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Review

Beyond Structural Similarity: Metabolic and Analytical Approaches to Differentiate α-PHP and α-PiHP

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Abstract

Synthetic cathinones (SCs) pose significant challenges to forensic and clinical toxicology. Among these, α-pyrrolidinohexanophenone (α-PHP) and α-pyrrolidinoisohexanophenone (α-PiHP) are closely related structural isomers whose similar structures and overlapping metabolic pathways complicate their differentiation in biological specimens. This critical review examines their pharmacological and toxicological properties, metabolism, biological matrices, sample preparation, analytical methods, and biomarkers of exposure. Emphasis is placed on distinguishing computationally predicted and in vitro-generated metabolites from those confirmed in human samples, and on evaluating their suitability as exposure markers. Conventional and emerging analytical approaches, including GC–MS, LC–MS/MS, high-resolution mass spectrometry, and NMR, are critically assessed, alongside the emerging role of toxicometabolomics in identifying endogenous metabolic perturbations beyond xenobiotic metabolism. The review highlights key challenges in isomer discrimination, biomarker validation, and analytical harmonization, and proposes an evidence-based framework for selecting biological matrices, analytical targets, and instrumental platforms according to forensic and clinical needs.

Keywords: synthetic cathinones; α-PHP; α-PiHP; drug metabolism; exposure biomarkers; isomer differentiation; forensic toxicology; high-resolution mass spectrometry

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1. Introduction

New psychoactive substances (NPS) remain a major challenge for forensic and clinical toxicology because their chemical structures, pharmacological properties, and patterns of availability evolve rapidly. Synthetic cathinones (SCs) are one of the largest and most dynamic groups of NPS monitored internationally and have become established in illicit stimulant markets as alternatives to, or substitutes for, controlled substances such as amphetamine, cocaine, and MDMA. Their continued emergence creates important analytical and toxicological challenges because newly introduced analogues may differ substantially in potency, metabolism, toxicity, and detectability despite sharing a common chemical scaffold. According to the European Union Drugs Agency (EUDA), reported seizures and

imports of synthetic cathinones in the European Union increased from 37 tonnes in 2023 to 48.5 tonnes in 2024, while 69 previously identified synthetic cathinones were detected on the European drug market in 2024. While compounds such as 2-MMC, NEP, 4-CMC, and MDPHP have been reported in European drug markets, less prevalent substances, including α-PiHP, remain of concern due to their potential association with localized health harms [1].

Among synthetic cathinones, pyrrolidinophenones are of forensic concern because of their potent psychostimulant effects and association with severe adverse outcomes. α-PHP and α-PiHP are positional isomers that differ in the branching pattern of their alkyl side chains. This structural variation may influence their interactions with monoamine transporters, metabolic susceptibility, and the formation of diagnostic metabolites, although compound-specific differences in pharmacological potency and toxicity require further experimental confirmation. From an analytical perspective, their closely related structures can produce overlapping mass spectra and fragmentation patterns, complicating identification based on mass spectrometry alone. Consequently, chromatographic separation and compound-specific reference standards are important for reliable discrimination. These considerations highlight the need to integrate structural, pharmacological, metabolic, and analytical evidence when interpreting exposure to either compound [2,3].

A major challenge in the toxicological investigation of α-PHP and α-PiHP is their extensive biotransformation. Depending on the biological matrix and the time elapsed between exposure and sampling, the parent compounds may be present only transiently, whereas Phase I metabolites and subsequently formed Phase II conjugates may persist for longer periods and therefore provide more suitable targets for exposure assessment. Hydroxylation, β-keto reduction, pyrrolidine-ring oxidation, further oxidation, and conjugation reactions have been described for pyrrolidinophenone cathinones. However, the available evidence is heterogeneous and includes metabolites proposed from computational predictions, generated in vitro using human liver models, and confirmed in authentic human samples. These levels of evidence should not be considered equivalent when assessing the forensic value of a candidate exposure marker [4].

The analytical differentiation of α-PHP and α-PiHP is further complicated by their close structural relationship and by the generation of related positional isomers during metabolism. Biological matrix selection is therefore critical. Blood may be particularly informative in acute intoxication, whereas urine generally provides a longer detection window and is more suitable for metabolite-based exposure assessment. Oral fluid may provide information on more recent exposure, while hair can offer complementary information on longer-term or repeated exposure. The analytical value of each matrix depends not only on the pharmacokinetic behaviour of the compounds but also on analyte stability, extraction efficiency, matrix effects, and the sensitivity and structural specificity of the analytical platform [5].

Sample preparation represents another critical component of the analytical workflow. Conventional approaches such as protein precipitation, liquid–liquid extraction, and solid-phase extraction remain widely used because of their practical applicability and established performance. However, increasing attention has been directed toward miniaturized and greener approaches, including microextraction, solid-phase microextraction, dispersive liquid–liquid microextraction, and microextraction by packed sorbent. These approaches may reduce sample and solvent requirements while improving enrichment and compatibility with automated workflows. Their suitability for synthetic cathinone analysis should nevertheless be assessed not only in terms of recovery and sensitivity but also with respect to matrix effects, selectivity, reproducibility, and forensic applicability [6].

The analytical differentiation of α-PHP and α-PiHP remains challenging because of their identical molecular formulas and closely related mass spectral fragmentation patterns. Although GC–MS, LC–MS/MS, and high-resolution mass spectrometry provide complementary analytical capabilities, unequivocal isomer discrimination generally requires appropriate chromatographic resolution, diagnostic product-ion ratios, or metabolite-specific analytical strategies. Recent studies have demonstrated that combining chromatographic separation, tandem mass spectrometry, and metabolic profiling can substantially improve the identification of these structurally related synthetic cathinones [7,8].

In parallel with conventional xenobiotic metabolism studies, toxicometabolomics has emerged as a complementary strategy for investigating endogenous metabolic alterations associated with exposure to NPS. Whereas xenometabolomic approaches primarily aim to identify drug-derived metabolites and elucidate their biotransformation pathways, toxicometabolomics investigates perturbations of the endogenous metabolic phenotype that may provide additional insight into mechanisms of toxicity and exposure-associated biological responses. A recent systematic review highlighted the potential of metabolomics to characterize the metabolic footprint of NPS, while also emphasizing the heterogeneity of experimental models, analytical platforms, and evidence levels available across the field [9].

Previous reviews have addressed the physicochemical, pharmacological, toxicological, and analytical characteristics of α-PHP and α-PiHP, including their forensic relevance and associated public health concerns [10]. However, a critical gap remains in the systematic evaluation and validation of compound-specific metabolic biomarkers capable of reliably differentiating these closely related structural isomers in biological specimens. However, despite the available metabolic and analytical evidence, a critical gap remains in the validation of compound-specific biomarkers capable of reliably differentiating these closely related structural isomers in biological specimens. The present review therefore does not aim to provide another descriptive overview of these two compounds. Instead, it adopts a critical and evidence-based perspective centered on the relationship between metabolic pathways, biological matrices, candidate exposure biomarkers, analytical strategies, and the specific challenge of distinguishing α-PHP from α-PiHP in biological samples. Emphasis is placed on the level of experimental evidence supporting proposed metabolites and biomarkers, the complementary roles of targeted and untargeted analytical approaches, and the limitations that currently prevent robust compound-specific biomarker validation. The review further integrates these aspects into a matrix- and objective-dependent analytical framework intended to support forensic and clinical toxicology applications.

2. Literature Search Strategy

This critical narrative review was supported by a structured literature search conducted in PubMed, Scopus, Web of Science, ScienceDirect, and Google Scholar, primarily focusing on studies published between 2021 and 2026. Earlier publications were also considered when they provided essential experimental evidence, methodological background, or relevant toxicological information. The literature search was last updated in August 2026. A summary of the literature search strategy and study selection criteria is provided in Table 1.

The search strategy combined the terms “α-PHP”, “alpha-PHP”, “α-PiHP”, “alpha-PiHP”, “synthetic cathinones”, “pyrrolidinophenones”, “metabolism”, “biotransformation”, “toxicokinetics”, “toxicometabolomics”, “metabolomics”, “biomarkers”, “forensic toxicology”, “biological matrices”, “sample preparation”, “LC–MS/MS”, “HRMS”, and “GC–MS”, using Boolean operators (AND and OR).

Priority was given to peer-reviewed studies directly addressing α-PHP and α-PiHP, including investigations of pharmacology, toxicology, metabolism, toxicokinetics, biological matrices, biomarkers, analytical detection, and metabolic profiling. Studies involving struc-

turally related pyrrolidinophenones and synthethic cathinones were also considered when they provided relevant mechanistic, analytical, or methodological information applicable to α-PHP or α-PiHP.

Table 1. Summary of the literature search strategy and study selection criteria.

Parameter	Description
Review design	Critical narrative review
Information sources	PubMed; Scopus; Web of Science; ScienceDirect; Google Scholar
Publication period	Primarily 2021–2026; earlier publications included when essential to experimental evidence, methods or toxicological context
Last search update	August 2026
Search terms	"α-PHP", "alpha-PHP", "α-PiHP", "alpha-PiHP", "synthetic cathinones", "pyrrolidinophenones", "metabolism", "biotransformation", "toxicokinetics", "toxicometabolomics", "metabolomics", "biomarkers", "forensic toxicology", "biological matrices", "sample preparation", "LC–MS/MS", "HRMS", "GC–MS"
Search operators	AND; OR
Priority evidence	Peer-reviewed research directly addressing α-PHP or α-PiHP, including pharmacology, toxicology, metabolism, toxicokinetics, matrices, biomarkers, analytical detection and metabolic profiling
Supplementary evidence	Studies of structurally related pyrrolidinophenones and synthetic cathinones where mechanistically, analytically or methodologically relevant
Exclusion criteria	Outside review scope; insufficient experimental or methodological detail for meaningful interpretation; duplicate or substantially overlapping records
Selection procedure	Title/abstract screening, followed by full-text eligibility assessment
Metabolite evidence categories	(i) Computationally predicted; (ii) generated in vitro in human biological models; (iii) detected and confirmed in authentic human specimens
Evidence synthesis	Qualitative critical synthesis of agreement, uncertainty, methodological limitations, isomer-specific differences and validation gaps
Review reporting and appraisal	SANRA-informed narrative review; no formal risk-of-bias assessment or quantitative meta-analysis
Identification and screening counts	420 identified; 52 duplicates removed; 368 screened; 228 excluded at screening; 140 full texts assessed; 72 full texts excluded; 68 studies included
Full-text exclusion reasons	Not specific to topic ($n = 40$); insufficient relevant data/detail ($n = 20$); duplicate/overlapping information ($n = 12$)

Records were screened by title and abstract, followed by full-text assessment of potentially relevant publications. Studies were considered outside the scope of the review when they did not address the compounds of interest or provide no relevant information on their pharmacological, toxicological, metabolic, or analytical characteristics. Publications were excluded due to insufficient experimental or methodological detail when the available

experimental or methodological information did not allow meaningful interpretation of their findings in relation to the review objectives.

