Journal of Chromatography B 1179 (2021) 122846

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

# Improved LC-MS/MS method for the determination of 42 neurologically and metabolically important molecules in urine

Zsolt Galla *, Gábor Rácz, Nóra Grecsó, Ákos Baráth, Magdolna Kósa, Csaba Bereczki, Péter Monostori

*Metabolic and Newborn Screening Laboratory, Department of Paediatrics, University of Szeged*

ARTICLE INFO

*Keywords:*
Kynurenine
Neurotransmitter
Pterin
LC-MS/MS
Urine

ABSTRACT

Simultaneous determination of kynurenines, neurotransmitters, pterins and steroids linked to various neurological and metabolic diseases have important diagnostic significance for related pathology and drug monitoring. An improved, sensitive and selective ultra-high performance liquid chromatography coupled to electrospray ionization triple quadrupole mass spectrometric (UHPLC-MS/MS) method, based on our earlier publication, has been proposed for the quantitative measurement of 42 metabolites in human urine. The assay covers a larger number of analytes, uses an advanced, Waters Atlantis T3 chromatographic column and similarly meets the guideline of European Medicines Agency (EMA) on bioanalytical method validation. Analytical performance met all the EMA requirements and the assay covered the relevant clinical concentrations. Linear correlation coefficients were all $> 0.998$. Intra-day and inter-day accuracy and precision were 87−118%, 81−120% and 2–20%, respectively including the lower limit of quantification (LLOQ). The assay is expected to facilitate the diagnosis and allows drug level monitoring from urine.

## 1. Introduction

Neurotransmitters, kynurenines and pterins play important role for various neurologic, metabolic and psychiatric disorders [1–3]. Concurrent measurement of these metabolites in human serum, cerebrospinal fluid (CSF) and urine helps to diagnose diverse diseases and gives opportunity to better understand differences in metabolic pathways. In addition, one can monitor complementary drug therapies on a broader spectrum. Not only the complementary drug levels can be measured, but the whole metabolic pathway, thus, the activity of metabolizing enzymes can also be examined (for example the determination of kynurenine (KYN)/triptophan (TRP) ratio suggest the indoleamine-2,3-dioxigenase (IDO) and triptophan-2,3-deoxigenase (TDO) activity. Most laboratories use disease-specific methods which contain a few (3 1 0) metabolites [4–8] which make method development easier but limit the usability of the assay.

95% of the tryptophan metabolism results in the formation of kynurenines. Differences in this metabolic pathway may play an important role in the course of diverse neurological diseases [9]. The remaining 5% of tryptophan is metabolized to serotonin and melatonin, which are essential for normal brain function [10]. The metabolic pathway starting from the amino acids phenylalanine and tyrosine (TYR) results in synthesis of several neurotransmitters that play a fundamental role in maintaining brain physiological function.

A quantitative determination of some diagnostically important molecules in human serum and CSF has already been presented in our previous study [11] based on the guideline of EMA on bioanalytical method validation [12]. Using this methodology we now aimed to develop an extended method for measuring urine samples using of an improved chromatographic separation and wider range of analytes. These include metanephrine (METANEP), normetanephrine (NORMETANEP), tyramine (TYRA), octopamine (OCT), phenetylamine (PEA), *N*-methylphenethylamine (NMPEA), quinaldic acid (QAA), picolinic acid (PA), methylserotonin (Me-5HT), dihydroneopterin (NH2), tetrahydrobiopterin (BH4), testosterone (TESTO), creatinine (CRN) and adenosine 3,5-cyclic monophosphate (cAMP). The determination of METANEP and NORMETANEP in addition to homovanillic acid (HVA) and vanillylmandelic acid (VMA) is a key laboratory test for the diagnosis of pheochromocytoma. NORMETANEP level is significantly elevated in patients treated with serotonin-norepinephrine reuptake

* Corresponding author at: Metabolic and Newborn Screening Laboratory, Department of Paediatrics, University of Szeged, Korányi fasor 14-15, H-6720 Szeged, Hungary.
*E-mail address:* galla.zsolt@med.u-szeged.hu (Z. Galla).

