2O]^+$	15	108
Phe	165	15	166	$[M+H]^+$	10	120*
					30	103
Tyr	181	15	182	$[M+H]^+$	10	136*
					20	91
DA	153	15	154	$[M+H]^+$	20	91*
		25	137	$[M+H-NH_3]^+$	20	91
3Me-Ty	167	10	168	$[M+H]^+$	20	91*
		20	151	$[M+H-NH_3]^+$	20	91
HVA	182	20	183	$[M+NH_4-NH_3]^+$	10	137*
Trp-d_5	209	15	210	$[M+H]^+$	10	192

5HT-d_4	180	10	181	$[M+H]^+$	5	164
5HIAA-d_4	195	25	196	$[M+H]^+$	20	150
Kyn-$^{13}C_6$	214	15	215	$[M+H]^+$	20	152
3OH-Kyn-$^{13}C_6$	230	15	231	$[M+H]^+$	10	214
KA-d_5	194	20	195	$[M+H]^+$	20	149
Phe-d_5	170	15	171	$[M+H]^+$	10	125
Tyr-d_4	185	15	186	$[M+H]^+$	10	140
DA-d_4	157	15	158	$[M+H]^+$	20	95
3Me-Ty-d_4	171	10	172	$[M+H]^+$	20	95

* Transition used for quantification

Table 2. Linearity and LOD for the selected analytes in the different matrices

		Human Urine			Human Plasma			Mice PFC		
		Linearity			Linearity			Linearity		
	Internal standard	Range (ng/mL)	r	LOD (ng/mL)	Range (ng/mL)	r	LOD (ng/mL)	Range [(a)] (ng/mL)	r	LOD [(a)] (ng/mL)
Val	Tyr-d_4	50-5000	0.998	10	1000-30000	0.992	100**	10-1000	0.998	1
Leu	Tyr-d_4	50-5000	0.993	25	1000-30000	0.994	100**	10-1000	0.999	5
Ile	Tyr-d_4	250-25000	0.996	25	1000-30000	0.993	100**	10-1000	0.998	5
Met	Tyr-d_4	50-5000	0.998	50	1000-30000	0.998	100**	10-1000	0.997	5
Trp	Trp-d_5	250-25000	0.993	25**	1000-30000	0.995	100**	10-1000	0.996	2
5HT	5HT-d_4	10-500	0.995	10	2-200	0.997	0.5	1-25	0.995	0.5
5HIAA	5HIAA-d_4	250-25000	0.991	25**	2-200	0.997	1	0.25-25	0.993	0.1
KYN	Kyn-$^{13}C_6$	50-5000	0.996	50	25-3000	0.995	2.5**	1-25*	0.997	0.5*
3OH-KYN	3OH-Kyn-$^{13}C_6$	25-500	0.983	25	2-200	0.982	2	1-25*	0.995	1*
KA	KA-d_5	1000-100000	0.996	100**	2-200	0.995	1	0.25-25*	0.994	0.1*
XA	Trp-d_5	50-5000	0.998	25	-	-	-	-	-	-
AA	5HIAA-d_4	5-500	0.998	5	0.5-50	0.994	0.3	1-25*	0.996	1*
3OH-AA	Trp-d_5	25-500	0.991	25	-	-	-	-	-	-
Phe	Phe-d_5	250-25000	0.998	25**	1000-30000	0.991	100**	10-1000	0.999	1
Tyr	Tyr-d_4	250-25000	0.998	25**	1000-30000	0.999	100**	10-1000	0.998	2
DA	DA-d_4	10-500	0.993	10	-	-	-	2.5-250	0.997	2
3Me-Ty	3Me-Ty-d_4	5-500	0.997	2.5	-	-	-	0.25-25*	0.996	0.1*
HVA	5HIAA-d_4	1000-25000	0.993	1000	-	-	-	-	-	-

"-" LOD > endogenous levels, * values obtained after preconcentration of the sample, ** LODs arbitrarily set at 1/10 of the lowest calibration point. [(a)] levels referred to mL of mice PFC extract.

