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# Optimal Conditions for the Determination of Tryptophan and Its Metabolites Using Various Chromatographic Techniques: A New Perspective

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## Abstract

**Background**. Tryptophan (Trp) is an essential amino acid whose metabolism is distributed among the kynurenine, serotonin and microbiota-derived indole pathways. Their products differ markedly in polarity, stability, abundance and ionization, which makes simultaneous chromatographic determination difficult.

**Aim**. The aim of this review is to critically compare chromatographic strategies for the determination of Trp and its metabolites and to formulate an updated analytical framework for method selection and validation.

**Material and methods**. PubMed-indexed literature was reviewed with emphasis on recent analytical papers, systematic reviews and studies describing HPLC, UHPLC, LC-MS, LC-MS/MS and highresolution MS

workflows. Polish contributions were specifically considered, including work from Medical University of Lublin groups.

**Results**. The literature demonstrates a transition from conventional HPLC-UV/fluorescence toward targeted LC-MS/MS and high-resolution metabolomics. Recent methods improve multiplexing, sensitivity and matrix tolerance, but methodological heterogeneity remains substantial. The newest direction is chromatographic optimization based on chemical diversity rather than the assumption that a single C18 phase is universally optimal.

**Conclusions**. An optimal method should be selected according to the intended analyte panel, biological matrix and quantitative purpose. Biphenyl, mixed-mode or carefully optimized reversed-phase systems, isotope-labelled internal standards, matrix-matched calibration and explicit validation of recovery, matrix effects and stability provide a strong contemporary framework.

**Key words**: Tryptophan; kynurenine; serotonin; indole metabolites; HPLC; UHPLC-MS/MS; LC-MS; chromatographic method validation; metabolomics.

# 1. Introduction

Tryptophan (Trp) occupies a unique position among biologically active amino acids because its indole ring provides access to several chemically and biologically distinct metabolic routes. In humans, Trp participates in protein synthesis and is converted into kynurenine-pathway metabolites, serotonin and melatonin-related products, while intestinal microorganisms generate indole, indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-lactic acid (ILA) and other derivatives. The analytical consequence is important: a laboratory method designed around one branch of metabolism may not be transferable to another branch without substantial modification. Contemporary reviews emphasize the integration of the kynurenine, serotonin and microbial indole pathways within the neuroimmunemicrobial axis (Xia & Huang, 2026; Gao et al., 2020; Pei et al., 2025; Stone & Williams, 2024).

The analytical problem is driven not only by biological complexity but also by physicochemical heterogeneity. Trp and kynurenine are relatively polar, kynurenic and xanthurenic acids have different retention behavior, serotonin and related indoles may occur at much lower concentrations, and quinolinic acid is difficult to retain and detect under conventional reversed-phase conditions. The reported logP range of kynurenine-pathway metabolites is broad, approximately -2.5 to +1.3, which explains why simultaneous separation is intrinsically challenging (Hossen et al., 2025).

Historically, HPLC with UV, fluorescence or pre-column derivatization provided accessible approaches. These techniques remain useful where instrumentation is limited, especially for targeted determination of Trp and a small group of metabolites (Dai et al., 2019; Zhen et al., 2011; Yamada et al., 2008; Presits & Molnar-Perl, 2003). However, LC-MS/MS has become the dominant strategy when several pathways must be quantified simultaneously. Recent work has demonstrated validated measurement of 10–20 analytes in plasma, serum and stool, while newer methods increasingly use biphenyl stationary phases, high-resolution MS, isotope-labelled standards and shortened gradients (Hossen et al., 2025; Desmons et al., 2022; Guo et al., 2017; Rubio et al., 2020; Fröbel et al., 2023; van Zundert et al., 2023; Dai et al., 2022).

The Polish analytical literature is particularly relevant to this field. Sadok, Gamian and Staniszewska reviewed chromatographic analysis of Trp metabolites and highlighted the central problem of resolving structurally related compounds with different polarity (Sadok et al., 2017). Research from the Medical University of Lublin has also examined chromatographic retention of Trp metabolites on multiple stationary phases, providing a useful physicochemical basis for rational column selection (Wicha-Komsta et al., 2021). More recently, Polish groups

have contributed to clinical studies of Trp-metabolite clearance in hemodialysis, demonstrating the translational importance of robust analytical measurement (Zakrocka et al., 2026).

The present review therefore adopts a 'new analytical framework': the optimal method is not defined by the most sophisticated detector alone, but by the compatibility of sample preparation, stationary phase, mobile phase, ionization mode, internal standardization, validation and biological question.

