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Chromatographic resolution and reliable quantification of eleven endogenous and microbiota-derived tryptophan metabolites in human plasma by HPLC–MS/MS

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ABSTRACT

Tryptophan metabolism plays a central role in host–microbiota interactions and immune regulation, yet the simultaneous quantification of metabolites from the kynurenine, serotonin, and indole pathways remains analytically challenging due to their diverse physicochemical properties and wide dynamic concentration ranges. Here, we report an efficient, derivatization-free HPLC–MS/MS workflow for the targeted quantification of eleven key tryptophan-derived metabolites in human plasma. The method employs a biphenyl stationary phase to achieve robust chromatographic resolution of structurally similar analytes, including compounds known to co-elute in C18 stationary phase, improving chromatographic selectivity and minimizing co-elution of structurally related analytes. Sample preparation requires only 50 μL of plasma and relies on a simple protein-precipitation step, allowing high throughput. The method demonstrated strong performance in terms of sensitivity, accuracy, and reproducibility, supported by extensive internal standardization. The combination of multi-pathway coverage, analytical cycle, and suitability for human plasma positions this workflow as a valuable tool for monitoring gut-related metabolic signatures in both research studies and potential clinical applications.

1. Introduction

Tryptophan (TRP) metabolism plays a central role in microbiota–host communication, immune regulation, and neuroimmune signalling [1,2]. Microbial and host-derived TRP metabolites influence intestinal barrier integrity, immune cell activation, oxidative balance, and neurotransmission, making these pathways highly relevant to chronic inflammatory, metabolic, and neurological diseases [3–5]. >90% of dietary TRP is metabolized via the kynurenine (KYN) pathway into metabolites such as kynurenine, 3-hydroxykynurenine (3-HK), and kynurenic acid (KYNA) [6]. These compounds exert neuroprotective or neurotoxic actions, as well as potent immunomodulatory effects mediated by receptors including the aryl hydrocarbon receptor and pregnane X receptor [7–9]. The remaining fraction of TRP is converted into serotonin (SER) or transformed by the gut microbiota into indole derivatives, such as indole-3-acetic acid (IAA), indole-3-lactic acid (ILA), indole-3-carboxaldehyde (I3C), and indoxyl sulfate (INS), which collectively contribute to gut–brain communication and mucosal immune homeostasis [10,11].

Because these metabolites span three interconnected biochemical branches (kynurenine, serotonin, and indole pathways), they offer valuable insights into host–microbiota interactions and disease pathogenesis [12,13]. However, their simultaneous quantification in biological matrices remains analytically challenging. TRP-derived metabolites differ substantially in polarity, aromaticity, ionization efficiency, and physiological abundance, complicating their extraction, chromatographic retention, and peak resolution within a single method. Several HPLC–MS/MS protocols have attempted broad TRP pathway coverage, yet important limitations remain. Eggersten et al. [14]. demonstrated the quantification of selected kynurenine and serotonin pathway metabolites; however, the method was restricted to a limited panel and did not include microbiota-derived indole metabolites, thereby limiting pathway comprehensiveness. Similarly, targeted LC–MS/MS approaches such as those reported by Tömösi et al. [15] focused on a small subset of key metabolites (e.g., TRP, KYN, KYNA), lacking broader pathway integration. More comprehensive methods achieved expanded

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metabolite coverage but relied on complex chromatographic conditions, such as ion pairing, and still faced challenges related to matrix effects and signal variability [16]. Other approaches prioritizing short analysis times enable high throughput but often compromise chromatographic resolution, increasing dependence on MS selectivity [17]. Overall, previously reported methods either target a limited subset of metabolites, require complex chromatographic strategies and rely on derivatization strategies to improve detectability and retention of polar TRP metabolites [18] or rely heavily on MS-based discrimination, highlighting the need for robust methodologies combining broad coverage with effective chromatographic separation.

To design a method compatible with routine and high-throughput applications, the choice of biological matrix is also crucial. Plasma and serum are the most common matrices for clinical targeted metabolomics; however, their biochemical behaviour differs markedly for some TRP metabolites.

In this context, we developed a derivatization-free HPLC–MS/MS workflow for the simultaneous quantification of eleven TRP metabolites spanning the kynurenine, serotonin, and indole pathways (Fig. 1). The method emphasizes chromatographic performance as a central design principle, employing a biphenyl stationary phase that offers superior selectivity for structurally similar aromatic and polar compounds compared with C18 columns. This strategy enabled baseline separation of challenging analyte pairs and minimized reliance on MS-only discrimination. A simple protein-precipitation protocol requiring only 50 µL of plasma was optimized to ensure high sample throughput and compatibility with translational studies. By integrating high chromatographic selectivity, streamlined sample preparation, and a biologically justified matrix choice, this method advances current analytical capabilities and supports clinical and translational investigations into gut health, immune activation, and microbiota–host metabolic interactions.

2. Materials and methods

2.1. Chemicals and reagents

All solvents were of LC–MS grade. Methanol (MeOH), acetonitrile (ACN), dimethyl sulfoxide (DMSO), acetone, and formic acid (FA) were purchased from Fisher Scientific (Segrate, MI, Italy). Ultrapure water was generated using a Milli-Q purification system (Merck Millipore, Darmstadt, Germany) and used for all mobile phases. Unlabelled analytical standards of 3-HK, SER, KYN, KYNA, TRP, INS, TRPA, IAM, IAA, I3C and ILA were obtained from Merck. Stable isotope-labeled internal standards (ISs) included serotonin-d4 hydrochloride (SER-d4), L-tryptophan-d5 (TRP-d5), L-kynurenine-d4 (KYN-d4) (Cayman Chemical, Ann Arbor, MI, USA), and indol-3-acetic acid-d4 (IAA-d4) and kynurenic acid-d5 (KYNA-d5) (Toronto Research Chemicals, Vaughan, ON, Canada).