Particular attention was given to the level of evidence supporting reported metabolites and biomarkers. Evidence was classified, where appropriate, according to whether metabolites were (i) computationally predicted, (ii) generated in vitro using human biological models, or (iii) detected and confirmed in authentic human specimens. This distinction was considered essential for assessing the forensic and clinical relevance of candidate exposure biomarkers.

The literature was critically analysed rather than exhaustively tabulated. Emphasis was placed on identifying areas of agreement and uncertainty, methodological limitations, differences between α-PHP and α-PiHP, and gaps in analytical validation. The synthesis specifically considered the relationship between biological matrix, sample-preparation strategy, analytical platform, metabolite specificity, and the intended toxicological application.

As this manuscript was designed as a critical narrative review rather than a systematic review or meta-analysis, no formal risk-of-bias assessment or quantitative meta-analysis was performed. The reporting and structure of the review were informed by the Scale for the Assessment of Narrative Review Articles (SANRA), particularly regarding the justification of the review, clarity of objectives, description of the literature search, appropriate referencing, and critical presentation of the available evidence [11]. A schematic diagram of the literature search and study selection process for the narrative review is presented in Figure 1.

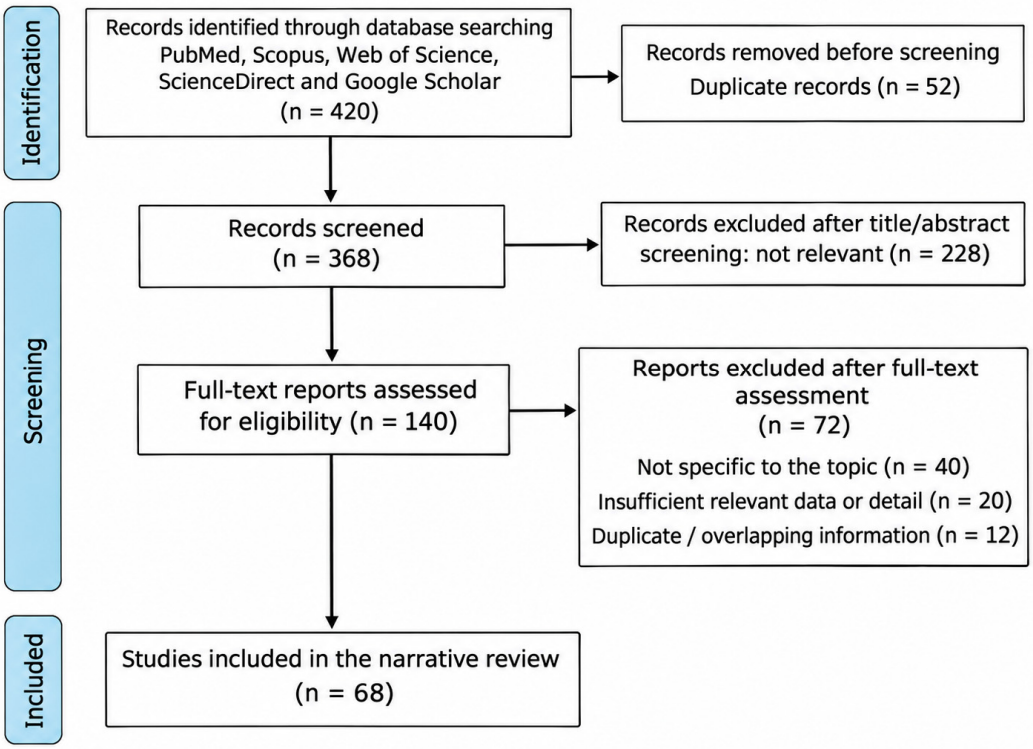


Figure 1. Flow diagram illustrating the identification, screening, eligibility assessment, and inclusion of studies in this critical narrative review.

3. α-PHP and α-PiHP: Chemical, Pharmacological and Toxicological Context

SCs are a structurally diverse class of new psychoactive substances derived from the β-ketoamphetamine scaffold. Structural modifications to the aromatic ring, amino group, or alkyl side chain generate compounds with markedly different physicochemical, pharmacological, metabolic, and toxicological properties [12–15]. Among these derivatives,

pyrrolidinophenones are of forensic relevance because of their potent psychostimulant effects and recurrent association with severe intoxication [16].

α-Pyrrolidinohexanophenone (α-PHP) and α-pyrrolidinoisohexanophenone (α-PiHP) are closely related pyrrolidinophenones that differ primarily in the architecture of their alkyl side chain. α-PHP contains a linear hexyl-derived side chain, whereas α-PiHP contains a branched isohexyl moiety. Although this structural difference is relatively small, it may affect molecular recognition, lipophilicity, pharmacokinetics, metabolism, and analytical behavior. Consequently, α-PHP and α-PiHP provide a relevant model for investigating how subtle structural modifications influence both biological activity and forensic detectability [17,18].

Both compounds primarily act at monoamine transporters, particularly the dopamine transporter (DAT) and norepinephrine transporter (NET), with substantially weaker activity at the serotonin transporter (SERT) [19–21]. Their predominant mechanism involves inhibition of monoamine reuptake rather than substrate-mediated neurotransmitter release, resulting in increased extracellular dopamine and norepinephrine concentrations. This pharmacological profile is consistent with their potent stimulant effects and with clinical manifestations including agitation, anxiety, tachycardia, hypertension, hyperthermia, psychosis, and seizures reported in cases of intoxication (Figure 2) [22–24].

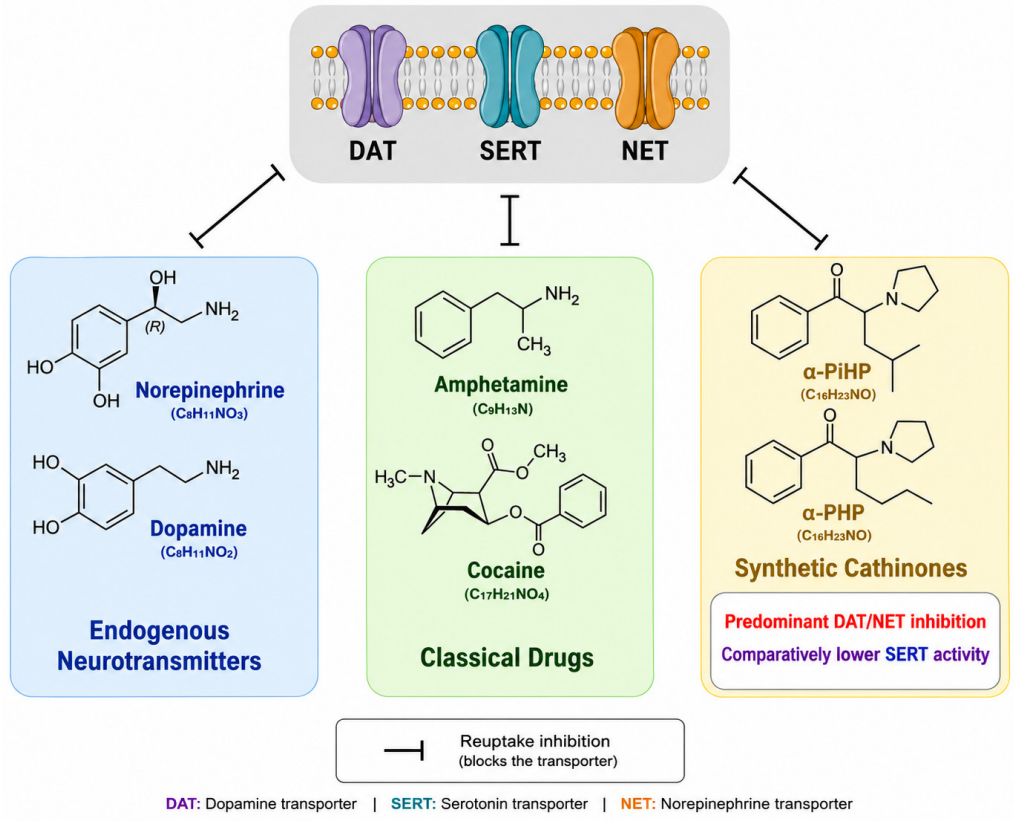


Figure 2. Schematic representation of the interactions of endogenous neurotransmitters, classical psychostimulants, and the synthetic cathinones α-PHP and α-PiHP with monoamine transporters (DAT, SERT, and NET). Both cathinones predominantly inhibit dopamine and norepinephrine reuptake, exhibiting substantially lower inhibitory potency at SERT. This pharmacological profile is supported by experimental monoamine uptake inhibition assays, including reported DAT IC_{50} values of 97 nM for α-PHP and 44 nM for α-PiHP, with markedly weaker SERT inhibition. The schematic is qualitative and does not depict the numerical differences in transporter inhibition or imply direct comparability between studies conducted using different experimental systems. Created by using Adobe Illustrator.

Importantly, the pharmacological similarities between α-PHP and α-PiHP should not be interpreted as evidence of identical biological behavior. Experimental studies indicate

that changes in the alkyl side-chain structure can modify transporter activity and behavioral effects. Recent work integrating computational, cellular, and in vivo approaches further supports the structure–activity sensitivity of the α-PiHP scaffold, demonstrating that relatively small structural changes can produce measurable differences in monoamine-transporter activity and toxicity [17,18]. This evidence reinforces the need for compound-specific rather than purely class-based toxicological assessment. Baarset et al. (2025) [25] examined the pharmacological effects and pharmacokinetics of α-PiHP in mice, using the structural analog α-PVP as a reference comparator drug. They investigated the in vitro ability of the drugs to inhibit uptake of tritiated monoamines at DAT, NET, and SERT, and then examined acute pharmacodynamic responses through locomotor stimulation and body temperature alterations following in vivo exposure. Additionally, they analyzed blood and brain concentrations of α-PiHP and α-PVP to characterize their pharmacokinetic profiles. The selective inhibition of DAT and NET suggests that α-PiHP has a high potential for misuse. An in vivo study showed that both drugs induced a significant increase in body temperature compared to the control; however, higher doses of α-PiHP were required to elicit locomotor activity compared to α-PVP. The dopamine transporter (DAT) and the norepinephrine transporter (NET) are the primary molecular targets of α-PHP and α-PiHP. Their affinity for the serotonin transporter (SERT) is significantly lower [20]. These transporters, which belong to the solute carrier family 6 (SLC6), mediate the reuptake of neurotransmitters into presynaptic neurons, thereby halting monoaminergic neurotransmission. Extracellular dopamine and norepinephrine concentrations are raised over time by α-PHP and α-PiHP, which primarily function as reuptake inhibitors rather than substrate-type releasers [25,26].