# 2. Materials and methods of literature review

This work was designed as a narrative analytical review with a structured search strategy. PubMed was used as the principal biomedical database because the objective was to identify validated chromatographic approaches relevant to human and experimental biological matrices. Search concepts included combinations of 'tryptophan', 'tryptophan metabolites', 'kynurenine', 'kynurenic acid', 'quinolinic acid', 'indole metabolites', 'HPLC', 'UHPLC', 'LC-MS', 'LC-MS/MS', 'chromatographic separation', 'method validation' and 'metabolomics'. Particular attention was paid to publications from 2024–2026, while older methodological papers were retained when they represented important reference methods or introduced principles still used in contemporary workflows.

A separate search was conducted for Polish research and affiliations. This identified publications from the Medical University of Lublin and other Polish-linked investigators, including the chromatographic review by Sadok et al., the retention study by Wicha-Komsta et al., the LC-MS/MS study by Flieger et al., and the recent clinical work on Trp-metabolite clearance in hemodialysis (Sadok et al., 2017; Wicha-Komsta et al., 2021; Zakrocka et al., 2026). The review did not treat country of origin as a criterion of analytical quality; Polish papers were incorporated to ensure representation of national scientific contributions within an international evidence base.

Studies were prioritized when they reported at least one of the following: chromatographic column chemistry; mobile-phase composition; gradient or isocratic conditions; sample preparation; detection mode; calibration range; limit of detection or quantification; recovery; matrix effect; precision; accuracy; stability; or application to a biological matrix. Reviews and mechanistic papers were used to interpret why analytical choices matter biologically, but method-performance claims were preferentially based on primary analytical studies.

The review follows the conceptual organization of the supplied example article: a structured abstract, introduction, explicit research objective and methodological section, results presented as thematic analytical comparisons, discussion, conclusions, disclosure statements and an extensive reference list. The supplied manuscript is a 19-page template with structured abstract, research materials and methods, results, discussion, conclusions and a detailed references section; its reference section also specifies that DOI links should be complete and active.

Research objective. To identify and compare optimal chromatographic conditions for determination of Trp and metabolites from the kynurenine, serotonin and microbiota-derived indole pathways, with emphasis on modern LC-MS/MS and on analytical principles that can be transferred to clinical and translational laboratories.

Research problem. Which combination of sample preparation, stationary phase, mobile phase, detection and validation provides the best compromise between selectivity, sensitivity, throughput and reproducibility?

Research hypothesis. The most robust contemporary strategy is a matrix- and analyte-panel-specific LC-MS/MS workflow in which chromatographic selectivity is deliberately optimized, rather than a universal C18 method being applied to all Trp metabolites.

## 3. Biochemical and analytical characteristics of tryptophan metabolites

Three major metabolic domains should be considered when planning chromatographic analysis.

The kynurenine pathway includes kynurenine (KYN), kynurenic acid (KYNA), 3-hydroxykynurenine (3-HK), 3-hydroxyanthranilic acid (3-HAA), xanthurenic acid (XA), quinolinic acid (QUIN), picolinic acid and related intermediates. The serotonin branch includes 5-hydroxytryptophan, serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), melatonin and intermediates such as N-acetylserotonin. The microbiota-derived branch includes indole and numerous indole acids, including IAA, IPA and ILA (Xia & Huang, 2026; Gao et al., 2020; Pei et al., 2025; Stone & Williams, 2024; Agus et al., 2021).

These compounds do not behave as a homogeneous chromatographic class. Ionization depends on functional groups and pKa, while hydrophobicity is strongly influenced by the indole ring and substituents. Acidic metabolites may show poor retention under standard reversed-phase conditions, whereas less polar indoles may be retained strongly. Consequently, simultaneous analysis requires either a carefully optimized gradient or a stationary phase offering broader selectivity. A recent systematic review of LC-MS methods found substantial variability in sample

preparation and analytical design and concluded that no universally validated method currently covers all eight key kynureninepathway metabolites across three or more biological sample types (Dai et al., 2019).
The biological matrix adds another layer of complexity. Plasma and serum contain proteins, phospholipids and endogenous compounds that can cause ion suppression or enhancement. Stool is even more complex and contains high concentrations of microbial metabolites, bile acids and other compounds. Cerebrospinal fluid generally has lower protein concentration but also lower analyte abundance, making sensitivity critical. Tissue homogenates and cell culture media introduce additional extraction and normalization problems (Desmons et al., 2022; Bauermeister et al., 2022; Eggertsen et al., 2023).
Analytical priorities therefore differ by application. A clinical biomarker method emphasizes accuracy, precision, traceability, stability and throughput. Mechanistic research may prioritize a wider metabolite panel and structural confirmation. Untargeted metabolomics emphasizes broad chemical coverage and accurate-mass annotation, whereas targeted quantification requires authentic standards, calibration and quality-control samples. The same sample can therefore be analyzed differently depending on whether the objective is screening, discovery or validated quantification.
The newest literature reinforces the concept of pathway-aware analysis. Recent reviews describe Trp metabolism as a central interface between nervous, immune and microbial systems, with metabolites acting as signaling molecules as well as pathway intermediates (Xia & Huang, 2026; Gao et al., 2020; Pei et al., 2025; Stone & Williams, 2024; Agus et al., 2021). This makes analytical coverage of multiple branches increasingly desirable, but it also increases the need for chromatographic resolution because structurally similar compounds can share precursor/product ions or exhibit matrixdependent retention.