2.2. Stock solutions and working solutions

Stock solutions were prepared individually at final concentrations of 0.5 mg/mL for 3-HK, 1 mg/mL for SER, KYN and KYNA, and 2 mg/mL for the remaining metabolites. Solvent selection was based on compound stability and solubility: TRPA, KYNA, KYNA-d5, ILA, I3C, IAA and IAA-d4 were dissolved in DMSO; KYN in ethanol; and INS in water. SER was dissolved in ACN; 3-HK and SER-d4 in water–acetonitrile (50:50 v/v); and TRP and TRP-d5 in ACN–water (9:1 v/v) (Suppl. Table 1). Internal standard stock solutions were prepared at concentrations comparable to the corresponding unlabeled metabolites. All stock solutions were pooled, diluted to working solutions immediately before use, and stored at −20 °C for further analyses (Suppl. Table 2). No signs of degradation were observed over a storage period of up to two years under these conditions.

2.3. Preparation of serum/plasma samples

To start, 50 µL of serum/plasma was mixed with 10 µL of working solution containing ISs followed by a brief vortex. Then, 400 µL of ice-cold solution acetonitrile/methanol/acetone (8:1:1 v/v/v) containing formic acid 1% (FA) was added. After vortexing, samples were incubated for 30 min at 4 °C and subsequently centrifuged at 4 °C for 15 min (12,000 g). Then, 325 µL of the surnatant was collected and evaporated to dryness using an Eppendorf 5301 vacuum concentrator (Merck). Dried samples were stored at −80 °C until analysis. Immediately prior to injection, samples were reconstituted in 50 µL of 0.1% formic acid (FA)

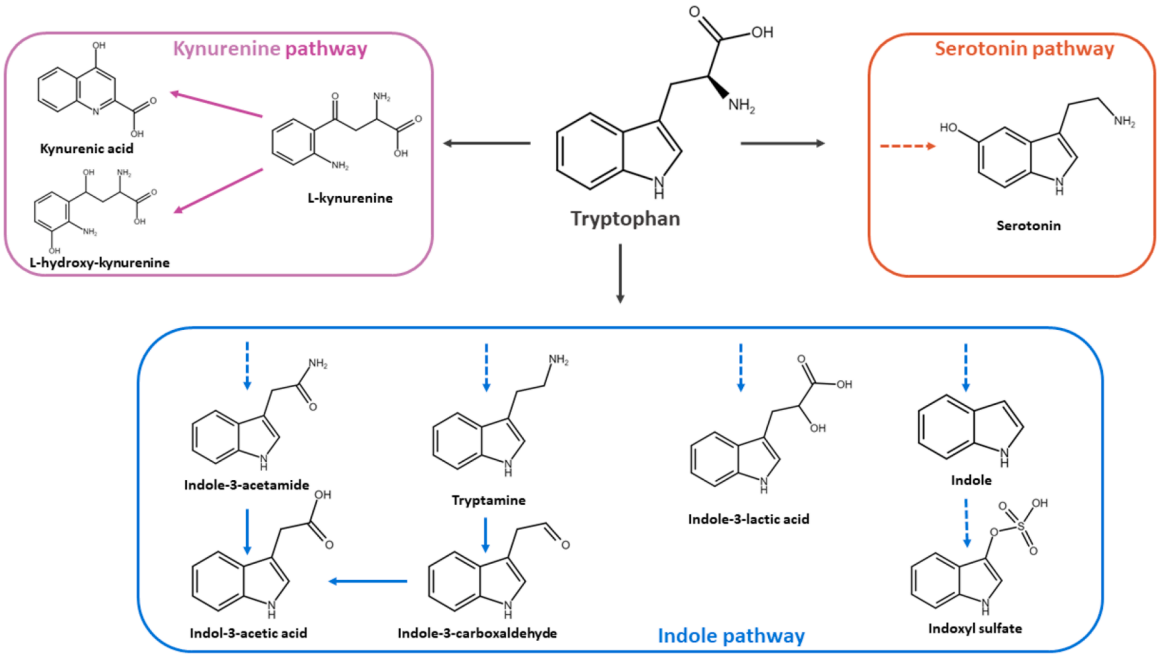
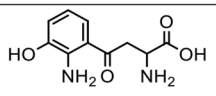
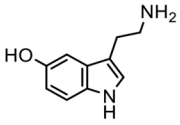
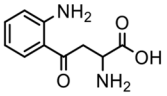
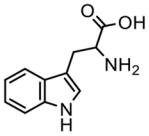
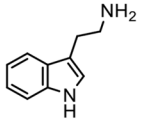
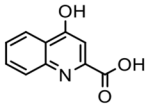
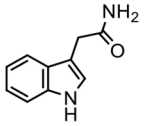
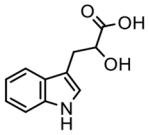
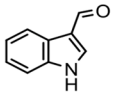
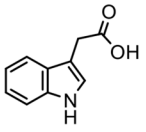
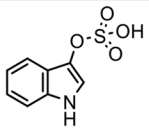


Fig. 1. Overview of tryptophan metabolism through the kynurenine (pink), serotonin (orange), and indole (blue) pathways. Chemical structures of tryptophan and all metabolites quantified using the developed LC–MS/MS method are shown, illustrating their distribution across the three metabolic routes.

Table 1
PRM settings for compound identification. The table lists the selected mass-to-charge (m/z) values for precursor and fragment ions, along with the optimized parallel reaction monitoring (PRM) parameters in positive ion mode and in negative ion mode for indoxyl sulfate. These parameters include normalized collision energy (NCE) and the retention time (RT) of each analyte, reported in minutes. Since some analytes undergo fragmentation directly in the ion source, the most intense primary fragment was selected as the precursor ion for subsequent PRM analysis. To standardize the process, the in-source CID energy was optimized to maximize its abundance.

