Quantitative Analysis of 4-Hydroxyphenylacetone in Biological Samples from Amphetamine Users

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Amphetamine is a widely abused psychostimulant that can be detected in various biological matrices for forensic purposes. Its metabolite, 4-hydroxyphenylacetone (4-HPA) is used as an informative biomarker of exposure in urine. This study quantified 4-HPA levels in blood and urine samples obtained from incarcerated individuals with documented histories of amphetamine use. Samples were stratified according to abstinence periods (24–48 h, 7–8 days, 15–16 days) and compared with drug-free controls and analysed with gas chromatography–mass spectrometry (GC–MS) coupled with liquid–liquid extraction (LLE). Calibration curves for 4-HPA demonstrated excellent linearity within the range of 1–250 μg/mL (r^2 = 0.999). Concentrations of 4-HPA in urine were higher than in blood, making it more sensitive biological matrix. Peak concentrations were observed in the 24–48 h group, with a gradual decline during prolonged abstinence; however, levels remained above control values after 15–16 days. These findings confirm that 4-HPA could be used as a biomarker for amphetamine intake and support its practical utility in forensic toxicology, with urine serving as the preferred matrix for screening and monitoring.

Keywords: amphetamine, 4-hydroxyphenylacetone, GC-MS, forensic toxicology, urine biomarker

Introduction

Amphetamine is one of the most commonly misused drugs worldwide. Its growing use, especially among young people, creates major health and social problems [1–4]. In forensic and clinical toxicology, different biological samples such as blood, plasma, urine, and hair are often tested to confirm amphetamine exposure [5–7]. Reliable and accurate detection methods are therefore essential for drug monitoring, workplace testing, anti-doping control, and post-mortem toxicology [8,9].

Among amphetamine metabolites, 4-hydroxyphenylacetone (4-HPA) is important as a main urinary marker. It offers practical advantages for analysis, such as a low detection limit and short retention time in gas chromatography [10–12]. Measuring 4-HPA not only helps to confirm amphetamine use but also gives useful data for pharmacokinetic and extrapolation studies [13,14]. In forensic toxicology, tracking metabolites like 4-HPA can improve interpretation in cases where the parent drug has already been extensively broken down [15,16].

Despite its forensic relevance, systematic data on 4-HPA concentrations in authentic human biological samples remain limited. Most published studies have focused either on the parent compound [17] or on other hydroxylated metabolites [18], while 4-HPA has been investigated primarily in animal models [19] or under in vitro experimental conditions [20]. The absence of steady-state concentration data in humans represents a major limitation for its use as a biomarker. Furthermore, the potential role of 4-HPA in differentiating recent from past drug use has not been systematically addressed [13,17].

Consequently, there is a strong need to establish validated analytical methods for the sensitive and reliable quantification of 4-HPA in human samples. Gas chromatography–mass spectrometry (GC–MS) is still considered the gold standard for this type of analysis because of its high selectivity, reproducibility, and ability to confirm chemical structures [8,9,11]. Combined with liquid–liquid extraction (LLE), GC–MS offers greater sensitivity for detecting metabolites at very low levels in complex biological samples [21,22].

The present study addresses this gap by developing and validating a GC–MS method coupled with LLE for the quantification of 4-HPA in blood and urine samples obtained from confirmed amphetamine users. Samples were stratified according to abstinence periods (24–48 h, 7–8 days, and 15–16 days) and compared with drug-free controls. To the best of our knowledge, this is among the first systematic investigations of 4-HPA levels in both blood and urine of real abusers, providing insights into its kinetics and matrix dependence. By comparing different biological matrices and setting reliable quantification parameters, this study shows the potential of 4-HPA as a solid biomarker of amphetamine use and emphasizes the value of GC–MS in forensic toxicology.