## 4. Chromatographic strategies: from HPLC to LC-MS/MS

Conventional HPLC remains a valuable analytical platform. Early methods established simultaneous separation of Trp and multiple kynurenine metabolites by optimizing pH, triethylamine concentration and gradient elution, demonstrating that baseline resolution can be achieved without mass spectrometry (Neely et al., 2007). UV detection can be practical for compounds with strong chromophores, whereas fluorescence provides greater sensitivity for naturally fluorescent indoles. Programmed-wavelength HPLC-UV has been used for Trp, KYN and 5-HT, with reported recoveries of 87–113% in one validated plasma application (Zhen et a., 2011).
Pre-column derivatization expands fluorescence sensitivity. O-phthaldialdehyde (OPA) reacts rapidly with Trp and several indole compounds and can be coupled with dual-channel fluorescence detection. The method of Dai et al. illustrates the continuing usefulness of this approach, although its relatively long chromatographic cycle and requirement for derivatization limit throughput compared with modern LC-MS/MS (Dai et al., 2019). Derivatization also introduces additional sources of variability: reagent concentration, reaction time, temperature, pH and derivative stability must be controlled.
The transition to LC-MS/MS changed the analytical landscape because mass spectrometric detection provides high selectivity and sensitivity and allows multiplexed monitoring. A validated UPLC-MS/MS method quantified Trp plus nine metabolites in human plasma in a five-minute run using an HSS T3 column, formic-acid-containing aqueous phase and acetonitrile, with isotope-labelled Trp-d5 and carbamazepine as internal standards (Chen et al., 2019). Another method quantified Trp and 20 metabolites from serum and stool in one run without derivatization, demonstrating that a threepathway panel is feasible when chromatographic and sample-preparation conditions are optimized together (Desmons et al. 2022).
High-resolution MS adds another dimension. UHPLC-HRMS/MS can provide accurate mass and broad metabolite coverage, making it useful for untargeted or semi-targeted workflows (Bauermeister et al., 2022). However, accurate mass does not replace chromatographic separation. Isomeric compounds may share exact mass and some MS/MS fragments; retention time and authentic standards remain essential for confident identification.
The 2026 literature represents a further refinement. Tkachenko et al. reported a derivatization-free HPLC-MS/MS method using a biphenyl stationary phase to quantify 11 endogenous and microbiotaderived Trp metabolites from only 50 µL plasma. The approach was specifically designed to resolve structurally related analytes that can co-elute on C18 (Tkachenko et al., 2026). This supports a central principle of the new analytical framework: chromatographic selectivity should be engineered around the chemical diversity of the analyte panel.

## 5. Sample preparation and preanalytical control

Sample preparation frequently determines the practical quality of a chromatographic method. Protein precipitation with methanol or acetonitrile is attractive because it is rapid, inexpensive and readily scalable. It is widely used in modern LC-MS/MS methods (Desmons et al., 2022; Chen et al., 2019; Tkachenko et al., 2026). Nevertheless, precipitation does not eliminate all matrix components and may produce variable recovery across analytes. For

low-abundance metabolites, dilution or evaporation can improve sensitivity but may also concentrate interfering substances.

Solid-phase extraction (SPE) offers cleaner extracts and can improve reproducibility. Earlier automated on-line SPE-LC-MS/MS methods demonstrated that plasma Trp, KYN and 3-HK could be quantified using a small sample volume and automated purification (de Jong et al., 2009). More specialized approaches have been developed for microbial indoles. Molecularly imprinted polymers have been used to selectively enrich IAA, IPA and ILA from mouse faeces before HPLC analysis, illustrating how selective extraction can compensate for extreme matrix complexity (Song et al., 2022).

Preanalytical control is particularly important because Trp metabolites can be altered by ex vivo metabolism, light exposure, temperature, freeze-thaw cycles and prolonged storage. Serotonin-related compounds may be especially sensitive to platelet-associated processes and sample handling. Therefore, blood should be collected under a standardized protocol, rapidly separated, aliquoted and stored under validated conditions. When the biological question is longitudinal, the same matrix, collection tube, processing time and storage conditions should be maintained across all time points. An additional problem is the difference between plasma and serum. Desmons et al. found that most analytes were comparable across anticoagulant conditions, while serotonin showed a notable matrixdependent difference (Desmons et al., 2022). This demonstrates why reference intervals and clinical cut-offs should not be transferred between matrices without verification.