More recently, Pazos et al. (2026) [3] investigated the structure–activity relationships of α-PiHP and five structurally related analogues (3-Me-α-PiHP, 4-Me-α-PiHP, 3-F-α-PiHP, 4-F-α-PiHP, and MDPiHP) by integrating in silico, in vitro, and in vivo approaches. The study focused on three key molecular targets involved in monoaminergic neurotransmission: the human dopamine transporter (hDAT), norepinephrine transporter (hNET), and serotonin transporter (hSERT). Using HEK293 cells expressing these transporters, the authors demonstrated that all investigated compounds potently inhibited dopamine and norepinephrine uptake, while exhibiting substantially weaker activity at hSERT, consistent with a predominantly psychostimulant pharmacological profile.

Molecular docking studies indicated that α-PiHP and its analogues interact with the orthosteric binding site of hDAT, adopting binding orientations comparable to that of cocaine. In particular, the protonated nitrogen of the pyrrolidine ring was predicted to establish an electrostatic interaction with Asp79, a key residue involved in ligand recognition. Structure–activity comparisons revealed that meta-substituted analogues generally exhibited higher hDAT binding affinity than their corresponding para-substituted derivatives, demonstrating the importance of aromatic substitution patterns in transporter recognition. Among the investigated compounds, MDPiHP displayed the highest hDAT affinity, approximately 70-fold greater than cocaine in the experimental binding assay.

4. Toxicokinetic, Metabolic and Biomarker Considerations in α-PHP and α-PiHP Exposure

4.1. Toxicokinetic and Biological Matrices

The toxicological interpretation of α-PHP and α-PiHP is strongly influenced by the relationship between exposure, distribution, metabolism, elimination, and the timing of biological sampling [17,27]. The route of administration can affect the rate of absorption, systemic exposure, onset of effects, and subsequent concentration-time profiles. However, because controlled human pharmacokinetic data remain limited for these emerging cathi-

nones, toxicological interpretation should avoid extrapolating extensively from established stimulants without direct experimental support (Figure 3) [2,28–31].

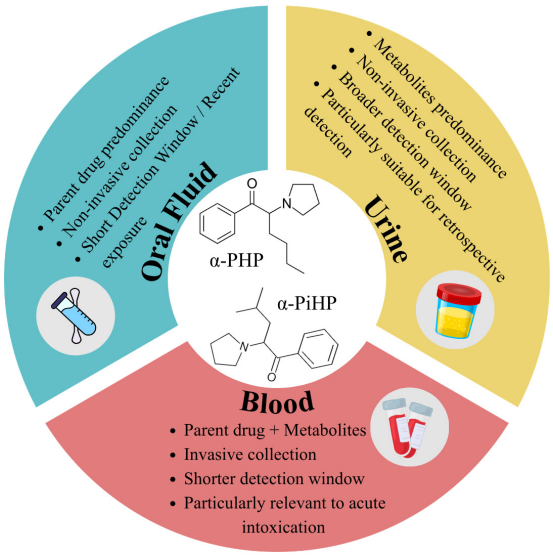


Figure 3. Overview of the main biological matrices used for synthetic cathinone detection and interpretation. Oral fluid is primarily associated with the detection of parent compounds and recent exposure due to its short detection window and non-invasive collection. Urine is mainly used for metabolite detection because of its longer detection window and suitability for retrospective exposure assessment. Blood analysis allows the detection of both parent compounds and metabolites and is particularly relevant for the evaluation of acute intoxication, although it requires invasive collection and generally provides a shorter detection window. Created by using Canva.

Biological matrix selection is therefore a critical component of exposure assessment. Blood is particularly relevant for investigating acute intoxication because it may contain both the parent compounds and their metabolites following systemic exposure. However, its relatively short detection window limits its value when sampling is delayed. Oral fluid provides a non-invasive alternative matrix that may be useful for detecting recent exposure, although low analyte concentrations, rapid clearance, variable salivary composition, and collection-related factors can affect analytical performance and interpretation [29,32–34]. Urine is generally preferred for retrospective exposure assessment because Phase I and Phase II metabolites may remain detectable after parent drug concentrations have declined. This creates both an analytical opportunity and a biomarker challenge: metabolites extend the detection window, but structurally related cathinones may generate overlapping metabolic products [35,36]. Hair provides a complementary matrix for investigating longer-term or repeated exposure. Its extended temporal window and ease of collection and storage can be advantageous in forensic investigations. However, interpretation is influenced by external contamination, cosmetic treatment, incorporation efficiency, hair characteristics, and potential differences in drug deposition. Sweat may also provide complementary information, particularly in monitoring recent or repeated exposure, but compound-specific toxicokinetic data remain limited. Other matrices, including semen, currently have insufficient evidence to support routine use for α-PHP or α-PiHP detection and should therefore be regarded as exploratory rather than established matrices [29,32,37,38].

Overall, biological matrix selection should be guided by the clinical or forensic objective, the expected detection window, and the availability of validated analytical targets. Table 2 provides a comparative overview of the biological matrices considered for α-PHP and α-PiHP exposure assessment, including their analytical relevance, advantages, limitations, and recommended analytical approaches.

Table 2. Biological matrices, analytical targets, and forensic applications for α-PHP and α-PiHP exposure assessment.

Biological Matrix	Parent Drug	Metabolites	Detection Window	Main Advantages	Main Limitations	Recommended Analytical Approach	Ref
Blood	Yes; particularly relevant in recent/acute exposure	Yes	Short; parent compounds may decline rapidly after exposure	Direct assessment of recent systemic exposure; relevant to acute intoxication and impairment; both parent drugs and metabolites can be investigated	Invasive sampling; relatively short detection window; concentrations may decrease rapidly due to distribution and metabolism	LC–MS/MS for targeted quantitative analysis; LC-HRMS for broader screening and metabolite identification	[29,30]
Oral fluid	Yes; particularly useful for recent exposure	Possible; compound-specific evidence limited	Short to relatively short	Non-invasive and easy collection; useful for recent exposure; can complement blood analysis	Low analyte concentrations; short detection window; variable matrix composition; xerostomia may compromise collection	LC–MS/MS or LC-HRMS; GC–MS/MS may be applicable for selected analytes	[17,37]
Urine	Limited/variable; often less informative than metabolites	Yes; major analytical target	Longer than blood and oral fluid	Metabolites may provide informative exposure markers; generally longer detection window than blood or oral fluid; non-invasive collection; suitable for retrospective exposure assessment.	Does not necessarily reflect impairment or current pharmacological effects; extensive metabolism can complicate parent-drug interpretation; dilution effects may affect concentrations	LC–MS/MS for targeted metabolite quantification; LC-HRMS for suspect/non-target screening and metabolite discovery	[35,36]
Hair	Potentially detectable; compound-specific evidence should be considered	Yes	Long; potentially weeks to months, depending on hair length, growth rate, and segment analyzed	Long retrospective detection window; useful for repeated or chronic exposure; easy storage and transport; segmental analysis may provide temporal information	External contamination; cosmetic treatment; differences in pigmentation and drug incorporation; interpretation is more complex than in urine or blood	LC–MS/MS or LC-HRMS; rigorous decontamination and matrix-specific validation required	[17,32]

Table 2. *Cont.*

Biological Matrix	Parent Drug	Metabolites	Detection Window	Main Advantages	Main Limitations	Recommended Analytical Approach	Ref
Sweat	Potentially detectable; limited compound-specific evidence	Potentially	Collection-dependent; extended monitoring may be possible using sweat patches	Non-invasive; useful for monitoring exposure over extended collection periods, particularly with sweat patches	Limited compound-specific pharmacokinetic evidence; collection strategy-dependent; environmental and physiological factors may influence results	LC–MS/MS or GC–MS/MS, depending on analyte and matrix	[37,38]
Semen	Limited evidence	Limited evidence	Not established	Potential alternative matrix in highly specific forensic contexts	Insufficient compound-specific evidence for α-PHP and α-PiHP; detection, disposition, and detection windows remain poorly characterized; not established for routine use	Exploratory LC–MS/MS or HRMS, further compound-specific validation required	[17]

Among these matrices, urine is particularly important for metabolite-based exposure assessment because extensive biotransformation frequently limits the usefulness of the parent compounds at delayed sampling times. The metabolic pathways underlying the formation of these urinary targets are discussed below [35,36].

4.2. Phase I and Phase II Biotransformation

α-PHP and α-PiHP undergo extensive biotransformation, with Phase I reactions generating more polar metabolites that may subsequently undergo Phase II conjugation. Reported Phase I pathways for pyrrolidinophenone cathinones include β-keto reduction, aliphatic hydroxylation and oxidation, pyrrolidine-ring oxidation, formation of lactam or 2′-keto derivatives, further oxidation, and, in some cases, ring opening and carboxylation. Phase II metabolism is dominated by conjugation reactions, particularly glucuronidation, with sulfation also reported for SCs (Figure 4) [20,35,36].