https://doi.org/10.1016/j.jchromb.2021.122846
Received 9 February 2021; Received in revised form 14 May 2021; Accepted 21 June 2021
Available online 25 June 2021
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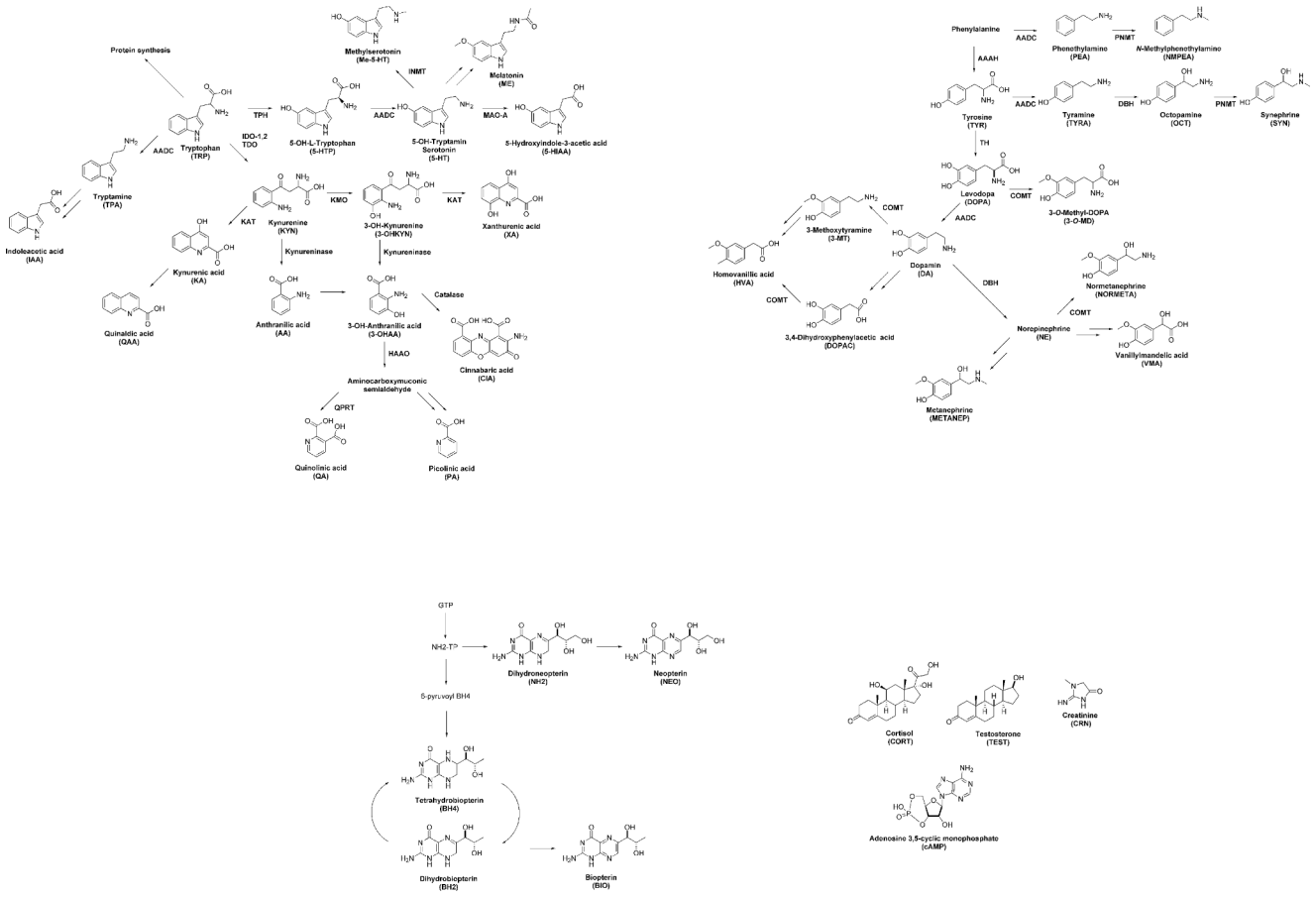


**Fig. 1. Scheme of the 42 metabolites.** TYR: tyrosine; DOPA: levodopa; 3-*O*-MD: 3-*O*-methyldopa; DA: dopamine; 3-MT: 3-methoxytyramine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; VMA: vanillylmandelic acid; METANEP: metanephrine; NORMETANEP: normetanephrine; TYRA: tyramine; OCT: octopamine; SYN: synephrine; PEA: phenethylamine; NMPEA: N-methylphenethylamine; TRP: tryptophan; KYN: kynurenine; 3-OHK: 3-hydroxykynurenine; XA: xanthurenic acid; KA: kynurenic acid; QAA: quinaldic acid; AA: anthranilic acid; 3-OHAA: 3-hydroxyanthranilic acid; CIA: cinnabaric acid; QA: quinolinic acid; PA: picolinic acid; TPA: tryptamine; IAA: indole-3-acetic acid; 5-HTP: 5-hydroxy-tryptophan; 5-HT: serotonin; 5-HIAA: 5-hydroxyindolacetic acid; Me-5HT: methylserotonin; ME: melatonin; NH2: dihydroneopterin; NEO: neopterin; BH4: tetrahydrobiopterin; BH2: dihydrobiopterin; BIO: biopterin; CORT: cortisol; TESTO: testosterone; CRN: creatinine; cAMP: adenosine 3,5-cyclic monophosphate. TPH: tryptophan hydroxylase; AADC: aromatic amino acid decarboxylase; MAO-A: Monoamine oxidase-A; INMT: indolethylamine *N*-methyltransferase; IDO-1: indoleamine-2,3-dioxigenase-1; IDO-2: indoleamine-2,3-dioxigenase-2; TDO: triptophan-2,3-deoxigenase; KMO: kynurenine 3-monooxigenase; KAT: kynurenine aminotransferase; QPRT: quinolinate phosphoribosyl transferase; PNMT: phenylethanolamine *N*-methyltransferase; DBH: dopamine beta-hydroxylase; TH: tyrosine hydroxylase; COMT: catechol *O*-methyltransferase.

inhibitors (SNRI) or tricyclic antidepressants (TCAs) [13,14].

The separate measurement of the individual pterin species (biopterin (BIO), dihydrobiopterin (BH2), BH4, neopterin (NEO) and NH2) could help the differential diagnosis of tetrahydrobiopterin metabolism disorders (autosomal recessive guanosine triphosphate cyclohydrolase I (GTPCH) deficiency, 6-pyruvoyl tetrahydropterin synthase (PTPS) deficiency, dihydropteridine reductase (DHPR) deficiency, pterin-4-α-carbinolamine dehydratase (PCD) deficiency) (Fig. 1) [15,16].