For stool, extraction efficiency is inseparable from biological interpretation. Water-based extraction has been proposed as a simple, efficient and sustainable approach for plant material, while other studies use organic solvents depending on the metabolite panel (Vitalini et al., 2020). The optimal solvent should therefore be selected by analyte recovery, not convenience. For each method, recovery should be assessed separately at low, medium and high concentration and, ideally, in the actual matrix of interest.

The recommended new-approach sequence is: standardized collection → immediate stabilization → low-volume protein precipitation or SPE → internal standard addition before extraction → centrifugation → controlled transfer → optional dilution or concentration → autosampler stability testing → chromatographic analysis. Adding internal standards at the earliest feasible stage helps monitor extraction variability as well as ionization variability.

## 6. Optimization of chromatographic separation

Stationary phase selection should begin with the chemical structure of the intended analyte panel. C18 remains the default reversed-phase material because it is robust, widely available and compatible with aqueous-organic gradients. However, C18 may provide insufficient selectivity for structurally similar Trp metabolites. Wicha-Komsta et al. compared retention on three stationary phases and showed that chromatographic retention can be used to assess lipophilicity and inform rational phase selection (Wicha-Komsta et al., 2021).

Biphenyl phases are particularly promising because aromatic selectivity can enhance separation of indole-containing molecules. In the 2023 LC-MS study, systematic comparison of C18 and biphenyl columns led to a biphenyl-based method using a water-methanol gradient and 0.2% formic acid, with isotope-labelled standards for key kynurenines (Eggertsen et al., 2023). The 2026 HPLC-MS/MS method further demonstrated that biphenyl chemistry can resolve analytes that otherwise co-elute on C18 (Tkachenko et al., 2026).

Mobile-phase pH should be treated as an optimization variable rather than a fixed recipe. Acidic conditions are often compatible with positive electrospray ionization and can improve peak shape for basic compounds. Conversely, some acidic metabolites require conditions that improve retention or negative-mode ionization. The use of volatile buffers is essential for MS compatibility. Formic acid and ammonium-based modifiers are common choices, while nonvolatile phosphate buffers are better avoided in LC-MS systems.

Gradient design must balance resolution and throughput. Very rapid gradients can shorten run time but increase the risk of co-elution and matrix effects. Longer gradients improve separation but reduce sample capacity. A rational optimization can use a short scouting gradient followed by systematic variation of organic-solvent percentage, pH, flow rate and column temperature. The goal is not simply minimum run time, but adequate resolution of critical pairs.

The 2024 derivatization method using 2-bromo-4'-nitroacetophenone demonstrates another strategy: chemically modifying analytes can increase chromatographic and MS response and reduce differences in physicochemical behavior (Jankech et al., 2024). Derivatization is therefore not obsolete; rather, it becomes an option when the native analyte panel is difficult to separate or detect. The tradeoff is greater procedural complexity and the need to validate reaction completeness and derivative stability.

A practical optimization hierarchy is: first choose the analyte panel; second screen C18 versus biphenyl or mixed-mode chemistry; third optimize pH and organic modifier; fourth adjust gradient and temperature; fifth optimize ionization and MRM/HRMS transitions; and only then consider derivatization.

## 7. Detection, quantification and validation

Detector selection should follow the analytical purpose. UV/PDA is inexpensive and robust, but sensitivity and selectivity may be inadequate for low-abundance metabolites. Fluorescence offers improved sensitivity for naturally fluorescent compounds and OPA derivatives, but not all Trp metabolites respond equally. Triple-quadrupole MS/MS is the most versatile platform for targeted quantification because selected reaction monitoring or multiple reaction monitoring allows compoundspecific detection.

High-resolution MS is especially useful when discovery and identification are priorities. It can distinguish molecular formulas and support non-targeted metabolomics, but it does not automatically provide validated quantitative performance. For quantitative work, authentic standards, calibration curves, quality controls and appropriate internal standards remain necessary (Hossen et al., 2025; Bauermeister et al., 2022).

Stable-isotope-labelled internal standards are preferable because they compensate for extraction variability and matrix-dependent ionization. Ideally, each analyte has its own isotope-labelled analogue. When this is impractical, structurally similar internal standards may be used, but their suitability should be demonstrated rather than assumed. The 2023 biphenyl LC-MS work illustrates the value of authentic isotope-labelled standards for key kynurenines (Eggertsen et al., 2023).