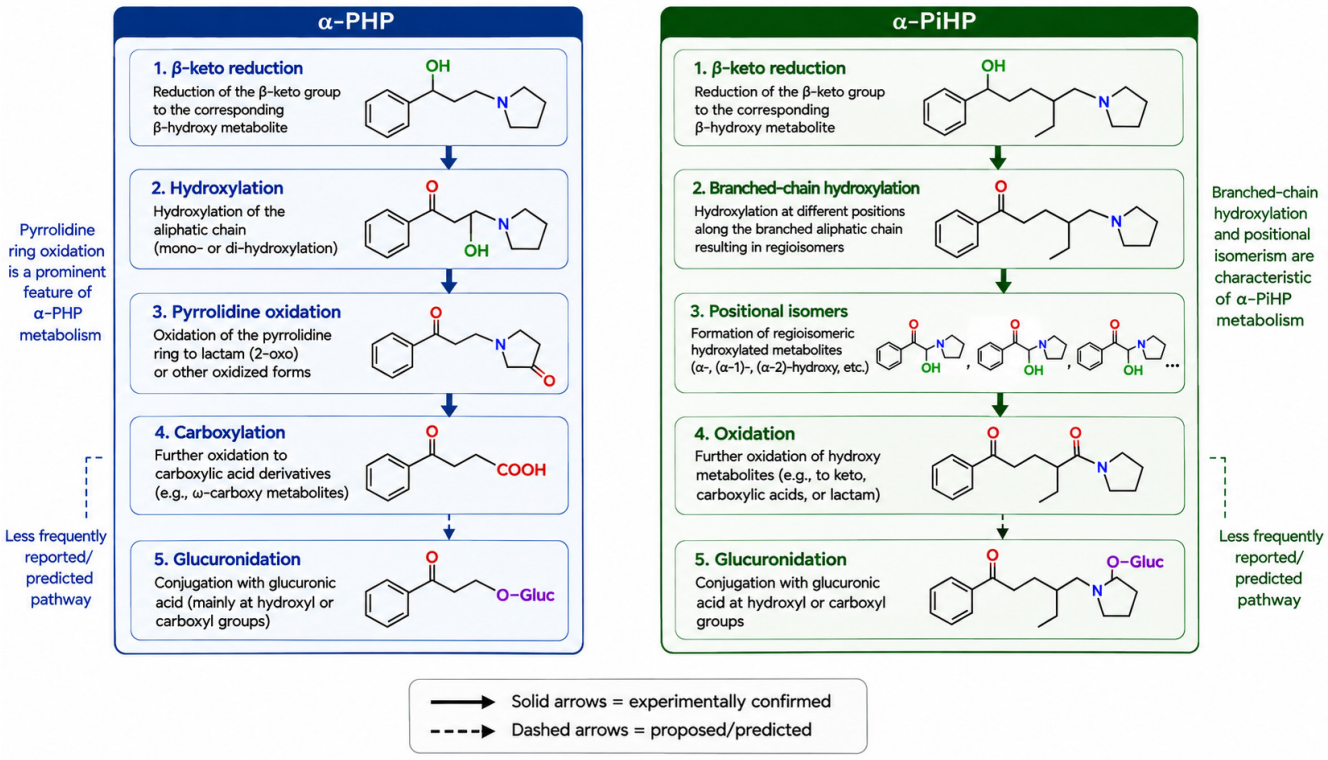


Figure 4. Comparative biotransformation pathways of α-PHP and α-PiHP. The schemes summarize the main transformation routes reported for α-PHP and α-PiHP metabolism, which is characterized by pyrrolidine-ring oxidation and subsequent carboxylation, whereas α-PiHP metabolism shows extensive branched-chain hydroxylation leading to positional isomers prior to further oxidation. Created by using BioRender.

The available evidence indicates that the overall metabolic pattern of α-PHP and α-PiHP is related but not identical. For α-PHP, studies using pooled human hepatocytes have identified several hydroxylated and pyrrolidine-oxidized products, including 2′-keto derivatives and hydroxylated side-chain metabolites. Several of these metabolites have subsequently been reported in authentic human urine, providing stronger evidence for their relevance as exposure markers [20,35].

Depending on the collection strategy, particularly when sweat patches are used, this matrix may provide a longer monitoring period than oral fluid. Nevertheless, pharmacokinetic data on SCs in sweat remains limited and is compound-specific [37,38]. In specific forensic contexts, other biological specimens, such as semen, may potentially be consid-

ered [37]. However, unlike hair and sweat, there is currently extremely limited evidence regarding the detection, disposition and detection window of SCs in semen. Further studies are therefore required before semen can be considered a reliable alternative matrix for SC detection.

Following systemic absorption, α-PHP and α-PiHP undergo extensive hepatic biotransformation broadly consistent with that described for other pyrrolidinophenone cathinones, including α-PVP (Figure 4). The metabolic pathway of these compounds is marked by sequential Phase I oxidation reactions and subsequent Phase II conjugation reactions that help in their elimination. Phase I metabolism involves CYP-mediated transformations, with CYP2D6, CYP2B6, and CYP1A2 reported to contribute to the oxidative metabolism of pyrrolidinophenone cathinones [20,39]. These reactions involve adding hydroxyl groups to the aliphatic side chain, oxidizing the pyrrolidine-ring, forming 2′-keto (pyrrolidone/lactam-type) derivatives, carrying out dihydroxylation, and further oxidation that results in carboxylated metabolites.

For α-PiHP, human liver microsome, S9-fraction, and authentic urine investigations have similarly identified β-keto reduction, pyrrolidine-ring oxidation, and aliphatic hydroxylation/oxidation pathways. The branched side chain of α-PiHP may generate additional positional isomers that complicate both metabolite annotation and analytical differentiation. The currently available evidence nevertheless indicates that some hydroxylated and oxidized products may provide discriminatory information when appropriately resolved and structurally characterized [36,40].

The level of experimental evidence should be considered when interpreting these metabolic findings. A metabolite detected only in an in vitro system demonstrates that the proposed biotransformation is chemically and biologically plausible, but it does not establish that the metabolite is formed, sufficiently abundant, or stable in humans. Conversely, detection in authentic urine provides direct evidence of occurrence following exposure, although the absence of a metabolite from a limited number of specimens cannot be taken as evidence that the metabolic pathway does not occur. Thus, metabolic evidence should be ranked according to experimental model and confirmation in authentic specimens rather than treating all reported metabolites as equivalent [20,35,36].

This distinction is particularly important for α-PHP and α-PiHP because their overlapping metabolic pathways may produce common metabolites. A metabolite should therefore be considered a useful biomarker for differentiation only when there is evidence that its formation pattern, chromatographic behaviour, structural characteristics, or relative abundance provides sufficient discriminatory power between the two isomers.

4.3. From Metabolite Identification to Exposure Biomarkers

The identification of a xenobiotic metabolite and its designation as a biomarker of exposure represent different levels of evidence. For α-PHP and α-PiHP, metabolic products can be broadly categorized according to the evidence supporting their occurrence and toxicological relevance.

Computationally predicted metabolites represent the lowest level of direct biological evidence. They are useful for hypothesis generation and for guiding targeted or suspect-screening experiments, but their formation in humans cannot be inferred solely from accurate mass or mechanistic prediction [5,40].

In vitro metabolites, particularly those generated using human liver microsomes, S9 fractions, hepatocytes, or recombinant enzymes, provide experimental evidence that a metabolic pathway can occur. These systems are highly valuable for pathway elucidation and for identifying candidate urinary targets, but differences in enzyme abundance, cellular

uptake, metabolic capacity, and incubation conditions mean that in vitro formation does not necessarily predict quantitative relevance in vivo [5,20,36,39].

Metabolites detected in authentic human specimens provide a substantially stronger basis for exposure interpretation. However, detection in a limited number of case samples is still insufficient to establish biomarker specificity or diagnostic performance. Factors such as dose, sampling time, co-exposure, matrix, metabolism, and analytical methodology may influence metabolite abundance [35,36].

A candidate exposure biomarker should therefore fulfil several criteria, including reproducible occurrence after exposure, analytical detectability at relevant concentrations, adequate stability, and a relationship with the parent compound. A discriminatory biomarker requires an additional level of evidence: it should provide information capable of differentiating α-PHP from α-PiHP or from other structurally related SCs.

The current literature suggests that several α-PHP and α-PiHP metabolites satisfy some, but not necessarily all, of these criteria. Consequently, candidate biomarkers should be interpreted according to their evidence level rather than as uniformly validated markers. A systematic distinction between predicted metabolites, experimentally generated metabolites, human-confirmed metabolites, and isomer-discriminating candidates is therefore essential for the future development of validated forensic biomarkers [20,35,36,40].

The currently reported metabolites of α-PHP and α-PiHP are summarized in Table 3. Importantly, the available evidence is heterogeneous, ranging from metabolites identified in in vitro metabolic systems to compounds detected in authentic human specimens. These levels of evidence should be distinguished when assessing the forensic relevance of individual metabolites and their potential suitability as exposure biomarkers [20,35,36].

Table 3. Reported α-PHP and α-PiHP metabolites and their level of experimental evidence.

Metabolite	Metabolic Pathway	Matrix	Analytical Platform	Reference
α-PHP				
α-PHP dihydroxy-pyrrolidinyl	Pyrrolidine-ring oxidation + dihydroxylation	Pooled human hepatocytes	LC-HRMS/MS	[20]
α-PHP hexanol	β-keto reduction (aliphatic chain hydroxylation)	Pooled human hepatocytes	LC-HRMS/MS	[20]
α-PHP 2′-keto-pyrrolidinyl-hexanol	Pyrrolidine-ring oxidation (2′-keto) + aliphatic chain hydroxylation	Pooled human hepatocytes	LC-HRMS/MS	[20]
α-PHP 2′-keto-pyrrolidinyl	Pyrrolidine-ring oxidation (lactam formation)	Pooled human hepatocytes	LC-HRMS/MS	[20]
Dihydroxy-pyrrolidinyl-α-PHP	Dihydroxylation (pyrrolidine-ring + chain)	Human urine (case series, $n = 29$)	LC-HRMS	[35]
Dihydro-α-PHP	β-keto reduction	Human urine (case series, $n = 29$)	LC-HRMS	[35]
2′-oxo-dihydro-α-PHP	Pyrrolidine-ring oxidation + β-keto reduction	Human urine (case series, $n = 29$)	LC-HRMS	[35]
2′-oxo-α-PHP	Pyrrolidine-ring oxidation (2′-oxo)	Human urine (case series, $n = 29$)	LC-HRMS	[35]
M1 (β-keto reduction)	Keto group reduction (primary metabolite)	Pooled HLMs; authentic urine ($n = 7$)	LC-QTOF-MS	[40]

Table 3. *Cont.*

Metabolite	Metabolic Pathway	Matrix	Analytical Platform	Reference
19 Phase I metabolites/9 Phase II glucuronides (overall profile)	β-keto reduction; aliphatic chain hydroxylation/oxidation; pyrrolidine-ring oxidation (lactam); glucuronidation	Human urine (single user)	LC-QTOF-MS	[35]
α-PiHP				
M01—keto group reduction	β-keto reduction	pHLMs, pS9, user urine ($n = 4$)	UHPLC-MS-QToF	[36]
M06—pyrrolidine-ring oxidation + isoalkyl chain hydroxylation/oxidation	Pyrrolidine-ring oxidation + branched chain hydroxylation/oxidation + keto reduction	pHLMs, pS9, user urine ($n = 4$)	UHPLC-MS-QToF	[36]
10 in vivo urinary metabolites (8 Phase I + 2 Phase II)	Overall profile: β-keto reduction, hydroxylation, pyrrolidine-ring oxidation, glucuronidation	pHLMs, pS9 (in vitro confirmation) + user urine ($n = 4$)	UHPLC-MS-QToF	[36]
M1 (β-keto reduction, isomer-specific)	Keto group reduction (primary metabolite)	Pooled HLMs; authentic urine ($n = 7$)	LC-QTOF-MS	[40]
9 identified Phase I metabolites (in vitro profile)	β-keto reduction; hydroxylation; pyrrolidine-ring oxidation	Pooled HLMs, 5h incubation (64.7% α-PiHP remaining unmetabolized)	LC-QTOF-MS	[40]

This leads to a wider range of Phase I metabolites and offers compound-specific metabolic fingerprints [17,41]. Even though α-PHP and α-PiHP possess the same basic skeleton, the presence of an alkyl branched side chain in α-PiHP leads to some discrepancies between their metabolic profiles.