Our aim was to develop a combined method to diagnose several diseases and monitor complementary drug therapies in a single run, such as DOPA (MADOPAR®) or BH4 (KUVAN®).

## 2. Materials and methods

### 2.1. Chemicals and reagents

Unlabelled standards as well as formic acid (FA), ascorbic acid, 1,4-dithioerythritol (DTE), dimethyl sulfoxide (DMSO), Sigmatrix Urine Diluent and methanol (MeOH) were purchased from Sigma (St. Louis, MO, USA) and acetonitrile (ACN) from Merck (Darmstadt, Germany). The solvents were LC-MS grade. The deuterated internal standard (IS) analogues DA-d4, TYR-d4 and DOPA-d3 were purchased from Cambridge Isotope Laboratories (Andover, MA, USA), KA-d5, CRN-d3, CORT-d4, HVA-d5, NORMETANEP-d3 and METANEP-d3 from Sigma, QA-d3 from Buchem BV (Apeldoorn, Netherland) and 5-HT-d4 from Medical Isotopes Inc (Pelham, NH, USA). Steroids depleted serum was purchased from BBI Solutions (Crumlin, UK). Ultrapure water (18.2 MΩ × cm), filtered through a 0.22-μm pore size membrane, was obtained from a Merck Millipore Direct-Q 3 UV system (Billerica, Mass., USA).

### 2.2. Instrumentation

The UHPLC-MS/MS system and MS parameters are the same as previously described [11], with the exception that a Waters Atlantis T3 (2.1 mm × 150 mm, 5 μm) column and an Atlantis T3 VanGuard Cartridge (Waters, Milford, MA, USA) were used for chromatographic analysis.

### 2.3. UHPLC-MS/MS conditions

For tuning, the analytes and IS in 0.2% FA were infused directly into the mass spectrometer and tuned for their molecular transitions in positive and/or negative ESI mode. Various percentages of acidified (0.2% FA) ACN/$H_2O$ and MeOH/$H_2O$ have been tested in order to check