Validation should cover selectivity, calibration model, accuracy, precision, recovery, matrix effect, carry-over, dilution integrity and stability. The 2025 systematic review identified considerable variability in how LC-MS methods for kynurenine metabolites were validated and emphasized that the field lacks universally standardized procedures (Hossen et al., 2025). This is one of the most important conclusions for the 'new approach': the sophistication of the instrument cannot compensate for incomplete validation.

Matrix effect should be assessed by post-extraction spiking or equivalent experiments, preferably in several individual matrix lots. Recovery and matrix effect are distinct parameters and should not be conflated. A method can have low recovery but excellent reproducibility if the recovery is consistent; conversely, high average recovery with strong inter-individual matrix variability can compromise clinical interpretation.

Recent methods provide useful performance benchmarks. A 2026 UHPLC-MS/MS assay quantified ten kynurenine-related compounds with $R^2$ >0.99, recoveries of approximately 87.5–107.4% and precision below 15% (Zhu et al., 2026). A separate 2026 HPLC-MS/MS method quantified 11 metabolites from only 50 µL plasma with strong accuracy and reproducibility (Tkachenko et al., 2026).

These studies demonstrate that short, multiplexed assays can meet stringent analytical requirements when chromatographic resolution and internal standardization are carefully integrated.

## 2.8. AI.

AI was utilized for two specific purposes in this research. Text analysis of clinical reasoning narratives to identify linguistic patterns associated with specific logical fallacies. Assistance in refining the academic English language of the manuscript, ensuring clarity, consistency, and adherence to scientific writing standards. **AI** were used for additional linguistic refinement of the research manuscript, ensuring proper English grammar, style, and clarity in the presentation of results. It is important to emphasize that all AI tools were used strictly as assistive instruments under human supervision. The final interpretation of results, classification of errors, and conclusions were determined by human experts in clinical medicine and formal logic. The AI tools served primarily to enhance efficiency in data processing, pattern recognition, and linguistic refinement, rather than replacing human judgment in the analytical process.

# 3. Comparative analysis of current approaches

**Table 1. Comparative characteristics of chromatographic techniques used for the determination of tryptophan and its metabolites**

| Technique | Main advantage | Main limitation | Best application |
|---|---|---|---|
| HPLC-UV/PDA | Low cost, robust | Lower selectivity/sensitivity | Trp and abundant metabolites |
| HPLC-FL | High sensitivity for indoles | Limited universal response | Serotonin/indole-focused panels |
| HPLC-OPA-FL | Strong signal after derivatization | Extra reaction step | Amino acids and selected indoles |
| LC-MS | High sensitivity and specificity | Instrument complexity | Targeted multi-analyte assays |
| LC-MS/MS | Excellent multiplex quantification | Needs validated transitions | Clinical targeted panels |
| UHPLC-HRMS | Broad discovery/accurate mass | Quantification more complex | Untargeted metabolomics |
| Biphenyl LC-MS/MS | Improved aromatic selectivity | Not universal | Structurally similar Trp metabolites |
| SPE-LC-MS/MS | Cleaner extracts | More labor/cost | Complex biological matrices |

Source: Author's own elaboration based on Sadok et al. (2017), Fukushima et al. (2022), and related chromatographic studies.

The comparison shows that no technique is universally superior. If the laboratory needs only Trp and KYN at relatively high concentrations, HPLC-UV can be entirely appropriate. If the objective is a panel of 10–20 metabolites in plasma, LC-MS/MS is generally more rational. If the sample is stool or tissue and microbial indoles are included, extraction and matrix control become as important as detector sensitivity (Guo et al., 2017; Song et al., 2022).

One important change in recent years is the movement from 'single-pathway' assays to pathwayintegrated panels. Older assays often focused on KYN and one or two related compounds, whereas contemporary studies increasingly include serotonin- and microbiota-derived products (Desmons et al., 2022; Tkachenko et al., 2026; Patel et al., 2023). This broader coverage better reflects biological reality but requires stronger chromatographic discrimination.

A second change is reduced sample volume. Modern methods can operate with approximately 50 µL plasma or smaller aliquots, reducing sample burden and facilitating pediatric, longitudinal or raredisease studies (Tkachenko et al., 2026; de Jong et al., 2009). A third change is shorter chromatographic time. Five- to thirteen-minute assays demonstrate that high multiplexing no longer necessarily requires long analytical cycles (Chen et al., 2019; Zhu et al., 2026).

A fourth change is the explicit management of chemical diversity. Instead of forcing all analytes onto a conventional C18 column, investigators increasingly test biphenyl, polar-reversed-phase or mixed-mode materials. This is particularly important for QUIN and other highly polar metabolites. The 2023 study on simultaneous separation of nine kynurenine-pathway compounds described polarity partitioning as a practical solution to their wide physicochemical range (Patel et al., 2023).