NAME	$[M+H]^+$	FORMULA	STRUCTURE	TRANSITION	NCE	RT
POSITIVE MODE						
3-hydroxy-L-kynurenine	225.08698	$C_{10}H_{12}N_2O_4$		225.087→208.060	10	1.51
Serotonin	177.10224	$C_{10}H_{12}N_2O$		In-source fragmentation CID28 160.100→115.054	95	2.72
L-kynurenine	209.09207	$C_{10}H_{12}N_2O_3$		209.092→192.066	20	6.24
Tryptophan	205.09715	$C_{11}H_{12}N_2O_2$		205.100→146.060	50	7.78
Tryptamine	161.10732	$C_{10}H_{12}N_2$		In-source fragmentation CID42 144.081→115.054	110	12.77
Kynurenic acid	190.04987	$C_{10}H_7NO_3$		190.049→162.055	50	16.93
Indole-3-acetamide	175.08659	$C_{10}H_{10}N_2O$		175.070→130.065	30	17.42
Indole-3-lactic acid	206.08117	$C_{11}H_{11}NO_3$		206.081→188.070	45	18.92
Indole-3-carboxaldehyde	146.06004	C_9H_7NO		146.060→118.065	50	20.85
Indole-3-acetic acid	176.07061	$C_{10}H_9NO_2$		176.071→130.060	35	21.72
NEGATIVE MODE						
Indoxyl sulfate	212.00175	$C_8H_7NO_4S$		212.002→132.044	55	8.95

in water, briefly vortexed, centrifuged, and transferred to HPLC vials. The study was approved by the Ethics Committee of Liguria Region, Italy (635/2020). Serum/plasma were collected by venipuncture from 46 subjects with multiple sclerosis (MS) enrolled in a study aimed at evaluating metabolomic signatures, after obtaining written informed consent.

2.4. Instrumentation

2.4.1. Liquid chromatography method settings

Chromatographic analyses were carried out using a Vanquish HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) composed of binary pump, auto-sampler and column oven. Separation of the target metabolites was achieved on a Raptor biphenyl analytical column [1.8 μm, 50 × 2.1 mm] equipped with a guard column [2.7 μm, 5 × 2.1 mm] (both Restek, Milan, Italy). The column temperature was maintained at 45 °C throughout the runs. Two microliters of extracted samples were injected via autosampler, kept at 8 °C. A binary gradient, consisting of mobile phase A (water with 0.1% formic acid, v/v) and mobile phase B (methanol with 0.1% formic acid, v/v) was employed. The flow rate was set at 0.3 mL/min. Gradient elution was optimized during method development to ensure adequate resolution of all 11 metabolites. The RP-HPLC gradient consisted of two main isocratic steps at different organic contents: an initial hold at 0% organic, followed by a rapid ramp to 22% with an isocratic hold of equal duration, and a final fast increase to 95% organic for column washing to prevent carryover. In details, 0–13 min, 0% B; 13–14 min, linear increase to 22% B; 14–29 min, held at 22% B; 29–30 min, ramped to 95% B; and 30–37 min, maintained at 95% B. The mobile phase was then returned to initial conditions at 37.10 min (0% B), followed by a re-equilibration step from 37.10 to 50 min at 0% B to ensure retention time stability and reproducibility for subsequent injections.

2.4.2. Mass spectrometry

A Q Exactive™Plus Hybrid Quadrupole-Orbitrap™mass spectrometer (Thermo Fisher Scientific,) was employed to perform parallel reaction monitoring (PRM) analyses in positive mode for all metabolites, except for indoxyl sulfate, which was analysed in negative mode. Polarity switching was therefore implemented within a single analytical run, eliminating the need for separate injections and improving overall analytical efficiency. For positive and negative mode acquisition, an electrospray voltage of 3.5 kV was applied, sheath, auxiliary and sweep gas flow rates were 48, 11 and 2, respectively. The transfer tube was kept at 256 °C and the auxiliary gas heater temperature was 413 °C. The full scan MS was operated at high resolution (70,000), with a S-lens RF level of 55. The scan range was from 100 to 250 *m/z*, with automatic gain control (AGC) set to 1×10^6, maximum injection time (maxIT) to 250 ms and isolation window to 1 *m/z*. PRM, at a mass resolution of 17,500 was applied for compound identification in the targeted analyses using AGC of 2×10^5 and maxIT of 100 ms. In negative ionization mode, full-scan MS analysis, consistent with the positive ion mode settings, was performed at a resolution of 70,000. The S-lens RF level was set to 55, and the scan range covered *m/z* 100–250. Automatic gain control (AGC) was configured to 1×10^6, with maxIT of 250 ms and an isolation window of 1 *m/z*. Targeted compound identification was carried out using parallel reaction monitoring (PRM) at a resolution of 35,000, with AGC set to 1×10^6 and a maximum injection time of 250 ms.

2.4.3. Data processing and statistical analysis

The primary HPLC/HR-MS data acquisition and first-pass processing were conducted through the native Xcalibur (Version 4.1, Thermo Fisher) interface to ensure full control over instrument settings and raw spectral evaluation. Subsequently, Skyline was employed to further refine chromatographic peak picking, verify transition consistency, and explore alternative quantification strategies in a transparent and reproducible environment [19].

2.5. Method validation

The analytical method described in this study was validated following the guidelines provided by the International Conference on Harmonisation (ICH) [20]. Validation of this bioanalytical procedure involved evaluating selectivity, linearity, lower limits of detection (LOD) and quantification (LOQ), precision and accuracy within and between days, recovery, matrix effects, and analyte stability.