4.4. Toxicometabolomics: From Xenobiotic Metabolism to Endogenous Metabolic Response

Conventional drug-metabolism studies and toxicometabolomics address related but distinct questions. Xenobiotic metabolism seeks to characterize the biotransformation of the administered compound and identify drug-derived metabolites, whereas toxicometabolomics investigates alterations in endogenous metabolic pathways associated with exposure. The latter may therefore provide information about the biological response to a synthetic cathinone rather than direct evidence of its metabolic fate [4,5].

Untargeted metabolomics approaches have been applied to investigate metabolic disturbances associated with synthetic cathinone exposure, particularly in experimental studies involving 3,4-methylenedioxypyrovalerone (MDPV) [42]. Available studies indicate alterations involving energy metabolism, mitochondrial function, lipid pathways, amino acid metabolism, and redox homeostasis. Such perturbations may contribute to the interpretation of mechanisms underlying cathinone toxicity, particularly where oxidative stress, mitochondrial dysfunction, or broader metabolic dysregulation are implicated [4,5,42–44].

Importantly, endogenous metabolic alterations should not automatically be interpreted as exposure biomarkers with the same forensic meaning as xenobiotic metabolites. A metabolomic feature may reflect a biological response to exposure without being specific to a particular cathinone. Consequently, toxicometabolomic signatures should be distinguished from direct exposure markers and evaluated according to their specificity, re-

producibility, dose- or time-dependence, and ability to distinguish exposure-related effects from confounding factors [44,45].

This distinction is particularly relevant for α-PHP and α-PiHP. Their close structural similarity may result in overlapping xenobiotic metabolic pathways, whereas differences in pharmacological activity or cellular response could potentially generate distinguishable endogenous metabolic signatures. However, the current evidence remains insufficient to establish robust α-PHP- or α-PiHP-specific toxicometabolomic biomarkers. Further controlled comparative studies and external validation are required before such signatures can be translated into forensic or clinical biomarkers [4,5,46].

Accordingly, toxicometabolomics should be regarded as a complementary strategy that may expand mechanistic understanding and support biomarker discovery, rather than as a replacement for targeted identification of parent compounds and their metabolites [44,46].

5. In Vitro Approaches to the Metabolism and Toxicological Evaluation of SCs

In vitro models are essential for investigating the metabolic fate and biological effects of emerging SCs, particularly when controlled human data are limited. However, these models provide complementary rather than interchangeable information, and their selection should be guided by the scientific question being addressed. In the context of α-PHP and α-PiHP, a particularly important distinction is whether the objective is to identify metabolic pathways, investigate enzyme-specific contributions, characterize cellular responses, or generate candidate biomarkers for subsequent confirmation in authentic human specimens [5,20,36,40].

5.1. Experimental Models and Their Contributions

Human liver microsomes (HLMs), S9 fractions, primary human hepatocytes, and recombinant enzymes provide complementary approaches for investigating the biotransformation of synthetic cathinones. Their main contribution is the identification of metabolic pathways and candidate metabolites that can subsequently be investigated in authentic human specimens [5,20,36,40].

HLMs are useful, particularly for investigating Phase I oxidative metabolism, including hydroxylation and pyrrolidine-ring oxidation. S9 fractions provide a broader enzymatic environment because they contain both microsomal and cytosolic components, allowing the investigation of additional metabolic reactions. Studies involving α-PiHP have used these systems to investigate β-keto reduction, aliphatic hydroxylation, and pyrrolidine-ring oxidation, contributing to the characterization of its metabolic profile [5,36,40].

Primary human hepatocytes provide a more comprehensive model because they retain a broad range of hepatic metabolic functions, including Phase I oxidation and Phase II conjugation [20]. Studies of α-PHP have identified several hydroxylated and pyrrolidine-oxidized metabolites, including 2′-keto derivatives and metabolites resulting from modifications of the aliphatic side chain. These findings have contributed to the identification of candidate urinary exposure markers [20].

Recombinant CYP enzymes provide a complementary mechanistic approach for investigating the contribution of individual enzymes to specific metabolic reactions. These types of experiments can assist in elucidating the enzyme-mediated reactions and the possible sources of individual variation in metabolism. However, the information regarding individual enzymes derived from other pyrrolidinophenone cathinones cannot be automatically generalized to α-PHP or α-PiHP without being confirmed in this case specifically [5].

Cell-based systems, such as HepG2 cells or two- and three-dimensional cell culture models, could provide further insight into cellular uptake, toxicity, oxidative stress, and

endogenous metabolism. Cell-based models are especially valuable for understanding mechanisms underlying toxicity which cannot be studied by isolated enzymatic systems. For instance, Silva et al. showed that 2D and 3D human hepatocyte-like cell cultures produced different yet complementary metabolic profiles for pentedrone and methylone. This highlights how the experimental model influences the characterization of synthetic cathinone metabolism. However, the metabolic capacity and physiological relevance of these models depend on the specific cellular model and experimental conditions used. Therefore, evidence obtained with other synthetic cathinones should be distinguished from findings directly demonstrated for α-PHP or α-PiHP [5,47].

5.2. Metabolite Identification and Confirmation in Human Biological Specimens

Although in vitro studies are valuable for predicting and characterizing metabolic pathways, confirmation in authentic human specimens is essential to establish the relevance of candidate exposure biomarkers [20,35,36].

Carlier et al. identified 24 α-PHP metabolites using pooled human hepatocyte incubations. Dihydroxy-pyrrolidinyl-α-PHP and α-PHP hexanol accounted for approximately 44% and 40% of the total metabolite peak area, respectively. These results emphasized pyrrolidine-ring dihydroxylation and β-keto reduction as significant metabolic transformations. Subsequently, Grapp et al. identified 11 Phase I metabolites and five glucuronides in human urine. They reported that dihydroxy-pyrrolidinyl-α-PHP and dihydro-α-PHP were the two most abundant metabolites, representing 45.1% and 25.5%, respectively, of the total metabolite peak area. The consistency between the metabolic pathways observed in hepatocyte incubations and in authentic human urine samples supports the relevance of these metabolites as candidate markers of α-PHP exposure [20,35].

Kemenes et al. tentatively identified ten urinary metabolites of α-PiHP, comprising eight Phase I and two Phase II metabolites. These findings were supported by parallel experiments using pooled human liver microsomes and S9 fractions. The two major metabolites were M01, associated with β-keto reduction, and M06, resulting from combined β-keto reduction, aliphatic hydroxylation, and pyrrolidine-ring oxidation. Other prominent metabolites included M04, associated with β-keto reduction and aliphatic hydroxylation; M09, associated with pyrrolidine-ring opening followed by carboxylation; and M07, corresponding to the glucuronidation of the reduced metabolite. These results highlight the importance of combining in vitro metabolic studies with analyses of authentic human specimens to identify relevant exposure markers [36].

The structural resemblance between α-PHP and α-PiHP complicates their unequivocal identification and differentiation using conventional analytical approaches. Their metabolic pathways may overlap, generating metabolites with identical elemental compositions. Consequently, accurate mass measurements alone may be insufficient to distinguish corresponding isomeric metabolites, necessitating additional chromatographic separation and structural characterization. In this context, Yeh et al. investigated a complementary strategy combining in vitro metabolic profiling using human liver microsomes, LC-QTOF-MS, and machine learning-based classification to improve the differentiation of α-PHP and α-PiHP in urine analysis [40].

Importantly, the detection of a metabolite in authentic human urine supports its potential relevance as an exposure marker but does not necessarily establish its specificity for either isomer. Therefore, the identification of discriminatory biomarkers requires direct comparative evidence demonstrating that the selected metabolite or metabolic profile can reliably differentiate α-PHP from α-PiHP [40].

5.3. Integration, Limitations, and Future Perspectives

No single in vitro model can accurately replicate the human metabolic and toxicological responses to α-PHP or α-PiHP. Therefore, a comprehensive understanding requires integrating complementary experimental systems [5,20,36,40].

Human liver microsomes (HLMs), S9 fractions, and recombinant enzymes contribute to elucidating metabolic pathways and characterizing enzyme involvement, whereas primary human hepatocytes provide broader information on Phase I and Phase II biotransformation. Cellular models, including 2D and 3D systems, offer additional insights into cellular toxicity and endogenous metabolic responses [5,20,36,40].

However, metabolites identified only in vitro should not automatically be classified as biomarkers of human exposure. Their relevance is more confidently established when metabolic products are consistently identified across complementary experimental systems and subsequently confirmed in authentic human specimens. Nevertheless, evidence of exposure does not necessarily demonstrate the ability to differentiate between structurally related cathinones [20,35,36].

Future investigations should prioritize direct comparative studies of α-PHP and α-PiHP under standardized experimental conditions that integrate metabolic profiling, toxicological assessment, and analysis of authentic biological specimens. These approaches may help identify more reliable exposure biomarkers and develop analytical strategies that can differentiate these closely related structural isomers.

6. Analytical Strategies for α-PHP and α-PiHP in Biological Matrices

The analytical determination of α-PHP, α-PiHP, and their metabolites in biological matrices is challenging because these compounds may occur at low concentrations, undergo extensive metabolism, and coexist with structurally related substances and a chemically complex endogenous matrix. Analytical performance therefore depends on the entire workflow, from sample collection and storage to extraction, chromatographic separation, detection, identification, and confirmation [48]. An overview of the analytical workflow, including biological matrices, sample preparation strategies, analytical techniques, and their main applications, is presented in Figure 5.

6.1. Sample Preparation

Sample preparation determines, to a large extent, analyte recovery, matrix clean-up, enrichment, and matrix effects. Conventional approaches include protein precipitation (PP), liquid–liquid extraction (LLE), and solid-phase extraction (SPE) [6,49].