Table 3. Accuracy, precision and matrix effect in the different matrices tested.

Analyte	Matrix	Intra-day (n=6)		Inter-day (n=12)		Matrix effect (n=6)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Recovery (%)	RSD (%)
Val	Urine	103	13	95	11	81	11
	Plasma	88	13	80	15	55	8
	PFC	97	4	104	13	51	8
Leu	Urine	95	4	91	7	85	4
	Plasma	92	10	93	11	73	3
	PFC	104	3	110	11	102	4
Ile	Urine	108	2	99	9	100	3
	Plasma	98	12	99	12	77	3
	PFC	111	4	116	7	110	5
Met	Urine	114	6	120	11	131	10
	Plasma	89	6	90	12	72	4
	PFC	117	22	105	35	68	30
Trp	Urine	85	10	82	13	101	7
	Plasma	93	13	86	13	87	4
	PFC	89	3	94	10	80	6
5-HT	Urine	101	6	98	10	87	14
	Plasma	105	6	96	12	59	11
	PFC	91	11	95	24	52	35
5HIAA	Urine	96	5	94	5	96	8
	Plasma	94	5	96	6	94	8
	PFC	83	6	87	17	61	18
Kyn	Urine	100	5	100	6	108	7
	Plasma	107	7	107	7	107	2
	PFC	113	9	116	14	290	11
3OH-Kyn	Urine	94	18	102	18	124	16
	Plasma	104	11	110	11	87	15
	PFC	102	11	98	10	192	12
KA	Urine	96	3	98	4	105	5
	Plasma	106	2	93	17	117	4
	PFC	114	4	108	6	173	6
XA	Urine	102	9	107	12	113	14
	Plasma	-	-	-	-	-	-
	PFC	-	-	-	-	-	-
AA	Urine	100	3	96	7	99	3
	Plasma	92	19	107	21	110	15
	PFC	91	6	86	9	128	8
3OH-AA	Urine	92	10	89	12	84	10
	Plasma	-	-	-	-	-	-
	PFC	-	-	-	-	-	-
Phe	Urine	100	6	106	12	113	12
	Plasma	89	9	92	12	75	6
	PFC	93	4	100	11	91	5
Tyr	Urine	99	5	94	7	91	6
	Plasma	89	11	89	12	72	5
	PFC	86	3	101	23	128	6
DA	Urine	92	10	88	12	55	22
	Plasma	-	-	-	-	-	-
	PFC	99	2	111	12	53	6
3Me-Ty	Urine	103	2	105	3	108	5
	Plasma	-	-	-	-	-	-
	PFC	100	8	101	6	64	17
HVA	Urine	100	8	96	8	97	8
	Plasma	-	-	-	-	-	-
	PFC	-	-	-	-	-	-