2.5.1. Linearity, limits of detection and quantification

Linearity was assessed using calibration standards ranging from 0,5 nM to 10 μM, except for TRP and INS which highest point was explored at 50 μM. Calibration intervals were selected based on measurements from pooled plasma samples and refined as needed. A linear regression model was used for quantification, incorporating a 1/x weighting scheme. Each concentration was prepared in triplicate to ensure reliability.

The LOD and LOQ represent the minimum concentrations at which the analytes can be reliably detected and quantified, based on the signal-to-noise ratio. Thus, LOD was accepted for a signal-to-noise ratio (S/N) ≥ 3 and the LOQ for a signal-to-noise ratio (S/N) ≥ 10. Both parameters were estimated from the chromatograms of each analyte at the lowest concentration evaluated.

2.5.2. Accuracy and precision

Accuracy, as well as intra-day and inter-day precision, were evaluated by analysing five replicates of low- and high-concentration quality control (LQC and HQC) samples ($n = 5 \times 2$) over two consecutive days, resulting in a total of 20 measurements.

Precision was considered acceptable when the relative standard deviation (RSD) did not exceed ±15%. Accuracy was assessed against the nominal concentrations, with the same ±15% acceptance criterion applied.

2.5.3. Recovery

Extraction recovery was evaluated to determine the efficiency of the sample preparation procedure and to assess potential analyte losses during extraction. For this purpose, isotopically labelled internal standards were spiked into neat samples prior to extraction and compared with samples at the same concentration injected directly for LC-MS/MS analyses. This approach allowed the estimation of analyte recovery by isolating the contribution of the extraction process from other analytical variables [17].

2.5.4. Matrix effects

Matrix effects were assessed by comparing the peak areas obtained from plasma samples spiked with the analyte mixture to those from neat samples spiked at equivalent concentrations and processed under identical extraction conditions. The matrix effect was further assessed with particular attention to the stability of the analytes' retention times.

2.5.5. Freeze-thaw stability

In order to evaluate the stability of tryptophan metabolites in human plasma under different storage conditions, we assessed the effects of freeze–thaw cycles and storage time on these metabolites. In the first condition, the sample was analysed immediately after plasma preparation, without freezing, serving as the baseline reference. A second aliquot of the same sample was subjected to one freeze cycle at −80 °C for 2 h, then thawed and analysed; immediately after withdrawing the volume required for analysis, the aliquot was refrozen and reanalysed after 6 days. The third analysis was therefore performed on this refrozen sample, while a fourth analysis was performed on a third "untouched" aliquot that had never been thawed and was kept frozen for 6 days prior to analysis.

2.5.6. Carryover

Carryover was assessed by injecting blank samples immediately after the highest concentration level of the calibration curve in order to evaluate potential residual analyte signals.

3. Results and discussion

Despite growing interest in tryptophan metabolism and its involvement in microbiota–host interactions, most analytical methods address only a single metabolic branch and often include derivatization or labor-intensive pre-treatment [18,21]. In this context, we developed a derivatization-free HPLC–MS/MS workflow enabling the simultaneous quantification of tryptophan and eleven downstream metabolites relevant to gut and immune health. The method emphasizes chromatographic selectivity as a central design principle, achieving effective separation across all three pathways while maintaining high sensitivity, precision, and suitability for clinical applications.

3.1. Chromatographic strategy and analytical advantages

A defining strength of this workflow is its chromatographic strategy, specifically engineered to separate, within the same run, metabolites that differ markedly in polarity, aromaticity, and ionization behaviour. The analytical panel encompasses highly polar kynurenines, intermediate-polarity serotonin derivatives, and hydrophobic microbiota-derived indoles, an exceptionally challenging combination for conventional C18 phases, where co-elution, poor retention, and distorted peak shapes are common [22]. Although LC–MS/MS is widely used for TRP metabolite analysis due to its sensitivity and selectivity [23,24], achieving simultaneous quantification across all three metabolic branches remains difficult because the analytes span such a broad physicochemical range.

To address this, we systematically optimized chromatographic and mass-spectrometric parameters using a Vanquish HPLC system. Method development focused on the key factors known to govern chromatographic resolution—column chemistry, mobile-phase composition, gradient design, column temperature, and flow rate. Guided by previous reports demonstrating the superior performance of phenyl-based stationary phases for aromatic and moderately polar compounds [14], a biphenyl column was selected. Its enhanced π–π interaction capacity provided markedly improved selectivity and peak shape, enabling baseline separation of structurally similar metabolites. This was especially evident for challenging pairs such as kynurenic acid and indole-3-acetamide, which often co-elute on C18 columns, as well as serotonin and tryptophan, which share a diagnostic product ion (*m/z* 115.05) but were resolved here by their widely spaced retention times and well-defined chromatographic peak profiles.