Protein precipitation is rapid, simple, inexpensive, and compatible with high-throughput workflows, but it generally provides limited matrix clean-up and may leave endogenous components capable of producing significant ion suppression or enhancement. LLE can provide efficient extraction of lipophilic compounds and partial removal of matrix components, but it commonly requires relatively large volumes of organic solvent and involves multiple transfer and evaporation steps. SPE offers enhanced selectivity and efficient sample clean-up and is amenable to automation, although it involves more complex method development and requires careful sorbent selection [12,15].

For basic SCs such as α-PHP and α-PiHP, mixed-mode or cation-exchange SPE can provide selective retention based on the basic character of the analytes. Nevertheless, the optimal procedure is matrix- and analyte-dependent, and extraction performance should be assessed together with recovery and matrix effects rather than recovery alone [12,15].

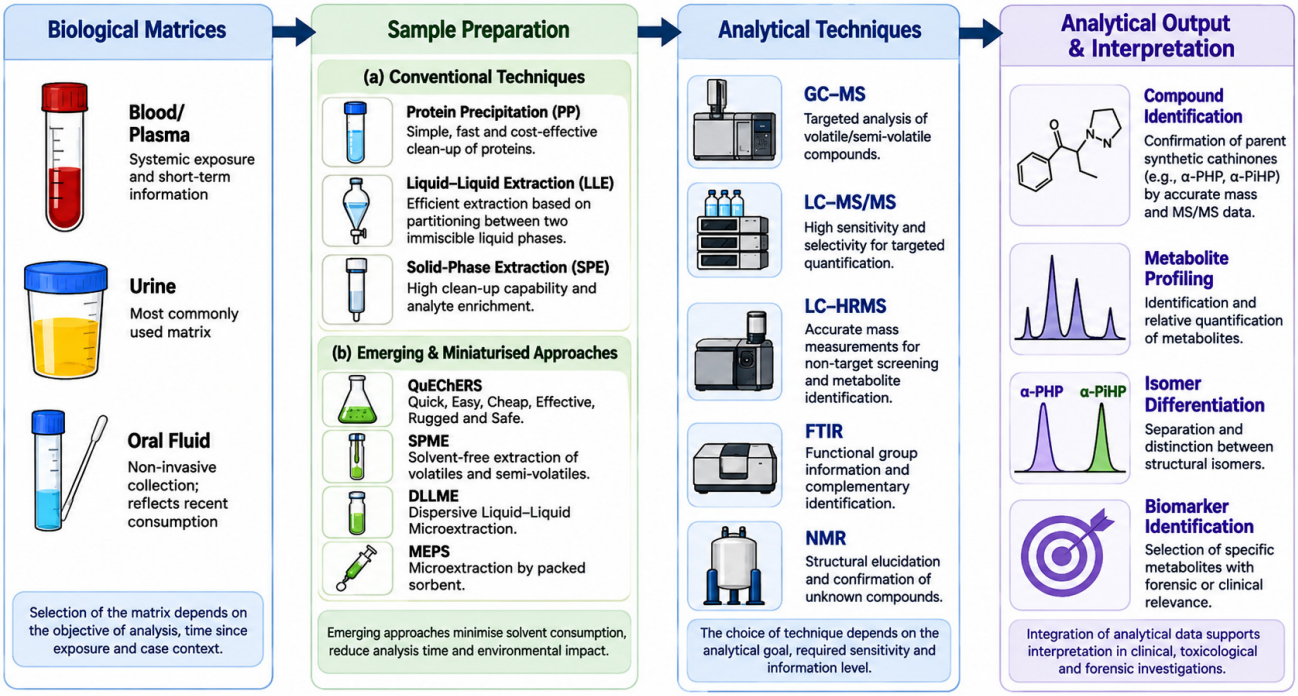


Figure 5. General workflow for the analysis of SCs in biological matrices, integrating conventional and emerging sample-preparation approaches with chromatographic, mass-spectrometric, spectroscopic, and structural characterization techniques (created by using Adobe Illustrator 2025 (version 29.0) and ChatGPT (OpenAI, GPT-6 Astra)).

6.2. Miniaturized and Greener Extraction Approaches

The need to reduce sample and solvent consumption has stimulated interest in alternative and miniaturized extraction strategies, including microextraction by packed sorbent (MEPS), solid-phase microextraction (SPME), dispersive liquid–liquid microextraction (DLLME), and QuEChERS, although their application and validation remain uneven across individual synthetic cathinones. These approaches may reduce extraction time, waste generation, and solvent consumption while providing analyte enrichment [50].

For forensic toxicology, however, "green" sample preparation should not be defined exclusively by solvent reduction. Recovery, matrix effects, reproducibility, automation potential, sample throughput, sorbent lifetime, and compatibility with validated quantitative workflows should also be considered. A miniaturized procedure with poor reproducibility or insufficient selectivity may provide limited practical advantage over a conventional extraction.

DLLME is particularly attractive because of its low solvent consumption and high enrichment potential, whereas MEPS combines SPE-type clean-up with reduced sorbent and sample requirements. SPME can provide highly selective extraction with limited solvent consumption and is particularly attractive for matrices in which volatile or semi-volatile compounds are of interest. Current evidence nevertheless remains more extensive for SCs as a broader class than for α-PHP and α-PiHP specifically [12,15,51,52].

6.3. Gas Chromatography–Mass Spectrometry

GC–MS remains an established technique in forensic analysis because of its robust chromatographic performance, reproducible electron-ionization fragmentation, and extensive spectral libraries. It is particularly useful for compound identification in seized materials and selected biological applications. However, cathinones may exhibit limited volatility or thermal stability, and derivatization can therefore be required. The use of GC–MS should accordingly be distinguished from GC-HRMS; conventional GC–MS does not inherently provide high-resolution accurate mass information [53–56]. For α-PHP and α-PiHP, GC-based strategies may be valuable where chromatographic and fragmentation behaviour provides sufficient discrimination, but the derivatization requirement and lower flexibility for highly polar or conjugated metabolites can limit their usefulness compared with LC-based methods. In comparison with LC–MS/MS, GC–MS is particularly useful for library-assisted identification and chromatographic profiling, whereas LC-based approaches are generally more suitable for the targeted determination of polar and conjugated metabolites in biological specimens [29,53].

6.4. LC–MS/MS

LC–MS/MS is currently one of the most suitable platforms for targeted quantitative analysis of SCs and their metabolites in biological matrices. It allows the analysis of polar, non-volatile, thermally labile, and conjugated analytes without the derivatization often required for GC-based approaches. Triple-quadrupole instruments operated in multiple-reaction-monitoring mode provide high sensitivity and selectivity and are therefore particularly appropriate for validated routine forensic methods [57–59]. The major limitation of targeted LC–MS/MS is its dependence on predefined analytes and appropriate standards. Unknown or unexpected metabolites may therefore remain undetected unless they are subsequently incorporated into the method. For the α-PHP/α-PiHP isomer pair, however, high analytical sensitivity does not necessarily ensure structural specificity, as shared precursor-to-product ion transitions may require adequate chromatographic resolution and confirmation using authentic reference standards [60].

6.5. High-Resolution Mass Spectrometry

HRMS provides a complementary strategy for broad screening, suspect and non-target analysis, accurate mass measurement, metabolite discovery, and retrospective interrogation of acquired data. Orbitrap and Q-TOF platforms can therefore be particularly valuable for emerging SCs for which analytical targets may change rapidly [40,48,61]. However, accurate mass alone does not necessarily establish molecular structure and cannot reliably distinguish all structural or positional isomers. For α-PHP and α-PiHP, this limitation is particularly important because closely related structures may generate highly similar precursor masses and product ions. Reliable differentiation may therefore require chromatographic separation, diagnostic fragmentation, authentic reference standards, or orthogonal structural techniques [29,40].

6.6. NMR and Complementary Structural Approaches

Nuclear magnetic resonance (NMR) provides highly informative structural data and is particularly useful for the characterization of newly emerging or previously unreported cathinones when reference materials are available in sufficient quantity. Its substantially lower sensitivity than MS-based techniques limit its direct application to trace analysis in biological matrices, but it provides valuable complementary information for structural elucidation, purity assessment, and confirmation of positional or constitutional isomers [62].

Consequently, NMR should be regarded as complementary to, rather than competitive with, MS-based toxicological analysis.

6.7. Analytical Performance and Method Validation

Comparisons between analytical methods should consider more than reported detection limits. Recovery, matrix effects, extraction efficiency, selectivity, calibration range, precision, accuracy, carry-over, stability, and the availability of authentic reference materials are all critical to forensic interpretation [63].

Published LC–MS/MS methods can achieve low ng/mL or sub-ng/mL detection limits for broad synthetic cathinone panels, but these figures should not be interpreted as compound-specific performance for α-PHP or α-PiHP unless these compounds were individually included in the validation. The current literature therefore reveals an important distinction between highly sensitive class-wide screening methods and genuinely validated, compound-specific quantitative methods for the α-PHP/α-PiHP isomer pair [29,48,53].

Overall, LC–MS/MS is generally preferable for targeted quantification, HRMS for broad screening and metabolite discovery, GC–MS for library-assisted identification and complementary chromatographic analysis, and NMR for structural confirmation when sufficient analyte is available. Nevertheless, the ability to distinguish α-PHP from α-PiHP depends primarily on demonstrated method selectivity and chromatographic or structural discrimination rather than instrumental sensitivity alone.

A qualitative meta-synthesis of the detection/quantification limits (LOD/LOQ) reported in the cited LC-MS/MS and GC-MS methods is presented in Table 4.

LC-MS/MS methods in biological matrices (urine, blood) achieve sensitivities in the 0.1–1 ng/mL range, suitable for detecting trace-level metabolites, while GC-MS methods targeting cathinones were validated mainly on seized samples (powder/tablets), not biological matrices. Their LODs (~1–5 µg/mL) reflect a pure-substance screening application rather than toxicological detection in biological fluids, a relevant limitation worth discussing in the text.

None of the reviewed LC-MS/MS methods report LOD/LOQ values specifically for α-PHP or α-PiHP; the values shown are ranges from broader cathinone panels, which reinforces the gap already identified in Section 7 of the manuscript (lack of compound-specific analytical validation for this isomer pair).

Table 4. Analytical strategies for α-PHP and α-PiHP.