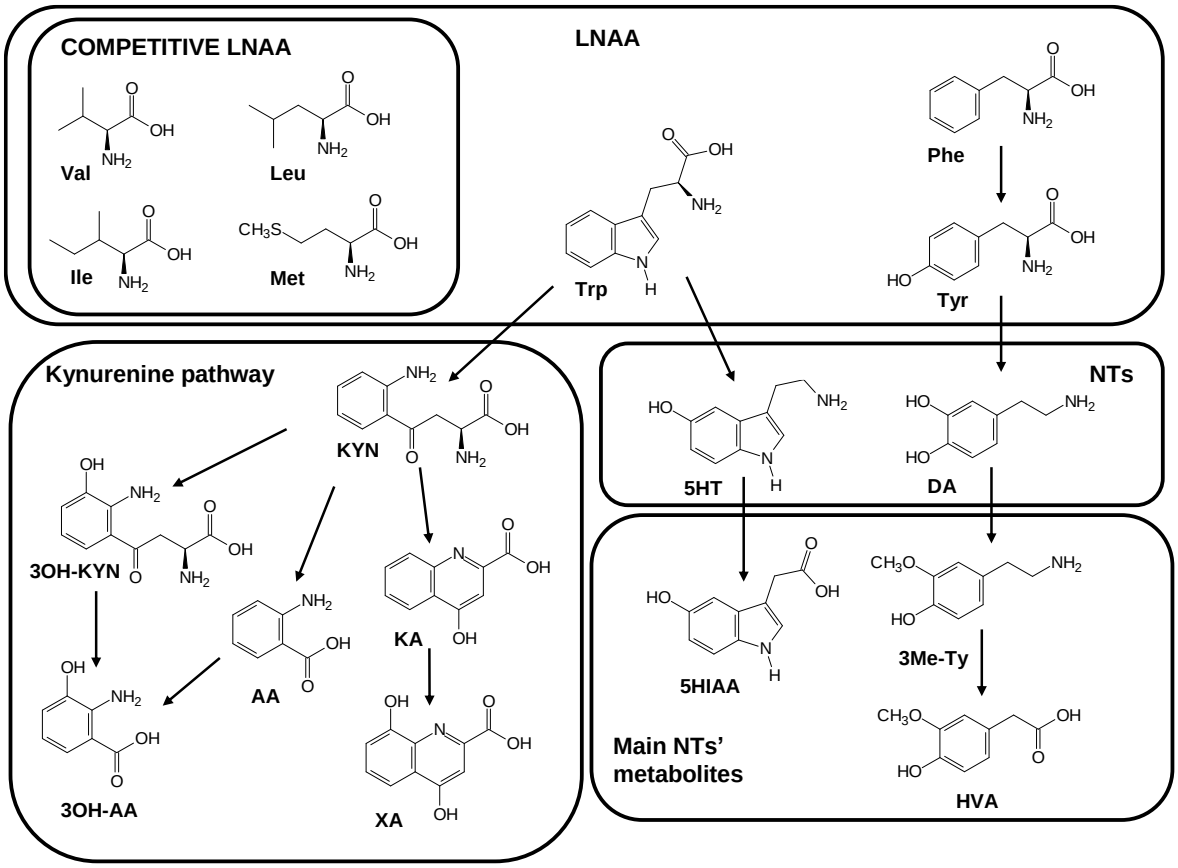
"-" LOD > endogenous levels

668 **Table 4.** Normal ranges obtained in this study in human adult urine compared with those previously reported**.**

	ng/mg creatinine			µg/24h					
	Present study	From references		Present study	From references		Present study	From references	
Analyte	**Range**	**Range**	**Ref.**	**Range**	**Range**	**Ref.**	**Average ± SD**	**Average ± SD**	**Ref.**
Val	1262-3974	1287-12424	[44-46]	1541-3771	-	-	2476±738	-	-
Leu	2986-11858	2318-18548	[44-46]	3204-18773	-	-	8003±4224	-	-
Ile	1460-4154	1158-10433	[44-46]	797-7367	-	-	3296±1633	-	-
Met	147-1797	1318-6592	[44-46]	148-1584	-	-	713±457	-	-
Trp	7483-24858	3060-25274	[44-46]	8755-37307	-	-	16506±8031	8874±2264	[52]
5-HT	44-211	15-366	[47,48]	47-219	60-167	[53]	132±48	733±341	[52]
5-HIAA	2219-7649	169-5696	[48,49]	2627-14399	382-9550	[53]	5388±3081	3800±1031	[52]
Kyn	266-1316	202-1988	[49]	321-1249	-	-	647±274	786±372	[52]
3OH-Kyn	23-433	-	-	17-678	0.4-7.4	[54]	249±177	828±380	[52]
KA	2017-6053	551-1923	[49]	3133-5744	-	-	4213±834	1884±489	[52]
XA	481-1634	-	-	574-2118	-	-	1051±426	565+-315	[52]
AA	3-56	-	-	3-59	-	-	25±17	143±65	[52]
3OH-AA	10-2957	-	-	14-2617	-	-	898±793	820±529	[52]
Phe	3326-13326	2145-18982	[44-46]	4949-15992	-	-	8086±3085	-	-
Tyr	3639-23290	2715-57663	[44-46]	4716-43842	-	-	15698±9931	-	-
DA	48-434	n.d.-812	[50]	35-520	65-400	[44]	248±143	-	-
3Me-Ty	19-80	-	-	26-95	227(mean)		55±23	-	-
HVA	2461-7146	n.d.-16106	[50,41]	4000-8119	0-7280	[51]	1250±8329	-	-