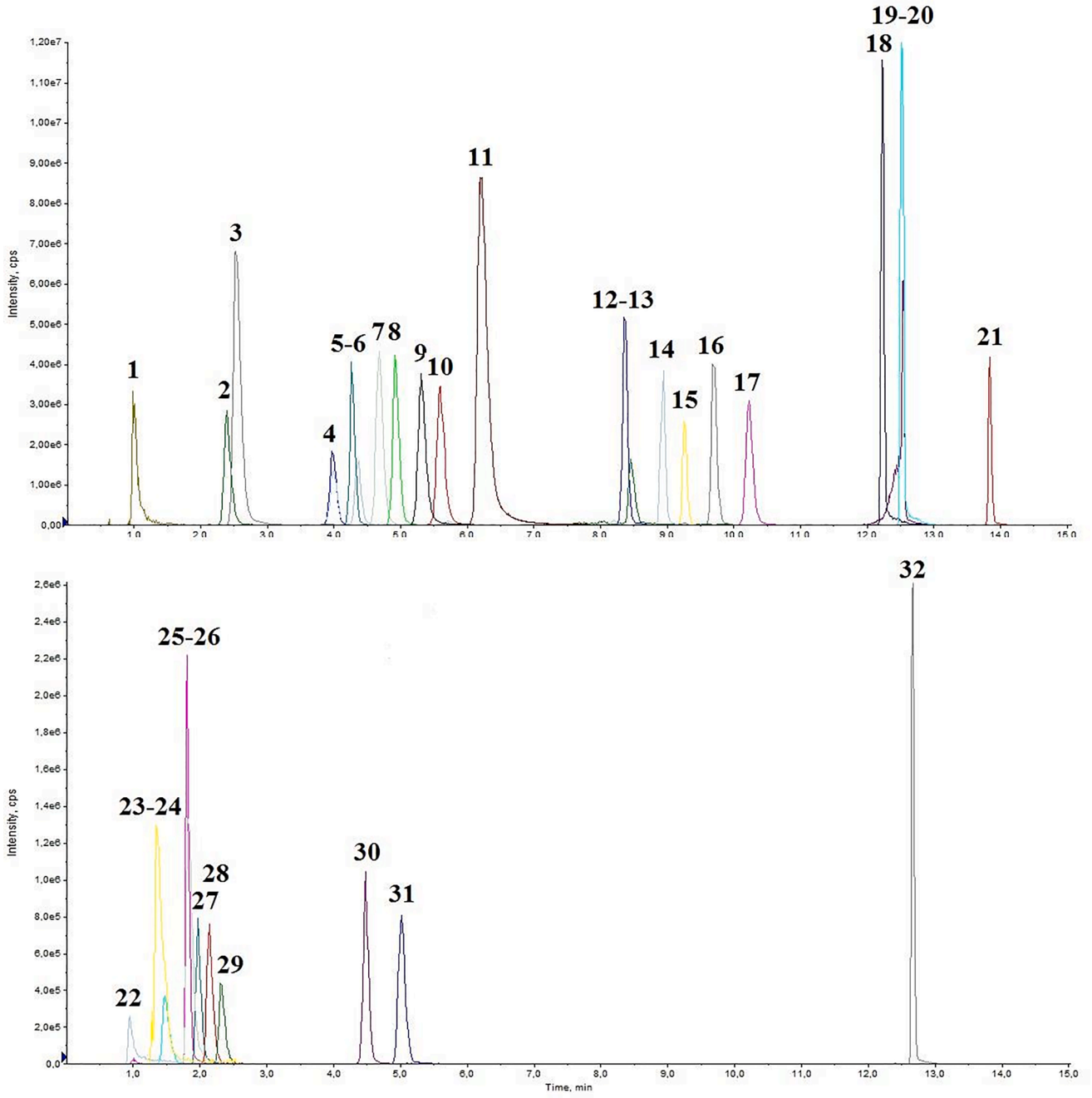


**Fig. 2.** Representative chromatogram of a medium QC spiked human urine, Part 1. 1: OCT $R_t = 0.99$; 2: 3-OHK $R_t = 2.39$; 3: TYRA $R_t = 2.53$; 4: 3-MT $R_t = 3.98$; 5: cAMP $R_t = 4.27$; 6: 5-HT $R_t = 4.36$; 7: KYN $R_t = 4.68$; 8: Me-5HT $R_t = 4.92$; 9: PEA $R_t = 5.31$; 10: 3-OHAA $R_t = 5.59$; 11: NMPEA $R_t = 6.21$; 12: QAA $R_t = 8.37$; 13: TPA $R_t = 8.46$; 14: XA $R_t = 8.94$; 15: KA $R_t = 9.26$; 16: 5–HIAA $R_t = 9.69$; 17: AA $R_t = 10.20$; 18: ME $R_t = 12.20$; 19: CIA $R_t = 12.50$; 20: IAA $R_t = 12.50$; 21: TESTO $R_t = 13.80$; 22: CRN $R_t = 0.94$; 23: PA $R_t = 1.36$; 24: NEO $R_t = 1.47$; 25: DA $R_t = 1.81$; 26: QA $R_t = 1.83$; 27: DOPA $R_t = 1.97$; 28: BH2 $R_t = 2.14$; 29: BIO $R_t = 2.32$; 30: 3-*O*-MD $R_t = 4.47$; 31: 5-HTP $R_t = 5.01$; 32: CORT $R_t = 12.70$.

for molecule-specific ionization.

Mobile phase A consisted of ultrapure water plus 0.2% FA (LC-MS grade). Mobile phase B consisted of ACN plus 0.2% FA (both LC-MS grade). Both eluents were prepared daily. The column temperature was 15 °C, flow rate was kept at 0.6 mL/min and the gradient profile was as follows: 0 – 1 min, 3% B; 1 – 5.5 min, 3% B – 7.5% B; 5.5 – 8.3 min, 7.5% B – 16.5% B; 8.3 – 10 min, 16.5% B – 23% B; 10 – 15 min, 23% B – 95% B; 15 – 16 min, 95% B; 16 min – 17 min 95% B – 3% B; 17 min – 20 min 3% B. The slope of the gradient changes was linear. The injection volume was 15 μL and the injection technique used was 'microliter pickup'. The sample compartment temperature was 10 °C.

### 2.4. Preparation of stock solutions

Stock solutions and IS were prepared individually, according to their solubility and stability. Finally, the solutions were diluted with $H_2O$/ACN/FA/ascorbic acid (96.9/3/0.2/0.02) (Diluting solution, DS) to two different mixtures (URINE MIX and IS MIX) according to their use (**STable 1**, **STable 2**). All standard stocks were stored at −75 °C in brown Eppendorf tubes in light protected containers.