Experimental part

Chemicals and reagents

Analytical standard of 4-hydroxyphenylacetone (4-HPA, ≥98%) was obtained from Sigma-Aldrich. Amphetamine sulfate (≥98%) from Cayman Chemical, Ann Arbor, MI, USA was used as a standard . Dichloromethane (DCM, HPLC grade), employed as the extraction solvent, was purchased from Merck (Darmstadt, Germany). Anhydrous sodium sulfate (Na□SO□, analytical grade) was obtained from BDH (Poole, UK) and used for drying organic extracts. Methanol and acetonitrile (both HPLC grade, Merck) were used for the reconstitution of dried residues prior to instrumental analysis. Ultrapure water Milli-Q (Millipore, Bedford, MA, USA) was used in all experiments. Stock solutions of 4-HPA (1 mg/mL) were prepared in methanol and stored at −20 °C in amber vials. Working standards were freshly prepared by serial dilution with methanol to obtain calibration solutions in the range of 1–250 μg/mL. Blank human plasma and urine samples, collected from drug-free volunteers, were used as negative matrices. These were spiked with known amounts of 4-HPA to prepare calibration standards and quality-control samples.

Instrumentation and GC–MS conditions

Instrumental analysis was performed using a gas chromatography–mass spectrometry (GC–MS) system equipped with an HP-5MS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min.

The oven temperature program was: initial temperature 100 °C (held for 2 min), ramped at 10 °C/min to 280 °C, and maintained for 10 min. The injector temperature was set at 250 °C, and injections were performed in splitless mode with an injection volume of 1 μL.

The mass spectrometer was operated in electron ionization (EI) mode at 70 eV. The ion source and quadrupole temperatures were maintained at 230 °C and 150 °C, respectively. Data acquisition was carried out in full scan mode (m/z 40–500), with selected ion monitoring (SIM) employed for quantification of 4-HPA.

Under the optimized conditions, 4-HPA had a retention time of about 8.5 min, with no interfering peaks from the biological matrix. The calibration curves were linear in the range of 1–250 μg/mL, with $r^2 = 0.999$.

Sample collection

Blood and urine samples were collected from incarcerated individuals with documented histories of amphetamine use. Sampling was carried out in collaboration with the Directorate of Narcotics and Psychotropic Substances Affairs in Wasit and the Prison and Deportation Division in Wasit. All procedures were conducted under judicial supervision, and samples were obtained only after official detention orders had been issued by the investigating judge.

The biological samples were stratified into four groups:

Group 1 (G1): 15 samples from addicts who had abstained from amphetamines for less than 24–48 h. This group represents recent use, with expected higher concentrations of amphetamines or metabolites.

Group 2 (G2): 15 samples from addicts who had abstained for 7–8 days. These individuals were expected to show decreasing drug levels, although metabolites could still be detectable.

Group 3 (G3): 15 samples from addicts who had abstained for 15–16 days. In this group, metabolite levels were expected to be low, with some subjects potentially showing signs of withdrawal or recovery.

Group 4 (Control): 15 samples from individuals with no history of drug use. This control group provided baseline values for comparison.

It should be noted that inmates testing positive for drugs upon incarceration were subjected to detention orders, and the length of incarceration depended on the documented onset of narcotic use.

Sample preparation

Sample preparation was carried out using the liquid–liquid extraction (LLE) technique, widely applied for the isolation of analytes from biological fluids [21, 22].

Whole blood was collected in anticoagulant-treated tubes, followed by centrifugation at low speed to separate plasma, buffy coat, and erythrocytes. The plasma layer was carefully pipetted off for analysis.

Plasma or urine aliquots were transferred to a separating funnel, followed by the addition of dichloromethane (DCM) at twice the volume of the biological sample. After gentle mixing and phase separation (2–3 min), the lower organic phase was collected. The extraction was repeated twice to maximize recovery.

The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The residue was reconstituted in methanol or acetonitrile prior to GC–MS analysis. Both acidic and basic extraction protocols were tested to optimize recovery.

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 25.0). Descriptive characteristics of the study subjects were assessed. Normality of data distribution was tested and showed significant deviations from normality; therefore, variables were expressed as medians with corresponding minimum and maximum values. Comparisons between control and time-dependent subgroups were conducted using the Kruskal–Wallis H test followed by Dunn's post hoc test. The Mann–Whitney U test was applied for pairwise comparisons between different sample type subgroups.

Results and discussion

Linearity and calibration

The calibration curve for 4-hydroxyphenylacetone (4-HPA) showed linearity in the range of 1–250 μg/mL, with the regression equation $y = 148201x + 429532$ and a correlation coefficient of $r^2 = 0.9996$.