669
670

671 **Figure 1**
672



673
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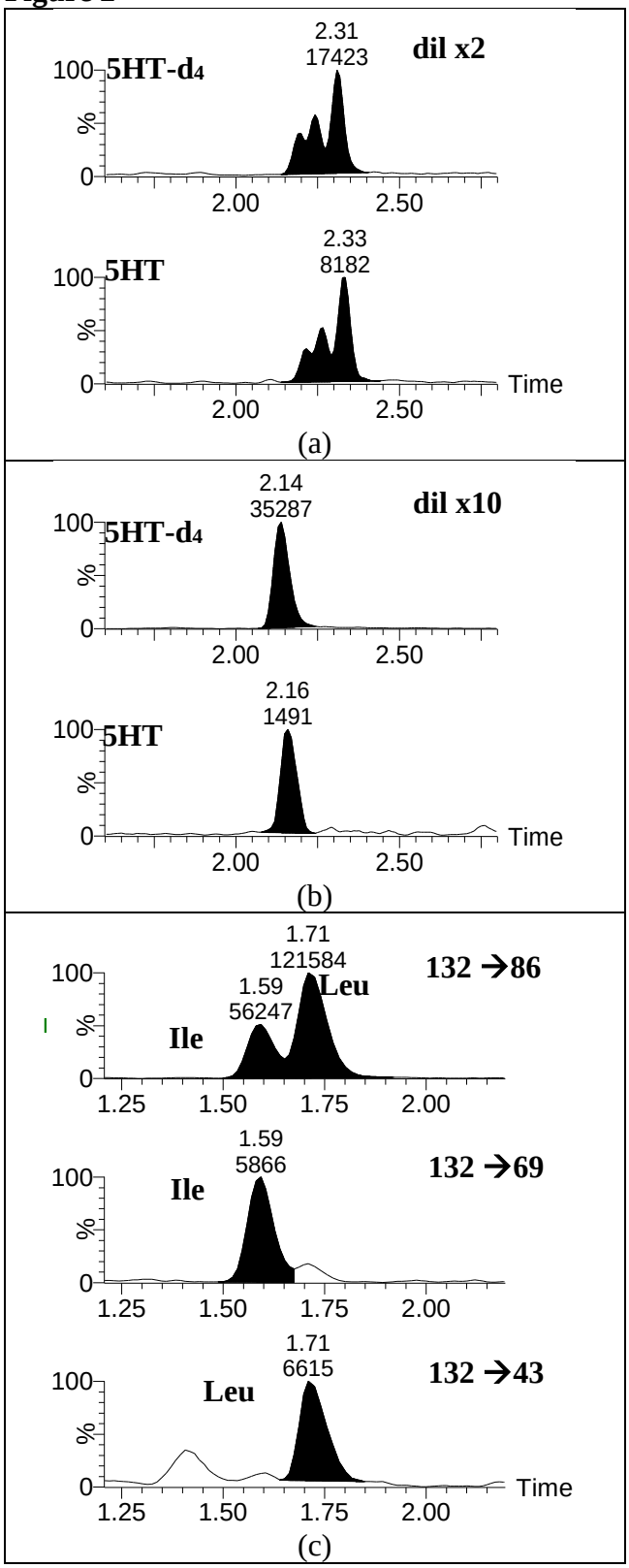
675 **Figure 2**