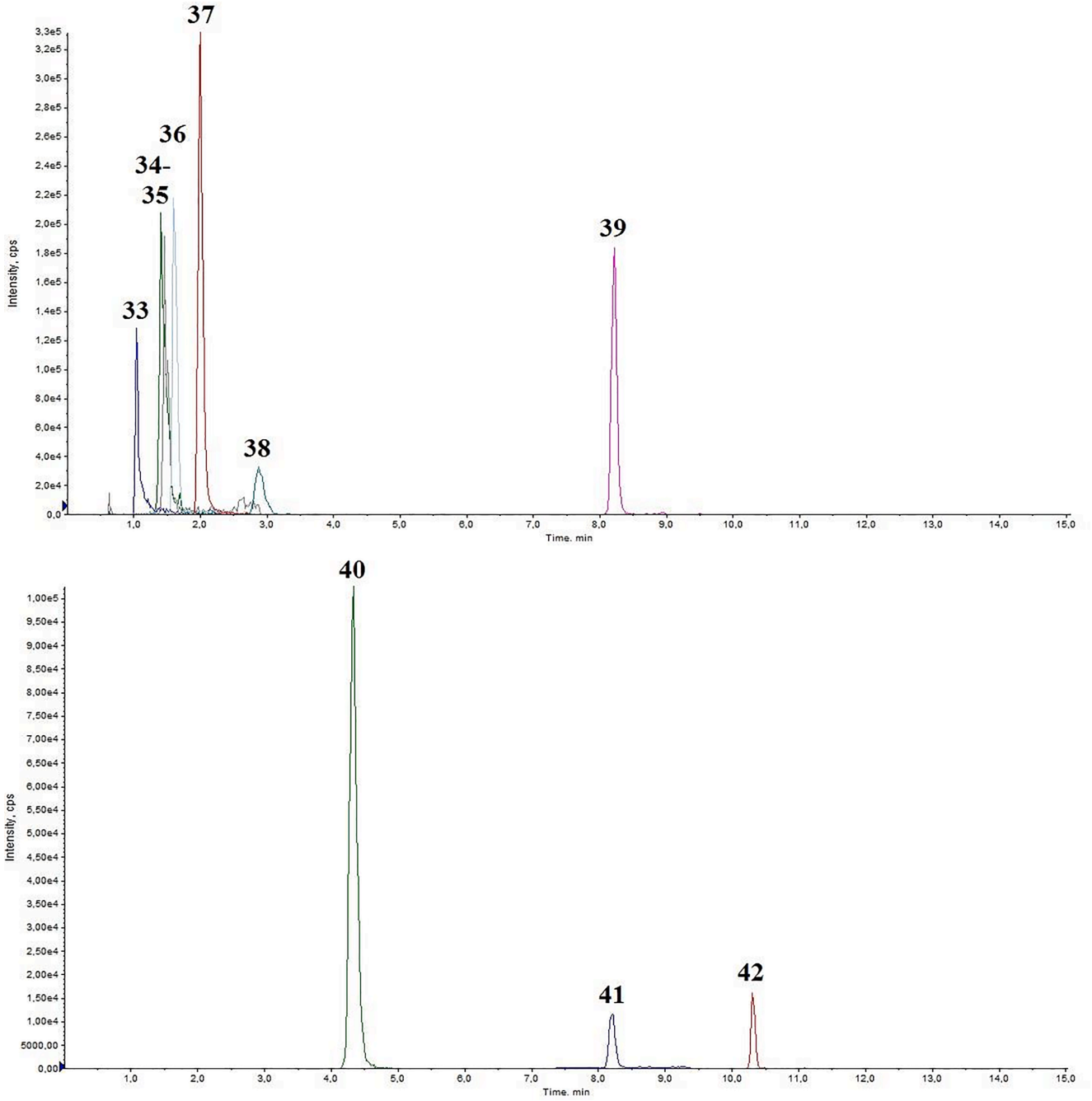


**Fig. 3.** Representative chromatogram of a medium QC spiked human urine, Part 2. 33: BH4 $R_t = 1.04$; 34: NH2 $R_t = 1.41$; 35: NORME $R_t = 1.46$; 36: SYN $R_t = 1.61$; 37: METANEP $R_t = 2.00$; 38: TYR $R_t = 2.88$; 39: TRP $R_t = 8.21$; 40: VMA $R_t = 4.32$; 41: DOPAC $R_t = 8.2$; 42: HVA $R_t = 10.30$.

### *2.5. Preparation of calibrators, quality controls (QC) and IS solutions*

The ranges of calibration standards were chosen to match the expected metabolite levels in human urine samples. The calibrators were prepared in a mixture of Sigmatrix Urine Diluent and 1 mg/mL DTE solution (1:3) and were spiked at nine concentration levels. The QCs were prepared as the calibrators but on different day and with different concentration levels as the calibrators and spiked at four levels of analytes (lower limit of quantitation (LLOQ), 3LLOQ, medium and high concentration).

A mixture of ISs was prepared by appropriate dilution of the stock solutions in DS (IS MIX). The calibrators, QCs and IS solutions were then aliquoted in brown Eppendorf tubes and stored at −75 °C in light protected containers until analysis.

### *2.6. Collection and preparation of samples*

This study was approved by ethical committees (No. 139/2018-SZTE and IV/8004–1/2020/EKU). All patients participated voluntarily and gave their informed consent at the time of admission to the hospital for medical examinations, and for collecting urine samples.

Urine samples from 10 healthy volunteers (30–48 years old) were obtained for the validation of the method. The patient samples were used for diagnosis and medication monitoring in the Metabolic and Newborn Screening Laboratory in Szeged.

The urine samples were placed immediately on ice and centrifuged (1000 × g at 4 °C for 10 min) within 60 min. 90 µL 1.0 mg/mL DTE solution was added to 30 µL urine samples in a brown Eppendorf tube then stored at −75 °C until analysis.