Further optimization involved systematic evaluation of gradient profiles, column temperatures, and solvent compositions. Notwithstanding, method was initiated under 100% aqueous conditions to ensure sufficient retention of the most polar tryptophan metabolites, no deterioration of column performance was observed over repeated analyses, probably due to the improved wettability and stability of biphenyl stationary phases, as demonstrated by stable retention times, consistent peak shapes, and reproducible signal responses (Suppl. Fig. 1). Raising the column temperature to 45 °C enhanced chromatographic efficiency, resulting in improved analyte resolution and more symmetrical peak shapes. In addition, the strategic incorporation of isocratic hold segments within the gradient program proved critical for overall method performance. These isocratic intervals stabilized the chromatographic environment during key elution windows, allowing better retention and separation of early-eluting metabolites while simultaneously improving resolution among structurally related compounds in the late-eluting branch. This approach increased selectivity, minimized co-elution, and enhanced reproducibility, thereby strengthening the robustness and reliability of the method for comprehensive metabolite profiling. The selected mobile phases—0.1% formic acid in water (A) and 0.1% formic acid in methanol (B)—yielded optimal signal-to-noise ratios and produced sharp, symmetrical peaks for all analytes in the panel. The behaviour of chromatographic separation during different LC condition attempts and the parameters tested are shown in Suppl. Fig. 2 and summarized in Suppl. Table 3. A simulated total ion chromatogram (TIC) depicting the elution order of all analytes is presented in Fig. 2. The resolution between the critical peak pair (kynurenic acid and indole-3-acetamide) was assessed using peak widths at half height, yielding a resolution (Rs) value of 5.76, indicative of excellent separation. When calculated using baseline peak widths, a lower Rs value of 1.18 was obtained (Suppl.Equation 1). To further support the evaluation of chromatographic performance, peak symmetry was quantitatively assessed by calculating tailing factors based on retention times at the peak apex and at corresponding left and right peak boundaries at 10% of peak height. The inclusion of these parameters provides an objective characterization of peak shape and enhances the transparency and reliability of the method evaluation. Peak symmetry was evaluated by calculating tailing factors for all analytes. The obtained values ranged from 0.87 to 1.75, indicating overall good chromatographic performance. All values were below the commonly accepted threshold of 2, confirming acceptable peak symmetry across the dataset. The majority of analytes exhibited tailing factors close to unity (0.9–1.3), reflecting highly symmetrical peaks and well-optimized chromatographic conditions. Slight fronting (values <1) and moderate tailing (up to 1.75) were observed for a limited number of analytes; however, these deviations were minor and did not adversely affect peak integration or quantification. Overall, the results demonstrate that the method provides reliable and reproducible peak shapes suitable for quantitative analysis. (Suppl. Table 4).

3.2. Internal standard strategy

To improve robustness and correct for extraction- and matrix-related variability, five deuterated internal standards were included into the workflow: SER-d4, TRP-d5, KYN-d4, KYNA-d5, and IAA-d4. Thus, KYN-d4 was used as the internal standard for 3-HK and KYN; SER-d4 for SER; TRP-d5 for TRP, TRPA, and INS; KYNA-d5 for KYNA; and IAA-d4 for indole derivatives, including I3C, IAA, IAM, and ILA. The use of isotopically labelled analogues—chemically and physically similar to their corresponding analytes—ensures accurate quantification by compensating for ion suppression, extraction efficiency differences, and instrumental drift [25]. Internal standards were added at the earliest stage of sample processing to control the entire analytical workflow.

Where possible, internal standards were matched to metabolites with similar chromatographic behaviour to maximize correction efficiency. Although slight chromatographic delays were occasionally observed for certain deuterated standards in plasma compared to neat solutions, this isotope effect is well documented and did not affect quantitative accuracy. All IS compounds displayed stable peak shapes, adequate co-elution, and consistent response factors.

3.3. Matrix choice: plasma vs. serum

Matrix selection was a critical aspect of this workflow. Serotonin is predominantly stored in platelets, and the coagulation process during serum preparation triggers platelet activation and degranulation, leading to massive release of serotonin into the matrix [26]. As a result, serum serotonin concentrations can be artificially inflated and highly variable, depending on clotting time and pre-analytical handling [27,28]. Such artefactual elevation can distort the physiological interpretation of circulating serotonin levels and their relationship within the tryptophan pathway. In contrast, plasma collected with appropriate anticoagulants minimizes platelet activation, thereby preserving the true circulating concentration of serotonin and providing a more reliable matrix for its quantitative assessment. As a consequence, serum does not

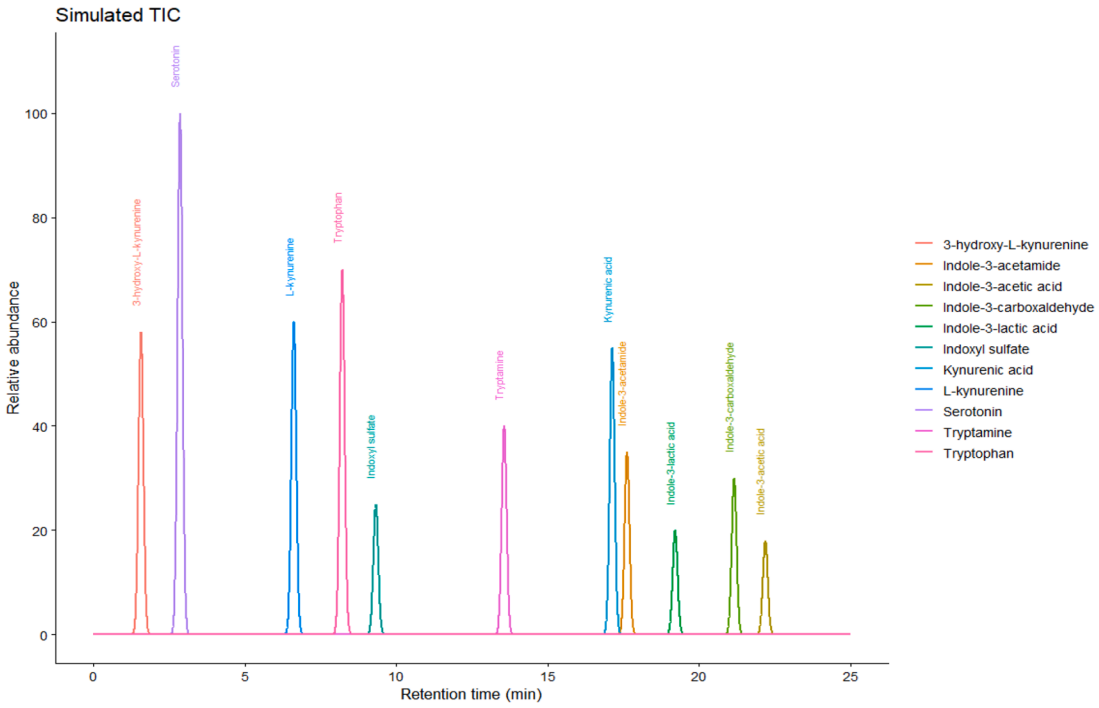


Fig. 2. Simulated total ion current (TIC) of the targeted analytes. Unlabelled analytical standards of SER, serotonin; KYN, L-kynurenine; TRP, tryptophan; KYNA, kynurenic acid; 3-HK, 3-hydroxykynurenine; indole-3-lactic acid (ILA), indole-3-carbaldehyde (I3C), and indole-acetic acid (IAA), indole-3-acetamide (IAM), indoxyl sulfate (INS), tryptamine (TRPA).