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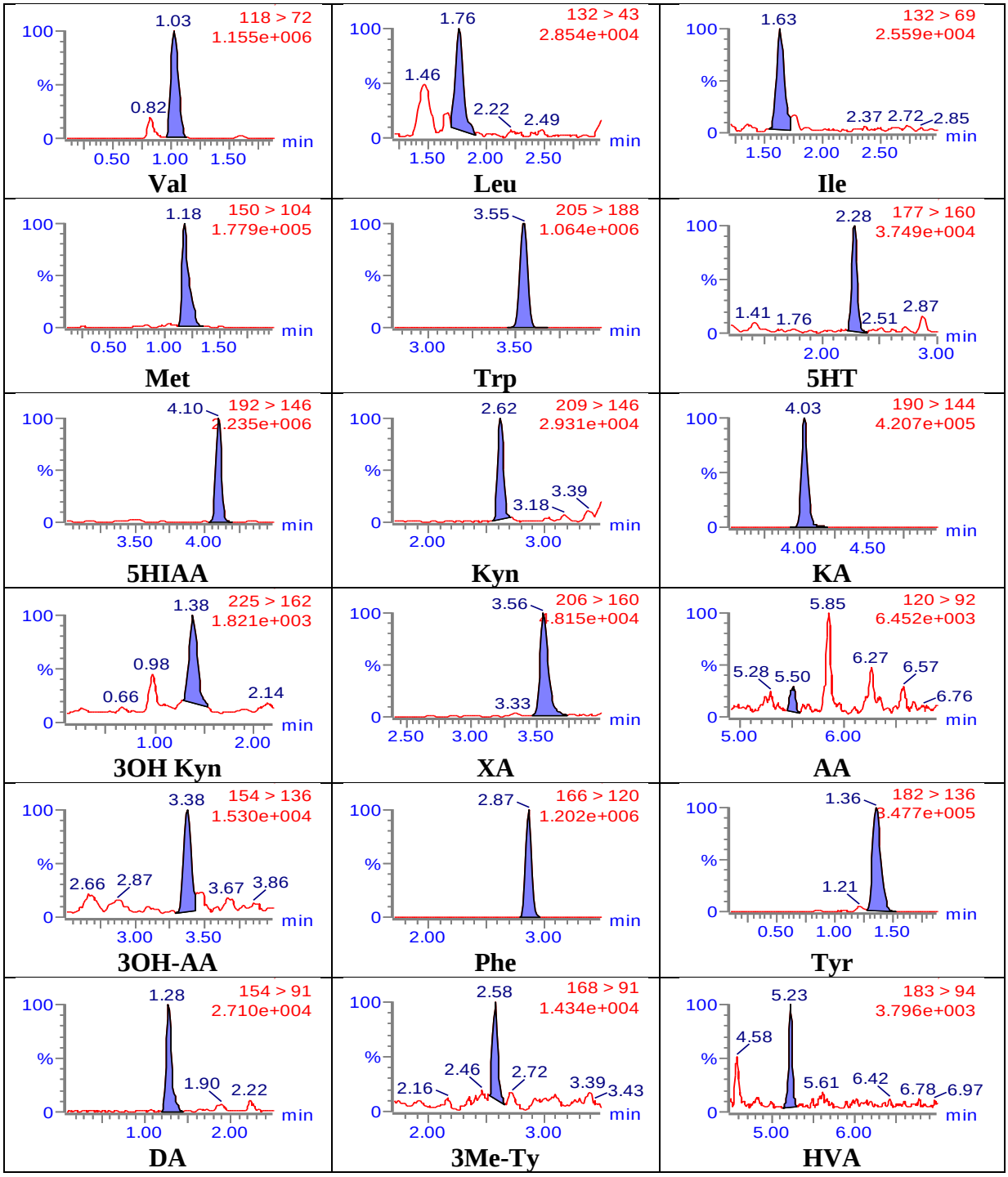
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Leu
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2.559e+004
1.63
2.37
2.72
2.85
Ile
150 > 104
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1.18
Met
205 > 188
1.064e+006
3.55
Trp
177 > 160
3.749e+004
2.28
1.41
1.76
2.51
2.87
5HT
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4.10
5HIAA
209 > 146
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2.62
3.18
3.39
Kyn
190 > 144
4.207e+005
4.03
KA
225 > 162
1.821e+003
1.38
0.98
0.66
2.14
3OH Kyn
206 > 160
3.56
3.33
XA
120 > 92