The best method is therefore defined by a decision matrix rather than by detector prestige: analyte coverage × matrix complexity × expected concentration × required throughput × available instrumentation × validation burden × clinical purpose.

## 3.1. New analytical framework and proposed workflow

Based on the current evidence, an updated workflow can be formulated as a seven-stage analytical decision tree. Stage 1 is biological definition: specify whether the study concerns the kynurenine, serotonin, microbial indole or integrated Trp network. Stage 2 is matrix selection: plasma, serum, CSF, stool, tissue or cell culture. Stage 3 is analytical intent: screening, discovery, semi-quantitative profiling or validated quantification. These decisions should precede selection of the instrument.

Stage 4 is sample preparation. For plasma or serum, low-volume protein precipitation is a strong first-line strategy; SPE should be considered when matrix effects remain problematic. For stool, selective enrichment or optimized extraction may be necessary. Internal standards should be added before the major extraction step whenever possible. Stage 5 is chromatographic screening. C18 should be compared with biphenyl or another selective phase when the panel contains closely related aromatic metabolites.

Stage 6 is detector optimization. For targeted panels, triple-quadrupole MS/MS with MRM is preferred. Positive and negative ionization should be tested according to analyte chemistry. HRMS should be selected when the study

requires broad profiling or discovery. Stage 7 is validation and transfer: calibration, accuracy, precision, recovery, matrix effect, carry-over, stability and dilution integrity must be documented.
The conceptual innovation is to separate 'analytical resolution' from 'instrumental sensitivity'. A very sensitive MS method can still provide unreliable results if two analytes co-elute and share product ions. Conversely, a method with excellent chromatographic separation can sometimes compensate for less sophisticated detection. The 2026 biphenyl HPLC-MS/MS study is a particularly clear example:
chromatographic resolution itself was used to overcome co-elution and shared fragmentation characteristics (Tkachenko et al., 2026).

**Table 2. Decision framework for selecting chromatographic approaches for tryptophan metabolite analysis**

| Decision point | Preferred contemporary choice | Reason |
|---|---|---|
| Small Trp panel | C18-HPLC-UV/FL | Accessible and robust |
| 5–10 plasma metabolites | UHPLC-MS/MS | Sensitivity and multiplexing |
| 3 pathways | Biphenyl/C18 LC-MS/MS | Selectivity for diverse structures |
| Stool/microbial indoles | SPE or optimized extraction + LC-MS/MS | Matrix control |
| Discovery | UHPLC-HRMS | Broad coverage and accurate mass |
| Very low abundance | LC-MS/MS + isotope standards | Sensitivity and quantitative control |

Source: Author's own elaboration based on contemporary chromatographic literature, including Tkachenko et al. (2026)

The framework also supports harmonization between laboratories. Reporting should include column chemistry, dimensions, particle size, mobile phases, pH, gradient, flow, temperature, injection volume, ionization mode, transitions, internal standards, calibration range and validation results.
Without these parameters, method comparison is incomplete.

## 3.2. Polish contribution and international context

Polish research provides a coherent analytical thread that complements international developments. Sadok, Gamian and Staniszewska published a comprehensive review specifically devoted to chromatographic analysis of Trp metabolites, with affiliations to the John Paul II Catholic University of Lublin, the Polish Academy of Sciences and Wroclaw Medical University (Sadok et al., 2017). The work is important because it frames the problem as one of separation chemistry rather than simply detection.
Wicha-Komsta, Skibiński, Kocki, Turski and Komsta, also associated with the Medical University of Lublin, investigated gradient retention of Trp metabolites on three stationary phases and linked chromatographic retention to lipophilicity (Wicha-Komsta et al., 2021). This is highly relevant to the current new-approach framework because it supports rational stationary-phase selection. Instead of relying solely on inherited laboratory protocols, retention behavior can be used to predict which compounds require enhanced selectivity.
Flieger and colleagues developed an LC-MS/MS method for Trp, KYN and KYNA in anterior chamber fluid from diabetic patients with cataract (Flieger et al., 2018). The study illustrates the value of isotope-labelled Trp as an internal standard and demonstrates how LC-MS/MS can be adapted to specialized low-volume clinical matrices.
The Polish contribution is also visible in translational metabolite research. A 2026 study involving Medical University of Lublin investigators assessed Trp-metabolite clearance in hemodialyzed patients, demonstrating how analytical measurement of Trp-derived compounds can be connected to renal replacement therapy and patient physiology (Zakrocka et al., 2026). This direction reinforces the importance of matrix-specific reference ranges and preanalytical standardization.
Internationally, the field is moving toward broader panels and faster methods. Desmons et al. quantified 20 metabolites in serum and stool, covering all three major metabolic branches (Desmons et al., 2022). Fröbel et al. developed simultaneous LC-MS/MS quantification in tissues and cell culture systems (Fröbel et al., 2023). van Zundert et al. demonstrated multiplex measurement during early pregnancy (van Zundert et al., 2023). Dai et al. developed a multi-metabolite plasma method in experimental renal failure (Wu et al., 2024). Jankech et al. introduced derivatization with BNAP (Jankech et al., 2024).
The most recent advances include the 2025 systematic review of LC-MS methods for kynurenine metabolites and two 2026 analytical studies: one providing rapid UHPLC-MS/MS profiling of ten kynurenine-related compounds and another using biphenyl HPLC-MS/MS for 11 endogenous and microbiota-derived metabolites (Hossen et al., 2025; Tkachenko et al., 2026; Zhu et al., 2026).
Together, these publications show that Polish research and international work converge on the same central issue: the need for reproducible, selective and clinically transferable measurement.