No interfering peaks were observed at the retention time of 4-HPA, confirming the high specificity of the method. These results demonstrate the reliability of GC–MS for quantitative analysis of this metabolite. The strong correlation between concentration and peak area highlights the reproducibility of the method and supports its use in forensic toxicology. The system proved capable of detecting trace levels of 4-HPA with high precision and sensitivity, fulfilling the requirements for forensic casework and drug monitoring studies.

Limit of detection (LOD) and limit of quantification (LOQ)

The sensitivity of the method was further evaluated through the determination of the limit of detection (LOD) and the limit of quantification (LOQ). Using the standard deviation of the response (σ) and the slope of the calibration curve (s), the following values were obtained:

LOD = 0.3 μg/mL; LOQ = 1.0 μg/mL

These values coincide with the lowest validated calibration point and confirm the ability of the method to detect and quantify very low concentrations of 4-HPA. In comparison with published data on amphetamine detection, where LODs are typically reported in the range of 1–10 μg/mL for biological samples, the present method demonstrates superior sensitivity. This ensures its practical utility in detecting metabolite traces long after the parent drug has been eliminated.

Distribution of study groups

A total of 60 biological samples were analyzed, comprising 15 samples from each experimental group (G1, G2, G3) and 15 samples from the control group. Equal representation ensured comparability of results. The grouping of subjects, stratified by abstinence period, is illustrated in Figure 1.

Blood concentrations of 4-HPA

The analysis of blood samples showed clear time-dependent changes in 4-HPA levels (Table S1). The highest median concentration (2410.33 ng/mL) was found in G1 (24–48 h after use), confirming blood as a reliable marker of recent amphetamine exposure. In G2 (7–8 days), median levels fell to 1028.37 ng/mL, more than 50% lower than G1, and dropped further in G3 (15–16 days) to 428.27 ng/mL. The control group showed only baseline values (32.86 ng/mL), consistent with no exposure. Statistical testing with the Kruskal–Wallis test revealed strong group differences ($p < 0.001$), and Dunn's post-hoc analysis confirmed that G1 differed significantly from both the control and G3 groups. These results indicate a rapid decline of 4-HPA in blood after cessation of use, though measurable amounts remain even after two weeks. This extends the detection window beyond that of the parent drug, which is usually traceable in blood for only 2–4 days [9,11,18]. The persistence of 4-HPA underlines its forensic value as a biomarker for monitoring amphetamine use over longer periods (Figure 2).

Urine concentrations of 4-HPA

In urine, 4-HPA levels were consistently much higher than in blood across all groups (Table S2). The highest median value appeared in G1 (6622.40 ng/mL), more than twice the blood concentration, showing that urine is the more sensitive matrix for metabolite detection. As with blood, concentrations declined with abstinence, dropping to 3022.95 ng/mL in G2 (7–8 days) and 1271.15 ng/mL in G3 (15–16 days), but still remained well above the control level (192.30 ng/mL). Kruskal–Wallis testing confirmed significant differences between groups ($p < 0.001$). These results indicate that urine offers a longer detection window for amphetamine use, with measurable 4-HPA present up to 15–16 days after intake (Figure 3). This agrees with evidence that urinary excretion is the main elimination route for amphetamine derivatives [7,10]. While earlier studies reported urinary metabolite detection for up to 10 days [12,19], our data extend this period, highlighting the value of 4-HPA as a reliable biomarker for longer-term monitoring of amphetamine exposure.