Matrix	Sample Preparation	Analytical Technique	LOD/LOQ	Key Notes	Reference
Blood	Solid-phase extraction (SPE)	GC–MS-EI	Not stated (method validated for medico-legal autopsy samples)	First method combining SPE + GC–MS-EI for α-PHP in blood; achieves unequivocal chromatographic separation of the α-PHP/α-PiHP structural isomers	[53]
Blood (500 µL)	Not specified	LC-MS/MS	LOD 0.2 ng/mL; LOQ 0.5 ng/mL	Cited within the GC-MS-EI validation paper as comparative LC-MS/MS performance	[32].
Blood, urine, bile, solid tissues (postmortem)	Not specified	LC-MS/MS	Quantitative (case-specific concentrations: blood 69 ng/mL; urine 2072 ng/mL; bile 341 ng/mL; tissues 7–478 ng/g)	Fatal intoxication case report; α-PiHP detected/quantified in all specimens except hair	[33]
Blood, urine (biological specimens, general)	Protein precipitation, liquid–liquid extraction (LLE), solid-phase extraction (SPE)	LC-MS/MS, GC-MS	Not specified (method-dependent)	Reviews the three main clean-up approaches (PP, LLE, SPE) used across synthetic cathinone analytical methods	[48]
Urine, blood	1-Chlorobutane extraction + MBTFA derivatization (GC-MS); direct injection (LC-MS/MS screening)	LC-MS/MS (screening) + GC-MS (confirmation) + UHPLC-QToF (metabolite ID)	Not stated	Combined screening (LC-MS/MS, validated for amphetamines/THC-COOH panel) with GC-MS confirmation via derivatization; UHPLC-QToF used for human metabolite identification	[36]
Urine	LC-HRMS/MS (cell media and intracellular contents, applied to 7 urine samples)	LC-HRMS/MS + machine learning (PCA, logistic regression) classification	Not stated (qualitative/semi-quantitative metabolite ID)	Novel dual strategy combining in vitro metabolite profiling with ML-based spectral classification to differentiate the α-PHP/α-PiHP isomer pair	[40]
Plasma (animal model)	Not specified	LC-MS/MS (MRM)	Linear calibration curves (residuals ≤ ±20%)	MRM transitions: α-PiHP m/z 246.2 – 140.1/91.1 (CE 20 eV); comparative pharmacokinetic study vs. α-PVP in mice	[25]
Method development, standard solutions	—	LC-MS/MS, isocratic, 4 min, biphenyl column	—	Biphenyl stationary phase (vs. conventional C18) selected for superior π–π selectivity in methanolic mobile phase, enabling rapid isomer confirmation	[64]

Complementary analytical techniques also have an important role in SC characterization. FTIR can quickly and safely identify functional groups in the substance in question, although it lacks the necessary specificity to confirm the presence of cathinones and must be combined with more complex chromatography analysis techniques [54,55]. Nuclear magnetic resonance (NMR) is still considered a superior method when it comes to understanding the structure of unknown cathinone derivatives, especially in cases where reference standards are not available. Despite being less sensitive than MS-based analyses, it gives detailed information about the compound's structure [62,65].

In summary, it is important that conventional and novel methods be viewed as supplementary to each other rather than conflicting to each other. Conventional extraction methods like LLE and SPE are still significant due to their reliability and universality, while miniaturization can help decrease the amounts of samples and solvents used, waste, and extraction time. Likewise, GC–MS is significant for routine forensic analysis, while LC–MS/MS can provide high selectivity and sensitivity to detect and identify SCs and their metabolites, and HRMS increases analytical capacity toward screening, metabolite identification, and retrospective data analysis. Thus, the combination of conventional and novel methods enables higher analytical versatility in coping with structural changes in SCs.

7. Exposure Biomarkers and the α-PHP/α-PiHP Differentiation Challenge

The identification of reliable biomarkers of synthetic cathinone exposure is central to forensic and clinical toxicology, particularly when the parent compound is no longer detectable. For α-PHP and α-PiHP, however, biomarker development involves two distinct objectives: confirming exposure and determining which closely related compound was responsible. These objectives should not be considered equivalent.

7.1. Parent Compounds and Metabolic Biomarkers

The unchanged parent compound provides direct evidence of exposure but may have limited detection value when sampling is delayed because of extensive metabolism and elimination. Metabolites can extend the detection window and may therefore provide more practical targets, particularly in urine [35,36].

For α-PHP, reported human metabolites include pyrrolidine-oxidized and hydroxylated products, several of which have been observed in authentic urine samples. For α-PiHP, reported metabolites include products arising from β-keto reduction, pyrrolidine oxidation, and side-chain hydroxylation or oxidation. These findings support their use as candidate exposure markers, but the strength of evidence differs substantially between metabolites and depends on whether they have been demonstrated in authentic human specimens [20,35,36].

7.2. Biomarker Specificity

The presence of a metabolite in urine is not sufficient to establish compound-specific biomarker status. A useful biomarker should ideally demonstrate a reproducible relationship with exposure, be analytically detectable at relevant concentrations, remain sufficiently stable during sample handling and storage, and provide adequate specificity.

This distinction is especially important for α-PHP and α-PiHP because common biotransformation reactions may generate metabolites with the same or closely related structures. A marker that confirms exposure to a pyrrolidinophenone cannot necessarily identify which isomer was consumed. Accordingly, candidate biomarkers should be classified according to whether they provide evidence of class-level exposure or can contribute to isomer-specific identification [40].

7.3. Analytical Differentiation of α-PHP and α-PiHP

The differentiation of α-PHP and α-PiHP is a central unresolved analytical problem. Their parent structures are closely related, and both undergo extensive metabolism through overlapping pathways. However, differences in side-chain branching can influence hydroxylation and oxidation patterns and may therefore create metabolite distributions that are analytically informative [40].

Current evidence suggests that some α-PHP and α-PiHP metabolites may contribute to differentiation, particularly hydroxylated or otherwise oxidized products. Studies combining in vitro metabolic profiling with authentic urine samples and mass-spectrometric analysis have demonstrated differences in metabolite formation that may be exploited analytically [40].

Nevertheless, accurate mass detection alone should not be considered sufficient evidence of isomer discrimination. Structural confirmation may require chromatographic resolution, diagnostic product-ion patterns, comparison with authentic standards, or orthogonal analytical techniques. Where positional isomers are generated, the interpretation of HRMS data should therefore distinguish between molecular-formula assignment, metabolite annotation, and definitive structural identification [20,40,54,55,64].

The main analytical and interpretative challenges associated with α-PHP/α-PiHP differentiation, together with recommended strategies to address these limitations, are summarized in Table 5.

Table 5. Main challenges in differentiating α-PHP and α-PiHP and recommended analytical strategies.

Differentiation Challenge	Analytical or Interpretative Limitation	Recommended Strategy	References
Structural isomerism	Identical molecular formulas and exact masses prevent differentiation based solely on precursor-ion measurements.	Chromatographic separation and comparison with authentic reference standards.	[29,64]
Similar mass spectral fragmentation	Shared fragment ions and overlapping MS/MS transitions may limit compound-specific identification.	Evaluation of diagnostic product ions, ion ratios, and retention times.	[55,56]
Overlapping metabolic pathways	Similar oxidative and conjugative reactions may generate metabolites common to both compounds.	Comparative metabolic profiling to identify potentially distinctive metabolites.	[20,36]
Isomeric metabolites	Metabolites with identical elemental compositions may differ in the position of functional groups or sites of biotransformation.	Chromatographic resolution, MS/MS fragmentation analysis, and complementary structural confirmation when necessary.	[40,60]
Limited biomarker specificity	Detection of individual metabolites may not reliably distinguish exposure to α-PHP from exposure to α-PiHP.	Prioritization of candidate biomarkers supported by comparative metabolic studies and authentic biological specimens.	[20,25]

Table 5. *Cont.*

Differentiation Challenge	Analytical or Interpretative Limitation	Recommended Strategy	References
Limited reference standards and analytical validation	Restricted availability of metabolite standards and insufficient comparative validation limit identification confidence.	Use of authentic reference materials and compound-specific method validation.	[53,59]
Biological and metabolic variability	Differences in biological matrices, sampling times, and individual metabolism may affect metabolite detection and interpretation.	Selection of appropriate matrices and sampling windows, supported by validation across representative biological specimens.	[35,66]

7.4. Matrix Dependence of Biomarker Performance

Biomarker performance is inherently matrix dependent. Urine is particularly attractive for metabolite-based detection because of its longer detection window and high metabolite concentrations, whereas blood is more closely associated with recent exposure and acute intoxication. Oral fluid may provide a shorter window for recent exposure, and hair may provide complementary information concerning longer-term exposure [28,32,33,40,67,68].

The same metabolite may therefore have different analytical and interpretative value according to the matrix, sampling time, degree of conjugation, and extraction method. Biomarker evaluation should consequently be based on the complete analytical context rather than solely on the presence or absence of a metabolite [28,35,36].

7.5. Validation Requirements

A major limitation in the current α-PHP/α-PiHP literature is the limited availability of fully validated compound-specific biomarkers. Candidate metabolites have frequently been identified using human liver models, HRMS-based approaches, or relatively small numbers of authentic urine specimens. Although these studies are essential for biomarker discovery, they do not by themselves establish diagnostic performance [20,36].

Robust biomarker validation should include authentic specimens from well-characterized exposures, appropriate negative controls, quantitative analytical methods, assessment of metabolite stability, determination of detection windows, evaluation of potential interference from structurally related cathinones, and, critically, demonstration of specificity for α-PHP or α-PiHP when isomer discrimination is intended [35,40,66].

7.6. Towards an Evidence-Based Biomarker Framework

The current evidence supports a hierarchical approach to biomarker interpretation. Parent compounds and shared metabolites can provide evidence of exposure, while metabolites confirmed in authentic human specimens provide stronger evidence of practical forensic relevance. Candidate isomer-discriminating biomarkers require an additional level of evidence demonstrating reproducible analytical separation or structural discrimination between α-PHP and α-PiHP [35,36,40]. A schematic framework integrating clinical scenarios, biological matrices, analytical targets, instrumental platforms, and levels of interpretation is presented in Figure 6.