reflect true circulating serotonin levels and may also alter downstream metabolites through enzymatic activity during clot formation.

In contrast, plasma collected with anticoagulants prevents platelet activation, preserving the physiological pre-release concentration of serotonin and ensuring a more reliable representation of in vivo levels. In our comparative assessments, serotonin concentrations in serum were markedly higher than in plasma; a nearly fivefold increase in peak intensity was observed ($1.83 \times 10^6 \rightarrow 7.76 \times 10^6$), consistent with platelet-derived release (Suppl.Table 5.)

3.4. Sample pre-treatment and workflow throughput

A major strength of the method is the streamlined sample preparation, designed for simplicity, low sample consumption, and high throughput. Only 50 µL of plasma is required, making the workflow particularly suitable for studies with limited sample availability or vulnerable patient cohorts.

Protein precipitation was selected as the extraction strategy, using a ternary organic mixture (acetonitrile:methanol:acetone 8:1:1 v/v containing 0.1% formic acid), which efficiently removed proteins in both serum and plasma (data not shown). The resulting supernatants were dried using a vacuum concentrator at room temperature, eliminating the need for nitrogen-assisted evaporation and reducing operational costs (Fig. 3). The mild extraction and concentration steps minimized analyte degradation while providing excellent peak shapes and low background.

3.5. Optimization of PRM parameters

To achieve optimal differentiation, MS/MS parameters were systematically optimized by monitoring precursor ions production and selecting the most intense product ions for quantification in positive and negative ionization modes. A Parallel Reaction Monitoring strategy was

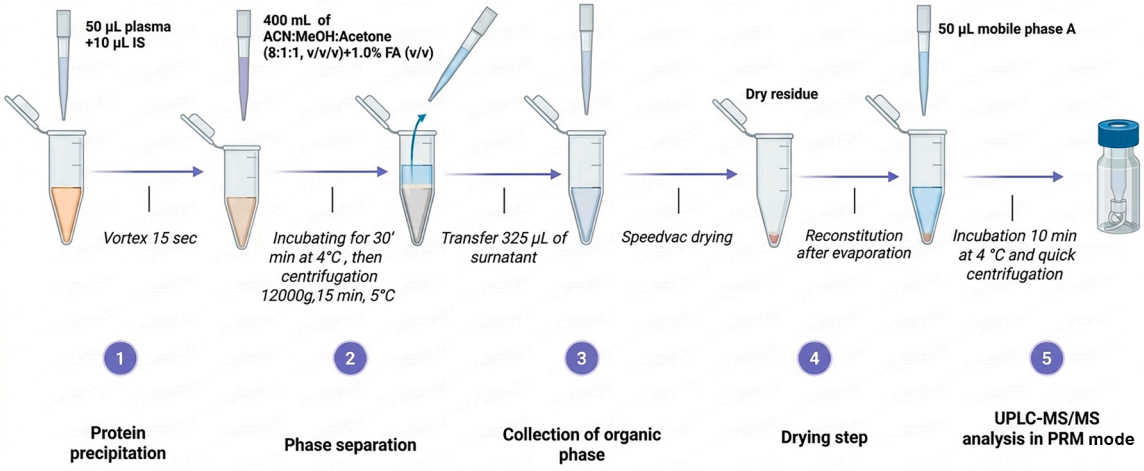


Fig. 3. Method overview of sample preparation for targeted quantification of tryptophan metabolites.

developed and refined to enable accurate quantification of 11 tryptophan pathway metabolites. The optimization process followed several key steps. First, precursor ion information for each metabolite was predicted from its molecular formula using publicly available databases. Next, a range of collision energies (20–110 eV) was systematically tested in order to achieve efficient fragmentation of the selected precursor ions. The optimized collision energies were applied in the HCD cell to generate high-quality MS/MS spectra, which were then examined to identify specific product ions that could reliably distinguish each metabolite. For every compound, the most abundant and selective product ions were chosen as diagnostic markers. The corresponding precursor ions, product ions, and collision energy values are reported in Table 1.

Although some structurally related metabolites generated identical PRM fragment ions, this did not compromise selectivity because chromatographic separation provided sufficient orthogonality. Compounds such as tryptamine and serotonin eluted at clearly distinct retention times, enabling unambiguous identification and reliable quantification. In addition, polarity switching was evaluated and found to have no significant impact on analytical linearity. Finally, the concentration of each compound in plasma was measured by the extracted ion current (EIC) peak area of the corresponding fragment ions (Suppl. Fig. 3).

3.6. Method validation

Currently, standardized guidelines for the validation of assays targeting endogenous compounds are not well established. The validation of LC–MS/MS methods for endogenous analytes is particularly challenging due to the lack of a true blank biological matrix [29]. This limitation complicates the evaluation of key analytical parameters, including accuracy, recovery, and matrix effects. In the present study, method validation procedures were therefore adapted to address these specific constraints and to ensure reliable quantification of tryptophan metabolites.