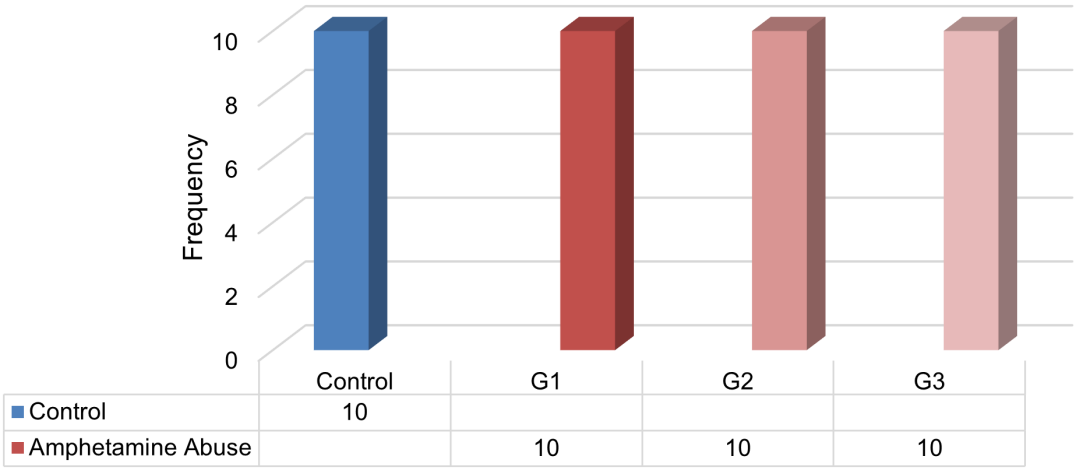

Figure 1. Distribution of participants in the four study groups: Control (drug-free), G1 (24–48 h abstinence), G2 (7–8 days abstinence), and G3 (15–16 days abstinence). Each group included 15 subjects, providing balanced representation for comparison.

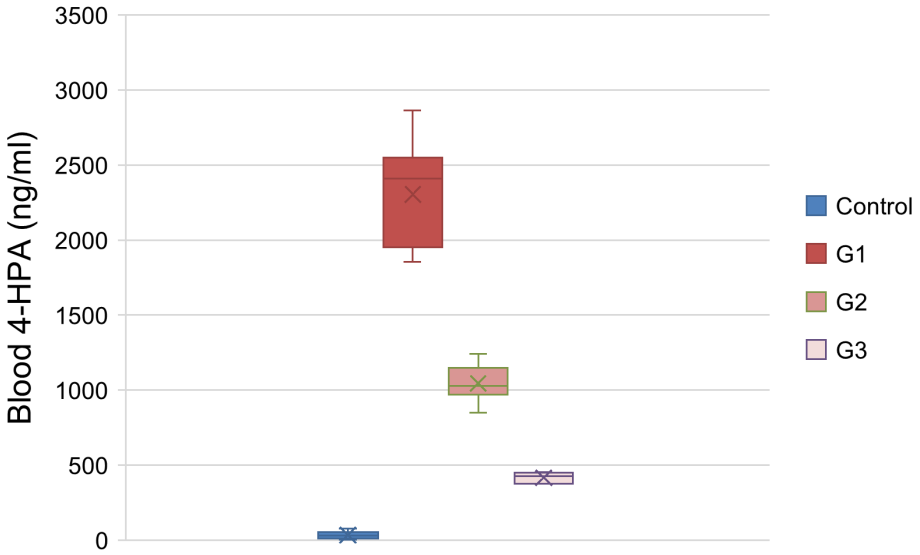

Figure 2. Box-plot of blood 4-hydroxyphenylacetone (4-HPA) concentrations across study groups (Control, G1, G2, and G3). Significantly elevated levels were observed in G1 (recent amphetamine users, 24–48 h) compared with the control group. Median values decreased progressively in G2 and G3, reflecting time-dependent clearance.

Comparison between blood and urine

Direct comparison of paired blood and urine samples (Table S3) showed that urinary concentrations of 4-HPA were always at least three times higher than those in blood. The largest difference appeared in G1 (recent users, 24–48 h), where urinary levels were more than threefold greater. Mann–Whitney U testing confirmed significant differences between the two matrices in all groups ($p < 0.001$). These results underline the diagnostic advantage of urine for monitoring amphetamine use. Blood is useful for confirming recent intake, while urine provides a wider detection window and reflects cumulative exposure. The persistence of urinary 4-HPA up to 15–16 days after last use highlights its role as a long-term biomarker, whereas blood is more suited to tracking short-term exposure. From a forensic and clinical standpoint, reliable quantification of 4-HPA adds evidentiary value: blood helps establish recent consumption, while urine gives retrospective confirmation. In addition, incorporating 4-HPA monitoring into therapeutic drug management could support compliance checks and reduce the risk of amphetamine misuse or diversion.