6.452e+003
5.85
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5.50
6.27
6.57
6.76
AA
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3.38
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2.87
3.67
3.86
3OH-AA
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2.87
Phe
182 > 136
1.36
1.21
Tyr
154 > 91
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1.28
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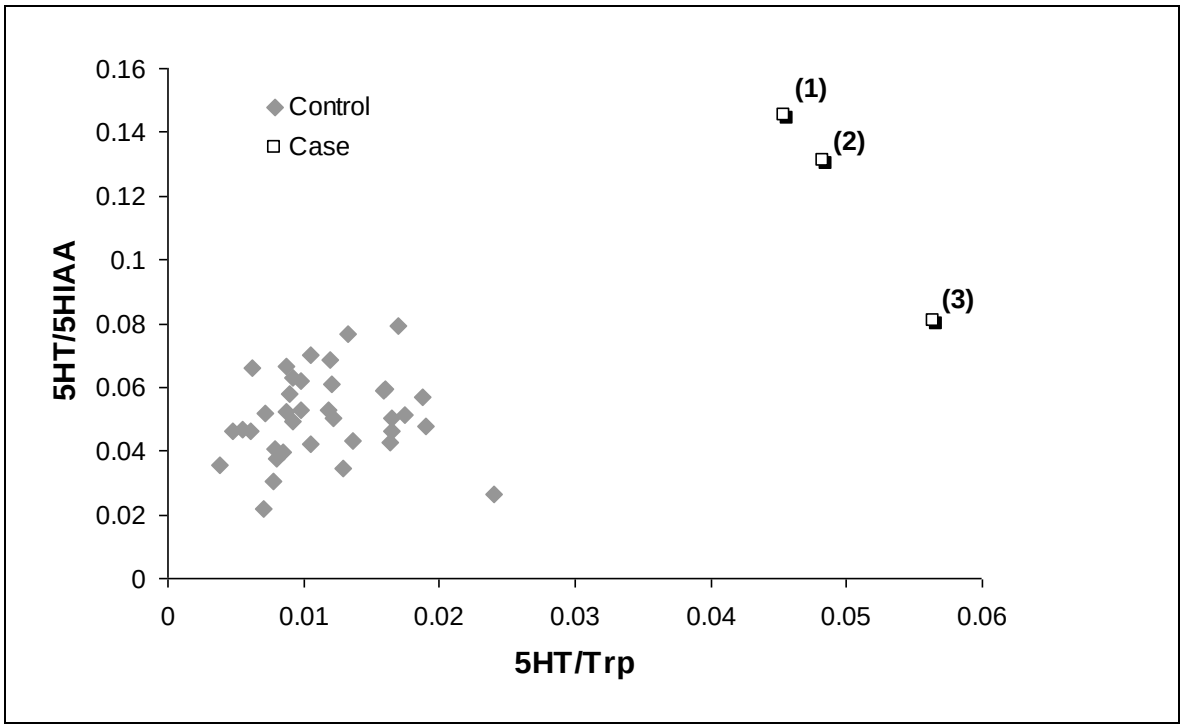
DA
168 > 91
1.434e+004
2.58
2.16
2.46
2.72
3.39
3.43
3Me-Ty
183 > 94
3.796e+003
5.23
4.58
5.61
6.42
6.78
6.97
HVA
100
%
0
min

Figure 3

680 **Figure 4.**

681



682

683