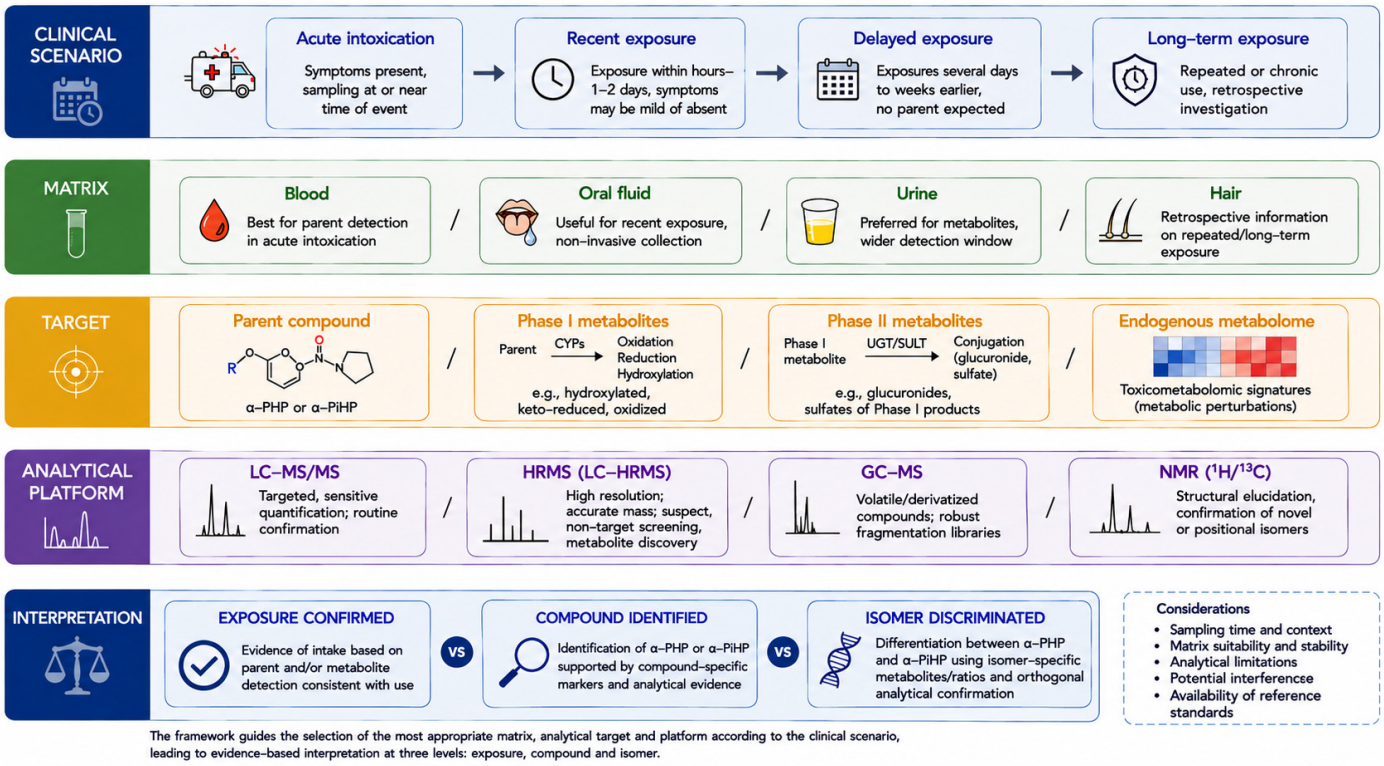


Figure 6. Evidence-based decision framework for α-PHP/α-PiHP toxicological analysis. Matrix selection, analytical target, and platform choice by clinical/forensic scenario (created by Adobe Illustrator and ChatGPT).

Future studies should therefore move beyond simple metabolite cataloguing toward quantitative and comparative biomarker validation. Integrating authentic human specimens, high-resolution mass spectrometry, chromatographic separation, reference standards, and orthogonal structural confirmation will be essential for establishing robust α-PHP/α-PiHP-specific biomarkers [40].

Untargeted toxicometabolomics may provide complementary information on endogenous biological responses, but such signatures should not be conflated with xenobiotic exposure biomarkers. Their future value will depend on demonstrating reproducibility, biological specificity, temporal stability, and external validation across independent populations and experimental settings [4,9].

The evidence hierarchy and current validation status of candidate α-PHP and α-PiHP exposure biomarkers are summarized in Table 6. This evidence-based framework supports the selection of biological matrices, analytical targets, and instrumental platforms according to the forensic or clinical objective, while distinguishing evidence of exposure from definitive compound- or isomer-specific identification (Table 6).

Table 6. Evidence hierarchy and current validation status of candidate α-PHP and α-PiHP exposure biomarkers.

Compound	Candidate Metabolite/Marker	Proposed Metabolic Pathway	Experimental Evidence	Authentic Human Specimens	Analytical Evidence	Evidence Level	Biomarker Status	Potential for α-PHP/α-PiHP Discrimination	Ref
α-PHP	α-PHP 2′-keto-pyrrolidinyl	Pyrrolidine-ring oxidation (lactam formation)	Human hepatocytes	Yes	LC-HRMS/MS/LC-HRMS	High	Candidate exposure biomarker	Low–Moderate; requires comparison with α-PiHP	[20,35]
α-PHP	α-PHP hexanol	β-Keto reduction and aliphatic hydroxylation	Human hepatocytes	Reported in authentic urine	LC-HRMS/MS/LC-HRMS	High	Candidate exposure biomarker	Moderate; hydroxylation pattern may provide discriminatory information	[20,35]
α-PHP	α-PHP 2′-keto-pyrrolidinyl-hexanol	Pyrrolidine oxidation + aliphatic hydroxylation	Human hepatocytes	Reported in authentic urine	LC-HRMS/MS/LC-HRMS	High	Candidate exposure biomarker	Moderate	[20,35]
α-PHP	α-PHP dihydroxy-pyrrolidinyl	Pyrrolidine oxidation + di-hydroxylation	Human hepatocytes	Yes; case series	LC-HRMS/MS/LC-HRMS	High	Candidate exposure biomarker	Moderate	[20,35]
α-PHP	Dihydro-α-PHP	β-Keto reduction	Human urine; HLM-related evidence	Yes	LC-HRMS/LC-QTOF-MS	Moderate–High	Candidate exposure biomarker	Low–Moderate	[20,35]
α-PHP	2′-Oxo-dihydro-α-PHP	Pyrrolidine oxidation + β-keto reduction	Human urine	Yes	LC-HRMS	High	Candidate exposure biomarker	Moderate	[20,35]
α-PiHP	M01	β-Keto reduction	pHLM, pS9	Yes; user urine	UHPLC-QTOF-MS	High	Candidate exposure biomarker	Moderate; reported as potentially isomer-specific	[36]

Table 6. *Cont.*

Compound	Candidate Metabolite/Marker	Proposed Metabolic Pathway	Experimental Evidence	Authentic Human Specimens	Analytical Evidence	Evidence Level	Biomarker Status	Potential for α-PHP/α-PiHP Discrimination	Ref
α-PiHP	M06	Pyrrolidine oxidation + branched-chain hydroxylation/oxidation + keto reduction	pHLM, pS9	Yes; user urine	UHPLC-QTOF-MS	High	Candidate exposure biomarker	Moderate–High; potentially informative for isomer differentiation	[36]
α-PiHP	Hydroxylated α-PiHP metabolites	Aliphatic hydroxylation/oxidation	Human liver systems	Yes, depending on metabolite	UHPLC-QTOF-MS/HRMS	Moderate–High	Candidate exposure biomarkers	Potentially high, particularly when positional isomers are chromatographically resolved	[36,40]
α-PiHP	Phase II glucuronide conjugates	Glucuronidation of Phase I metabolites	Human liver systems/human urine	Yes	UHPLC-QTOF-MS	Moderate	Candidate exposure biomarkers	Uncertain; structural specificity requires further confirmation	[36]
α-PHP/α-PiHP	Shared hydroxylated/oxidized metabolites	Common Phase I pathways	In vitro and/or human samples	Yes for some metabolites	HRMS-based approaches	Variable	Exposure markers rather than specific biomarkers	Low when metabolite occurs in both isomers	[20,36,40]
α-PHP/α-PiHP	Endogenous toxicometabolomic features	Exposure-associated metabolic perturbation	Cell/animal metabolomics	Generally no direct human confirmation specific to either isomer	HRMS/metabolomics	Low–Moderate	Candidate response markers	Currently unvalidated	[42–44]

Evidence level reflects the highest level of experimental support currently available and should not be interpreted as formal clinical or forensic validation. High indicates detection in authentic human specimens supported by experimental metabolic evidence. Moderate indicates evidence primarily from in vitro systems and/or limited human specimens. Low indicates predominantly putative or indirect evidence. "Candidate exposure biomarker" refers to a metabolite with potential utility for documenting exposure, whereas "discriminatory biomarker" requires demonstrated ability to distinguish α-PHP from α-PiHP and should be supported by appropriate chromatographic, mass-spectrometric, and preferably authentic reference standard evidence.

8. Conclusions

α-PHP and α-PiHP illustrate the analytical and toxicological challenges posed by closely related synthetic cathinones. Their structural similarities and overlapping metabolic pathways complicate isomer differentiation, despite potential differences in pharmacological activity, biotransformation, and analytical behaviour.

Although both compounds undergo extensive Phase I and Phase II metabolism, the evidence supporting individual metabolites varies considerably. Metabolite identification does not equate to biomarker validation, and robust human evidence supporting reliable discrimination between the two isomers remains limited. LC–MS/MS and HRMS offer complementary capabilities for targeted quantification, metabolite identification, and screening, while GC–MS and NMR provide additional analytical and structural information. Toxicometabolomics may further elucidate exposure-related biological responses but currently lacks sufficient evidence to support compound-specific biomarkers.

Future research should prioritize comparative studies using authentic human specimens, validated analytical methods, appropriate reference standards, and complementary structural confirmation. The key priority is to establish validated, isomer-discriminating biomarkers that enable reliable differentiation of α-PHP and α-PiHP in forensic and clinical settings.

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Abbreviations

The following abbreviations are used in this manuscript:

cAMP	Cyclic adenosine monophosphate
CYP450	Cytochrome P450
DAT	Dopamine transporter
DLLME	Dispersive liquid–liquid microextraction
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
GC-MS	Gas chromatography mass spectrometry
HLMs	Human liver microsomes
HRMS	High-resolution mass spectrometry
HS-SPME	Headspace solid-phase microextraction
LC-MS	Liquid chromatography-mass spectrometry
LLE	Liquid–liquid extraction
MAPK	Mitogen-activated protein kinase
MDMA	3,4-methylenedioxymethamphetamine
MEPS	Microextraction by packed sorbent
MS	Mass spectrometry
NET	Norepinephrine transporter
NMR	Nuclear magnetic resonance
NPS	New Psychoactive Substances
PP	Protein precipitation
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, Safe
SCs	Synthetic cathinones
SERT	Serotonin transporter
SLC6	Solute carrier family 6
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
UGTs	Uridine 5′-diphospho-glucuronosyltransferases
α-PHP	α-pyrrolidinohexanophenone
α-PiHP	α-pyrrolidinoisohexanophenone

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