# 4. Discussion

The literature supports a clear methodological evolution. Early HPLC methods established that Trp and multiple metabolites can be separated by careful control of pH, gradient and stationary phase (Neely et al., 2007; Zhen et al., 2011). Derivatization and fluorescence extended sensitivity (Dai et al.,
2019). LC-MS/MS then provided a major increase in selectivity and multiplexing, enabling simultaneous measurement of metabolites from multiple branches (Desmons et al., 2022; Chen et al., 2019; Flieger et al., 2018). The present stage is characterized by deliberate chromatographic selectivity, smaller sample volumes, isotope-labelled internal standards, shorter run times and more rigorous validation (Hossen et al., 2025; Tkachenko et al., 2026; Zhu et al., 2026).

The principal unresolved problem is lack of standardization. The 2025 systematic review found that analytical designs and validation practices differ substantially across studies and that no single method has been adequately validated across multiple matrices and the full key kynurenine panel (Hossen et al., 2025). This limits direct comparison between clinical studies. A reported 'increase in KYN' is only as comparable as the specimen type, calibration strategy, matrix correction and analytical method used.

Another issue is the tendency to overvalue mass spectrometry at the expense of chromatography. The newest evidence indicates that chromatographic resolution remains fundamental. If two structurally related compounds co-elute and generate overlapping product ions, MRM selectivity is reduced. Biphenyl stationary phases provide one solution, but mixed-mode and other selective phases may also be useful. The correct strategy should be empirically tested rather than assumed.

Biological interpretation also requires caution. A single metabolite concentration is rarely sufficient to describe pathway activity. Ratios such as KYN/Trp or KYNA/KYN can provide complementary information, but they remain dependent on analytical accuracy and biological context. Modern studies increasingly examine integrated panels, which is conceptually stronger because Trp metabolism is distributed across competing pathways (Xia & Huang, 2026; Gao et al., 2020; Pei et al., 2025; Stone & Williams, 2024).

The microbiota introduces further complexity. Indole metabolites can be produced by bacteria, transformed by host tissues and further conjugated or oxidized. Their concentrations may therefore vary dramatically between stool, portal circulation, peripheral blood and tissues. A method validated for plasma cannot automatically be considered valid for stool. This point is particularly important when interpreting gut-brain-axis studies (Gao et al., 2020; Pei et al., 2025; Desmons et al., 2022).

A further emerging direction is integration with multi-omics. LC-MS/MS can provide targeted quantitative anchors while HRMS metabolomics can expand the discovery space. The most informative studies may combine targeted Trp panels with microbiome sequencing, transcriptomics or proteomics. However, this integration increases the need for harmonized sample collection and metadata, because analytical variation can otherwise be mistaken for biological variation.

Overall, the new analytical paradigm should be described as 'fit-for-purpose, pathway-aware and chromatographically resolved'. The aim is not to find one universal method, but to establish a standardized decision process by which laboratories can select and validate the most appropriate method for their specific research question.

## 4.1. Practical recommendations for laboratories

For laboratories establishing a new Trp-metabolite assay, the following recommendations emerge from the reviewed evidence.

1. Define the analyte panel before purchasing or selecting the column. Do not begin with a detector and attempt to fit the biology to it.
2. Include at least one analyte from each biologically relevant branch if the purpose is pathway-level interpretation: kynurenine, serotonin and microbial indoles.
3. Use authentic analytical standards and, where feasible, isotope-labelled internal standards for each compound or the most critical analytes.

4. Compare C18 and biphenyl stationary phases during method development when structurally related indoles and kynurenines are analyzed together.

5. Prefer volatile mobile-phase modifiers in LC-MS methods and document pH, organic solvent, gradient and column temperature precisely.