**Table 1**
Creatinine normalized urine metabolite levels of 10 healthy volunteers and 5 treated PTPS patients.

| Analyte | Control (n = 10) Mean ± SD (nmol/mmol CRN) | PTPS patients (n = 5)Mean ± SD (nmol/mmol CRN) |
|---|---|---|
| TYR | 15823 ± 13904 | 28655 ± 15942 |
| DOPA | 26 ± 23 | 19892 ± 24346 |
| 3-*O*-MD | 244 ± 150 | 63675 ± 52304 |
| DA | 254 ± 161 | 24322 ± 25277 |
| 3-MT | 89 ± 71 | 2588 ± 1962 |
| DOPAC | 542 ± 369 | 31809 ± 16209 |
| HVA | 4011 ± 2048 | 52914 ± 18246 |
| VMA | 4006 ± 2294 | 1713 ± 1212 |
| METANEP | 27 ± 18 | 16 ± 9 |
| NORMETANEP | 26 ± 21 | 19 ± 15 |
| TYRA | 331 ± 192 | 26 ± 20 |
| OCT | <LLOQ | <LLOQ |
| SYN | 4 ± 3 | 5 ± 3 |
| PEA | 2 ± 1 | 1 ± 0.5 |
| NMPEA | <LLOQ | <LLOQ |
| TRP | 12783 ± 7648 | 14116 ± 4659 |
| KYN | 747 ± 409 | 788 ± 539 |
| 3-OHK | 176 ± 100 | 256 ± 133 |
| XA | 862 ± 511 | 320 ± 92 |
| KA | 2022 ± 1099 | 624 ± 195 |
| QAA | 16 ± 11 | 6 ± 2 |
| AA | 13 ± 11 | 6 ± 3 |
| 3-OHAA | 727 ± 434 | 475 ± 417 |
| CIA | <LLOQ | <LLOQ |
| QA | 7258 ± 5270 | 5694 ± 2014 |
| PA | 410 ± 300 | 249 ± 117 |
| TPA | 117 ± 84 | 2 ± 1 |
| IAA | 2889 ± 2331 | 3442 ± 962 |
| 5-HTP | 20 ± 11 | 46 ± 31 |
| 5-HT | 110 ± 79 | 50 ± 12 |
| 5-HIAA | 1090 ± 741 | 245 ± 95 |
| Me-5HT | <LLOQ | <LLOQ |
| ME | <LLOQ | <LLOQ |
| NH2 | 1055 ± 708 | 5201 ± 3366 |
| NEO | 302 ± 178 | 928 ± 212 |
| BH4 | 1061 ± 762 | 220 ± 177 |
| BH2 | 525 ± 185 | 264 ± 243 |
| BIO | 110 ± 54 | 107 ± 71 |
| CORT | 13 ± 4 | 25 ± 18 |
| TESTO | 0.8 ± 0.6 | 0.5 ± 0.3 |
| cAMP | 673 ± 352 | 288 ± 101 |

TYR: tyrosine; DOPA: levodopa; 3-*O*-MD: 3-*O*-methyldopa; DA: dopamine; 3-MT: 3-methoxytyramine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; VMA: vanillylmandelic acid; METANEP: metanephrine; NORMETANEP: normetanephrine; TYRA: tyramine; OCT: octopamine; SYN: synephrine; PEA: phenethylamine; NMPEA: N-methylphenethylamine; TRP: tryptophan; KYN: kynurenine; 3-OHK: 3-hydroxykynurenine; XA: xanthurenic acid; KA: kynurenic acid; QAA: quinaldic acid; AA: anthranilic acid; 3-OHAA: 3-hydroxyanthranilic acid; CIA: cinnabaric acid; QA: quinolinic acid; PA: picolinic acid; TPA: tryptamine; IAA: indole-3-acetic acid; 5-HTP: 5-hydroxy-tryptophan; 5-HT: serotonin; 5-HIAA: 5-hydroxyindolacetic acid; Me-5HT: methylserotonin; ME: melatonin; NH2: dihydroneopterin; NEO: neopterin; BH4: tetrahydrobiopterin; BH2: dihydrobiopterin; BIO: biopterin; CORT: cortisol; TESTO: testosterone; CRN: creatinine; cAMP: adenosine 3,5-cyclic monophosphate, PTPS: 6-pyruvoyltetrahydropterin synthase.

To 120 µL of DTE diluted urine 30 µL of IS-MIX was added in a brown 1.5 mL Eppendorf tube. After vortexing for 4 sec, 120 µL was collected in a 96-well round-bottom microtiter plate (Corning 3365; Sigma (St. Louis, MO, USA)), sealed with aluminium foil and shaken for 30 min. Fifteen µL was injected into the UHPLC-MS/MS system.

### 2.7. Validation of the method

For the validation of the urine analysis with UHPLC-MS/MS the following parameters were investigated: linearity, limit of detection (LOD), lower limit of quantitation (LLOQ), calibration curve, carry-over, intra and interday precision and accuracy, matrix effect, selectivity and stability. Recovery has not been calculated since the preparation of the samples contains only dilution steps. The validation process was evaluated according to the EMA guideline on bioanalytical methods [12] and as described in our previous study [11].

## 3. Results & discussion

### 3.1. Sample management

Prolonged light, oxygen or temperature exposure is critical for the stability of the measured analytes [17,18]. For this reason, the urine samples were transported to the laboratory on ice in light protected container after collection. The samples were centrifuged (3000 RPM, 4 °C for 10 min). The supernatants were diluted with 1 mg/mL DTE solution (1:3) then aliquoted and stored at −75 °C until use.

### 3.2. LC-MS/MS optimization

The UHPLC-MS/MS parameters and the preliminary experiments of LC and MS were the same as described previously [11]. The Waters Atlantis dc18 column was also tested here, but the chromatographic challenges set by the addition of further biomarkers and the use of urine specimens could only be addressed by the T3 column. MS parameters, polarities, retention times, related ISs and quantifiers are shown in **STable 3**. The representative chromatograms of medium QC spiked human urine are shown in the Supplementary material (Figs. 2–3).

### 3.3. Method validation

#### 3.3.1. Linearity, LOD, LLOQ and calibration curve.

Linearity, LOD and LLOQ were determined in a mixture of 1 mg/mL DTE, Sigmatrix Urine Diluent and IS MIX (3:1:1) then 15 µL was injected to the UHPLC-MS/MS system. LOD and LLOQ were calculated using the Analyst 1.6.2. software. The LLOQ represented the lowest concentration where the desired accuracy and precision were met. The determination coefficients were excellent ($R^2$: 0.998 – 1.000). The calibration curves contained at least seven calibrator points for each analyte. The results are shown in **STable 4**.