3.6.1. Linearity, LOD and LOQ

The validated analytical method demonstrated high sensitivity and specificity, making it suitable for routine application. Linearity was assessed across seven calibration levels, and regression parameters, including slope and R^2 values, are reported in Suppl. Table 6. Calibration curves generated for analyte concentrations ranging from 0.5 nM to 10 μM (50 μM for TPR and INS); the upper limits of 10 μM and 50 μM were selected because higher concentrations were not relevant for the intended application. Excellent linearity was observed with correlation coefficients (R^2) exceeding 0.999 for all analytes, except tryptamine (0.998), confirming a strong linear response within the tested concentration range. The method demonstrated high analytical sensitivity across the evaluated tryptophan metabolites. LOD were below 1 nM for all analytes, indicating excellent detectability. Similarly, limits of quantification were below 10 nM for the majority of analytes, and below 1 nM for 3-HK and SER, confirming the suitability of the method for trace-level determination in plasma.

3.6.2. Precision and accuracy

Accuracy and precision were evaluated in accordance with FDA guidance for bioanalytical method validation. Pooled human plasma samples were fortified with two concentration levels: 1.6 and 8.3 μM for 3-HK, SER, KYN, KYNA, IAM, I3C, ILA, IAA, INS; 36.6 and 43.3 μM for TRP of the analyte mixture using a standard addition approach. Each validation level was analysed in five replicates per run, across at least two independent, consecutive days, enabling assessment of both intra-day and inter-day accuracy and precision (Suppl. Table 7).

Overall, the method demonstrated good repeatability and reliability, with most analytes showing precision (CV%) below 11% and accuracy within the commonly accepted range of 80–120%. Compounds such as 3-hydroxykynurenine, serotonin, kynurenine, and tryptophan exhibited excellent performance, with low variability and accuracy values close to 100% at both QC levels.

For certain analytes, deviations from optimal performance were observed, particularly at high concentration levels, therefore, considering the performance of the remaining analytes, we recommend prompt analysis of samples immediately after thawing to minimize potential variability.

As deuterated internal standards were not available for all analytes, normalization was performed using surrogate internal standards selected based on structural similarity and comparable retention times. This approach proved suitable for the majority of compounds, supporting consistent quantification across the panel. For indoxyl sulfate (INS), a higher variability was observed, suggesting that the use of a dedicated isotopically labelled internal standard could further improve analytical performance. Similarly, the inclusion of labelled standards for IAM and I3C may provide additional refinement in quantification accuracy.

3.6.3. Recovery

The efficiency of the protein-precipitation step and of the subsequent extraction procedure was evaluated using internal standards-spiked plasma samples. As outlined above, the selected protocol is easier and milder than conventional liquid-/solid-phase extraction approaches. Recovery values indicated robust performance in the blood matrix, and all internal standards were successfully retrieved (ranging from 91.8 to 108.1%), with the results summarized in Suppl. Table 8. The final volumes of extracted and non-extracted samples were standardized to ensure comparability. Most metabolites exhibited mild ion suppression while one IS—kynurenic acid-d5—showed the largest fluctuation, displaying both suppression and slight enhancement across replicates (Suppl. Figure 4). This behaviour is consistent with previously published analyses of tryptophan metabolites [10,[14]].

3.6.4. Matrix effect

Most previously validated methods for endogenous metabolite quantification rely on charcoal-stripped matrices to approximate a blank biological background. However, such matrices do not fully reproduce the intrinsic variability, protein binding, or residual enzymatic activity present in native plasma. As a result, they may underestimate matrix-related effects and pre-analytical influences that occur under real clinical conditions. To better reflect physiological complexity, we deliberately performed validation experiments directly in untreated human plasma. This approach allowed us to account for endogenous baseline levels, matrix interactions, and potential metabolic activity, thereby providing a more realistic assessment of method performance and robustness in clinically relevant samples.

Matrix effects were assessed to determine the extent of ion suppression or enhancement caused by co-eluting plasma components and to verify the suitability of the isotope-dilution strategy for quantitative correction. In accordance with established LC–MS/MS practices for endogenous metabolites, matrix effects were evaluated by comparing the responses of stable isotope-labeled ISs spiked into water with those of the same IS spiked into plasma samples. Because each deuterated IS co-elutes with its corresponding analyte and exhibits nearly identical ionization behaviour, variations in IS signal provide a reliable estimate of the matrix-induced changes affecting each metabolite.

The observed matrix effect values (86.4–105.4%) fell within the commonly accepted ±20% interval for quantitative LC–MS/MS assays (Suppl.Table 9). Despite these localized effects, the use of structurally matched, co-eluting isotope-labeled standards effectively compensated for matrix-induced variability. Quantitative performance remained within validation criteria. Overall, the matrix effect profile observed in this study is consistent with values reported in existing LC–MS/MS methods for tryptophan pathway metabolites, confirming that the optimized biphenyl column and mobile-phase composition offer sufficient selectivity and chromatographic resolution to mitigate plasma-

derived interference.

Interestingly, tryptamine showed a noticeable increase in retention time in plasma, even after protein precipitation, which may be attributed to matrix-induced chromatographic effects. Other analytes exhibited smaller retention shifts, whereas late-eluting compounds were largely unaffected. This pattern is likely due to the influence of residual matrix components, such as phospholipids and salts, which may more strongly affect early-eluting, polar analytes, while late-eluting compounds are comparatively less sensitive because of their inherently stronger interactions with the reversed-phase stationary phase. This hypothesis was confirmed by diluting the sample before injection, which reduced the interaction with possible "ligands", the shift of tryptamine retention time, under different matrix conditions, is provided in Suppl. Figure 5.