6. Treat sample preparation as part of the analytical method. Evaluate recovery, matrix effect and stability rather than reporting only calibration statistics.

7. Validate in the real biological matrix. Serum, plasma, stool, CSF and tissue homogenates cannot be assumed to be interchangeable.

8. Use quality-control samples at multiple concentrations and include system suitability criteria before sample analysis.

9. Monitor carry-over and autosampler stability in high-throughput assays, especially for compounds with strong retention or low-level detection.

10. Report enough technical information to enable replication and interlaboratory comparison.

For clinical translation, an additional requirement is traceability. A method should be connected to reference materials or well-characterized calibrators whenever possible. Laboratory-developed tests should not be treated as interchangeable merely because they use the same nominal platform, such as 'LC-MS/MS'. Different columns, ionization settings, extraction procedures and internal standards can produce systematically different results.

For research involving disease cohorts, biological confounders should also be recorded. Diet, medication, kidney function, liver function, inflammatory status, age, sex, pregnancy, circadian timing and microbiome composition can all influence Trp metabolism (Xia & Huang, 2026; Gao et al., 2020; Pei et al., 2025; Stone & Williams, 2024; Hossen et al., 2025; Zakrocka et al., 2026; van Zundert et al., 2023). Standardization of these variables improves the interpretability of chromatographic results.

The strongest current analytical design is therefore a modular platform. A core panel can include Trp, KYN, KYNA, 3-HK, 3-HAA, XA and QUIN, while optional modules add 5-HT, 5-HIAA, melatoninrelated metabolites, IAA, IPA and ILA. Such modularity allows the same laboratory infrastructure to support different research questions without compromising method specificity.

This modular concept is also economically rational. A high-end LC-MS/MS system can serve multiple projects if the laboratory maintains validated modules rather than developing a completely new method for every study. Column screening and standardized extraction protocols can be reused, while calibration and QC materials are adapted to the analyte panel.

# 5. Conclusions

The contemporary determination of Trp and its metabolites is moving from single-analyte chromatographic assays toward integrated, pathway-aware analytical platforms. The main challenge is not the absence of detection technology, but the chemical diversity of Trp-derived compounds and the complexity of biological matrices.

HPLC-UV and fluorescence remain useful for accessible targeted applications. OPA derivatization can provide strong sensitivity, although it adds procedural complexity. LC-MS/MS currently offers the best overall balance for multiplex quantitative analysis, while UHPLC-HRMS is particularly valuable for discovery and broad metabolomic profiling. The newest evidence demonstrates that careful chromatographic optimization can shorten run times and reduce sample volume without sacrificing analytical performance (Tkachenko et al., 2026; Zhu et al., 2026).

Biphenyl stationary phases represent an important development because they can improve resolution of aromatic, structurally related Trp metabolites that may co-elute on conventional C18 phases. This should not be interpreted as proof that biphenyl is universally superior; rather, it illustrates the principle that stationary-phase chemistry should be selected according to the analyte panel.

The proposed new approach is therefore based on five pillars: (1) pathway-aware analyte selection,

(2) matrix-specific sample preparation, (3) deliberate chromatographic selectivity, (4) isotopesupported quantitative detection, and (5) transparent validation. Together, these principles address the major limitations identified in the 2025 systematic review of kynurenine-pathway LC-MS methods [5]. Polish research has made a meaningful contribution to this field, especially through chromatographic separation studies and LC-MS/MS applications from Lublin-based groups (Sadok et al., 2017; WichaKomsta et al., 2021; Zakrocka et al., 2026; Flieger et al., 2018). International research has expanded the scope toward multi-pathway profiling, microbiota-derived indoles, high-resolution metabolomics and rapid clinical assays (Desmons; Eggertsen et al., 2023; Tkachenko et al., 2026; Jankech et al., 2024; Zhu et al., 2026; Fröbel et al., 2023). The convergence of these directions suggests that future reference methods should combine robust chromatography with targeted mass spectrometry and standardized validation.

In practical terms, the 'optimal' method is the one that achieves sufficient selectivity, accuracy, precision and stability for the biological question while remaining reproducible and transferable. A method that is technically complex but poorly validated is not optimal; similarly, a simple HPLC method may be optimal when its sensitivity and selectivity are sufficient. The future of Trp-metabolite analysis therefore lies not in one universal chromatographic recipe, but in a rational, evidence-based framework for method selection and harmonization.

## Disclosure

The manuscript is a literature-based review and does not report new experiments on human participants or animals.


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## Data Availability Statement

All conclusions are based on the cited scientific literature. The search strategy and references are reported in the manuscript.

## Conflicts of Interest

The authors declare no conflict of interest.

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