#### 3.3.2. Carry-over

Carry-over was investigated by injecting three different, high-level spiked human urine (prepared from 30 µL urine (0.96 mM creatinine; 3.1 mM creatinine and 5.0 mM creatinine), 30 µL URINE MIX and 90 µL 1.0 mg/mL DTE solution) and analyte-free DS alternately. After each injection, 5 needle wash cycles with DS were used to eliminate carry-over, which was defined as blank samples displaying peaks with a height > 1% of the peak of the corresponding peak. Carry-over was less than 0.5% for all analytes and did not exceed 15% of the value of the area of LLOQ.

#### 3.3.3. Precision and accuracy

Intra-day accuracy and precision was determined by analysing 10 URINE QC samples at four concentration levels in a single run (LLOQ, 3LLOQ, medium and high spiked QCs) while interday accuracy and precision was determined in 5 QC samples at four concentration levels in three different days (**STable 5**). According to EMA regulation [12], accuracies were acceptable when the mean concentrations were within 15% of the nominal values (except for the LLOQ spiked samples where ≤ 20%) and precision was satisfactory when the RSD values ≤ 15% (LLOQ:≤ 20%). All analytes met the requirements.

#### 3.3.4. Matrix effect, recovery and selectivity.

Matrix effects were investigated in two different human urine specimens (QCM_1: CRN 0.96 mM; QCM_2: CRN 3.29 mM), spiked at two levels (medium and high QC) and measured five times in a row. Matrix factor (MF) was calculated for each analyte as the ratio of the analyte/IS

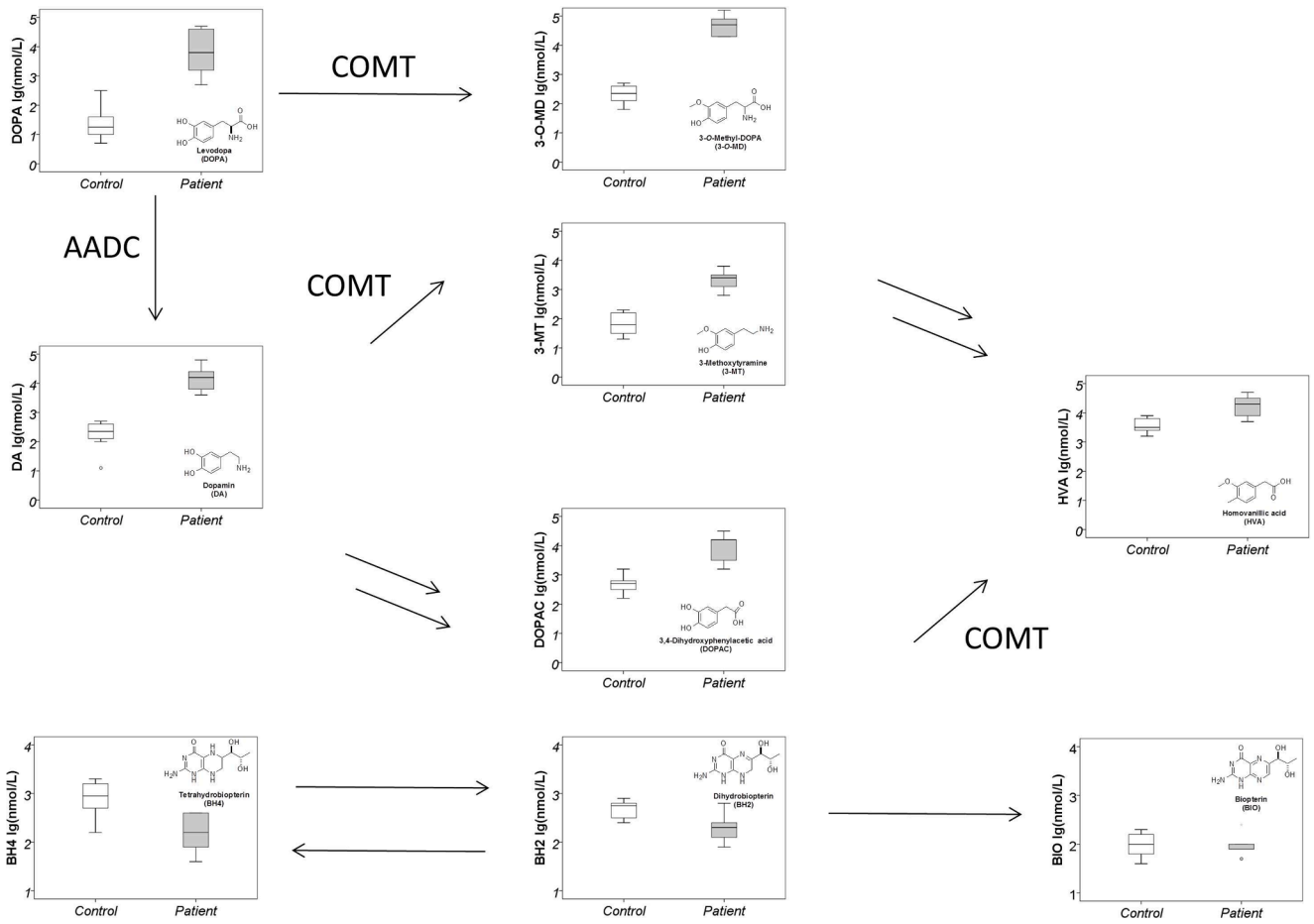


**Fig. 4. Creatinine normalized lg values of DOPA, 3-*O*-MD, DA, DOPAC, 3-MT, HVA, BH4, BH2 and BIO of 5 DOPA and BH4 treated PTPS patients and 10 non-treated healthy patients. Results are presented in an arrangement that corresponds to the biochemical routes.** DOPA: levodopa; 3-*O*-MD: 3-*O*-methyldopa; DA: dopamine; 3-MT: 3-methoxytyramine; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; NH2: dihydroneopterin; NEO: neopterin; BH4: tetrahydrobiopterin; BH2: dihydrobiopterin; BIO: biopterin; AADC: aromatic amino acid decarboxylase; COMT: catechol *O*-methyltransferase.