3.6.5. Freeze-thaw stability

Comparison of the four selected conditions demonstrated substantial analyte stability, indicating that neither the freeze–thaw cycle nor short-term storage at −80 °C resulted in significant changes in the concentrations of the investigated tryptophan metabolites. Changes in analyte concentrations were expressed as percentage recoveries relative to freshly prepared samples with %RSD values ranging from 5.48% to 11.87% (Suppl. Table 10).

3.6.6. Carryover

Carryover was assessed by injecting a neat sample following the highest calibration sample (50 ng/mL). No signals exceeding the detection threshold were detected at the analyte retention times, confirming the absence of carryover.

3.7. Application of method to clinical human plasma sample analysis

Following analytical validation, the method was applied to plasma samples obtained from patients diagnosed with multiple sclerosis (MS) to demonstrate its clinical applicability in a proof-of-concept setting. MS is a chronic, immune-mediated neurological disorder characterized by inflammation, demyelination, and neurodegeneration within the central nervous system [30]. Although its precise pathogenesis remains incompletely understood, MS is believed to result from a complex interplay between genetic predisposition, environmental triggers, and immune dysregulation [31]. Increasing evidence also implicates alterations in gut microbiota composition and systemic metabolic pathways in modulating disease activity and progression [32].

Given the well-established role of the tryptophan–kynurenine pathway in immune regulation and neuroinflammation [33], the validated method was applied to plasma samples from patients diagnosed with relapsing–remitting MS (RRMS) or secondary progressive MS (SPMS).

To ensure analytical robustness during the runs, a biological quality control (QC) sample was generated by pooling equal aliquots from all study samples. For analytes quantified within the validated range, coefficients of variation in the pooled QC remained below 14%, confirming the reproducibility and suitability of the method for clinical metabolomic investigations. Pooled quality control samples analysed in quintuplicate, intra-day %RSD values ranged from 2.14% to 13.83%, while inter-day %RSD values at two and six days ranged from 2.78% to 13.35% and 3.72% to 12.72%, confirming the high reproducibility of the assay under routine analytical conditions (Suppl. Table 11). These ranges fall well within commonly accepted criteria for bioanalytical precision, which typically consider %RSD values below >15% as indicative of acceptable repeatability and intermediate precision. These results demonstrate the robustness of the method for reliable metabolite profiling in clinical practice. A notable strength of the present proof-of-concept MS dataset lies in the consistency and robustness of the analytical results, despite the relatively small cohort size and the absence of adjustment for potential clinical confounders. Importantly, metabolite measurements remained highly reproducible across different analytical days, underscoring the stability and reliability of the developed method when applied to real-world clinical samples. The ability to generate consistent metabolic profiles under these conditions further supports the suitability of the assay for future, larger-scale studies incorporating comprehensive clinical stratification.

4. Conclusions

A simple, selective, and reproducible LC–MS/MS method was developed for the simultaneous quantification of eleven tryptophan-related metabolites in human plasma. Comprehensive validation included systematic evaluation of matrix effects, extraction recovery, limits of quantification, and analytical challenges inherent to endogenous compounds. Critical factors affecting robustness—such as retention time shifts observed for tryptamine, plasma protein binding, and elevated serotonin concentrations in serum—were carefully investigated and addressed to ensure reliable and reproducible quantification.

The simultaneous measurement of metabolites from both the kynurenine and indole pathways within a single analytical run represents a major strength of the method. Importantly, polarity switching between positive and negative ionization modes was successfully implemented without compromising linearity, sensitivity, or overall analytical stability. This confirms the robustness of the workflow and its suitability for routine application.

A further key advantage lies in the high-quality chromatographic resolution achieved. Clear peak separation was ensured not only by mass selectivity through PRM detection, but also by optimized chromatographic conditions, enhancing confidence in analyte identification and quantification. This strong chromatographic performance increases methodological versatility and facilitates adaptation to alternative detection platforms when needed.

The method requires minimal sample preparation, uses only 50 µL of plasma, and does not involve derivatization, thereby simplifying the analytical workflow and reducing potential sources of variability. All validation parameters met FDA bioanalytical method acceptance criteria. Collectively, these features demonstrate that the method provides sensitive, robust, and fit-for-purpose quantification suitable for both clinical and research applications.

5. Abbreviations

3-HK, 3-hydroxykynurenine; ACN, acetonitrile; AGC, automatic gain control; DMSO, dimethyl sulfoxide; EIC, extracted ion current; FA, formic acid; HQC, high quality control; HR, high resolution; I3C, indole-3-carbaldehyde; IAA, indole-3-acetic acid; IAA-d4, indole-3-acetic acid-d4; IAM, indole-3-acetamide; ILA, indole-3-lactic acid; INS, indoxyl sulfate; IS, internal standard; ISs, internal standards; KYN, L-kynurenine; KYN-d4, L-kynurenine-d4; KYNA, kynurenic acid; KYNA-d5, kynurenic acid-d5; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; LQC, low quality control; maxIT, maximum injection time; MeOH, methanol; MS, mass spectrometry; NCE, normalized collision energy; PRM, parallel reaction monitoring; QC, quality control; RRMS, relapsing–remitting multiple sclerosis; RT, retention time; SER, serotonin; SER-d4, serotonin-d4 hydrochloride; SPMS, secondary progressive multiple sclerosis; TRP, tryptophan; TRP-d5, L-tryptophan-d5; TRPA, tryptamine.

CRediT authorship contribution statement

Kateryna Tkachenko: Writing – original draft, Investigation, Data curation. **Alice Laroni:** Writing – review & editing, Resources, Funding acquisition. **Marco Ponassi:** Validation. **Davide Maioli:** Writing – review & editing. **Camillo Rosano:** Supervision. **Aldo Profumo:** Writing – review & editing, Methodology, Conceptualization.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2026.467035.

Data availability

Data will be made available on request.

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