peak area in the presence of matrix divided by the ratio analyte/IS peak area in post-extraction samples without matrix, multiplied by 100%. MF was regarded as acceptable when the CV of MF $\leq$ 15%. (**STable6**).

No significant endogenous matrix interferences were observed and there were no noticeable co-eluting compounds in the human urine samples.

#### 3.3.5. Stability

The stability of the compounds was investigated at 3LLOQ and in the high QC at different storage conditions in different matrices. The compounds were regarded stable when recovery and accuracy was 100 $\pm$ 20%.

Stock solutions, urine samples were stable at least 24 h at 4 °C and 3 months at −80 °C. Prepared urine samples were stable in the autosampler (10 °C) at least for 24 h. Stability tests after 3 freeze–thaw cycles showed no decomposition.

### 3.4. Evaluation of blank matrix

*The blank matrix (Sigmatrix Urine Diluent) was injected ten times in the UHPLC-MS/MS system. There were no interferences with the measured analytes and the blank matrix except for CRN which is in the Sigmatrix Urine Diluent. Besides that before every measurement 1 blank matrix was injected into the UHPLC-MS/MS system.*

### 3.5. CRN measurement and CRN normalized metabolite levels

In the routine diagnostics, urinary metabolite levels normalized to CRN are more appropriate than quantifying analytes in absolute units (e.g. ng/mL or nmol/L) in compensating differential urinary concentrations [19]. The classical method for the determination of urinary creatinine is the Jaffe's reaction, where creatinine produces quantitatively an orange colour with picric acid in alkaline medium [20]. CRN determined with the same UHPLC-MS/MS method as the measured analytes can shorten sample preparation time.

### 3.6. Application of the method: Healthy human samples and substitution drug therapy monitoring measurements

Of potential options to prove the clinical effectiveness of the method (e.g. diagnosis of BH4 deficiency [21], aromatic L-amino acid decarboxylase deficiency [22], neuroblastoma [23] etc.), therapy monitoring of 6-pyruvoyltetrahydropterin synthase deficiency (PTPS) patients was chosen. Substitution treatment with neurotransmitter precursors such as DOPA and BH4 result in marked elevation of certain analytes (3-*O*-MD, DA, DOPAC, 3-MT, HVA, BH2 and BIO) in the metabolic pathway which makes their analytical determination challenging. Our calibration ranges cover metabolite levels in health and disease for a reliable clinical diagnosis but analyte levels during substitution therapy can be outside of these ranges. Thus, urine samples of patients receiving adjunctive therapy was diluted 10- and 30-fold before the sample preparation. In PTPS due to the malfunction of the 6-pyruvoyltetrahydropterin synthase

enzyme the production of BH4, BH2 and BIO is decreased while NEO and NH2 increased. BH4 is an essential cofactor of vital enzymes (phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, alkylglycerol monooxygenase and nitric oxide synthase) which makes DOPA treatment also necessary. Table 1 contains the CRN normalized urinary levels in ten healthy controls and five DOPA and BH4 threated PTPS patients. Despite the treatment, it was shown that the NEO and NH2 urinary levels in the PTPS patients were still elevated and BH4, BH2 and BIO levels did not reach the values of the control group. Examination of the treatment is shown in Fig. 4, where the logarithmical values of metabolite concentrations are plotted for better comparability.

## 4. Conclusion

An improved, sensitive and selective UHPLC-MS/MS method, based on our earlier publication has been developed and validated for the quantitative measurement of TYR and TRP and their metabolites, pterins and steroids in human urine. The adequacy of the method has been proved through the guideline of EMA on bioanalytical method validation. The new method can be used to extend the quantitative study of metabolic pathways. Using new column and modified gradient allows to measure 42 analytes instead of 30. From a clinical point of view, the method is suitable for making a diagnosis for some metabolic and neurological several diseases and allows drug level monitoring from urine. The suitability of the method for measuring serum and CSF samples should be investigated in the future.

## CRediT authorship contribution statement

**Zsolt Galla:** Conceptualization, Investigation, Methodology, Validation, Formal analysis, Writing - original draft. **Gábor Rácz:** Conceptualization, Resources, Writing - review & editing. **Nóra Grecsó:** Conceptualization, Resources, Writing - review & editing. **Ákos Baráth:** Conceptualization, Resources, Writing - review & editing. **Magdolna Kósa:** Conceptualization, Resources, Writing - review & editing. **Csaba Bereczki:** Resources, Writing - review & editing, Supervision. **Péter Monostori:** Conceptualization, Investigation, Resources, Writing - review & editing, Project administration.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgement

Anita Zádori for the technical assistance in sample preparations. Article Processing Charges were covered by the Institutional Account Program of the University of Szeged, Szeged, Hungary (Open Access Fund, Grant number: 5196). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122846.

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