Interpretation of findings and forensic relevance

The results of this study show that 4-hydroxyphenylacetone (4-HPA) is a reliable biomarker of amphetamine use. A clear time trend was seen: the highest concentrations appeared within 24–48 h after intake (G1), with a steady decline at 7–8 days (G2) and 15–16 days (G3). Despite the decrease, 4-HPA was still detectable in both blood and urine two weeks after last use, confirming its value for forensic monitoring. Urine consistently contained higher concentrations of 4-HPA than blood in all groups, showing it is the more sensitive matrix for detecting exposure. Blood, on the other hand, is useful for confirming recent use. Together, both matrices provide a fuller picture of drug intake, which is important in forensic and clinical settings. These findings are consistent with published data on amphetamine metabolism, where urinary excretion is the main elimination pathway. However, earlier studies mostly focused on the parent drug or other metabolites, while systematic data on 4-HPA in real human samples are still scarce. This study adds new reference values for blood and urine across different abstinence periods.

Some limitations should be noted. The study included only young incarcerated males (18–25 years), which restricts broader application. The sample size (n = 60) was also modest, and larger, more diverse cohorts would help confirm the findings. In addition, only one analytical platform was used; further studies with LC–MS/MS or high-resolution MS could provide stronger validation. Future work could also examine stereoisomeric forms of 4-HPA and conjugated metabolites, which may give more insight into metabolic pathways and extend the detection window.

Conclusion

This study investigated the concentrations of 4-hydroxyphenylacetone (4-HPA), a major metabolite of amphetamine, in human blood and urine using GC–MS analysis. The results demonstrated clear time-dependent variations: the highest levels were observed within 24–48 h after drug intake, followed by a gradual decrease over 7–8 and 15–16 days of abstinence.

Urine consistently showed higher concentrations of 4-HPA compared to blood across all groups, confirming its greater sensitivity as a biological matrix

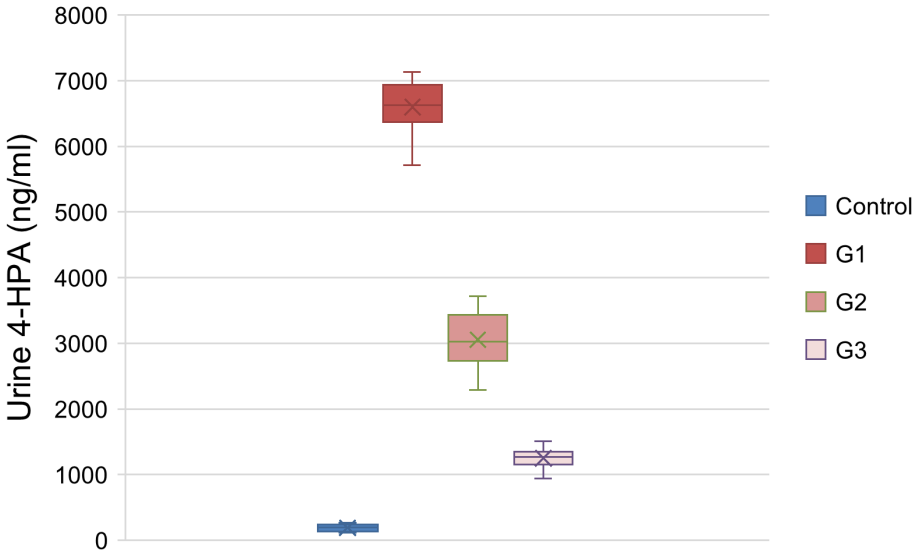

Figure 3. Box-plot of urinary 4-hydroxyphenylacetone (4-HPA) concentrations across study groups (Control, G1, G2, and G3). The highest levels were observed in G1, with progressive declines in G2 and G3, but still above control values.

for monitoring amphetamine exposure. Importantly, measurable levels of the metabolite were still detectable up to two weeks after last use, thereby extending the detection window beyond that of the parent drug.

Overall, these findings suggest that 4-HPA can be a reliable indicator of amphetamine use. By analyzing blood, we can see recent intake, and urine analysis gives us a longer history of prior use. Combining both methods makes forensic interpretation more accurate and could be useful in situations like verifying abstinence, workplace drug testing, and clinical or legal investigations.

References

1. Richards, I. N.; Richards, J. R. Does Manganese Contribute to Methamphetamine-Induced Psychosis? *Curr. Emerg. Hosp. Med.* **2020**. Springer.
2. Russel, G.; DeLucia, B. Monitoring and Successful Treatment of ADHD with Dextroamphetamine in a Patient with Progressive Familial Intrahepatic Cholestasis Type 3: A Case Report. *J. Clin. Psychopharmacol.* **2024**.
3. Drabińska, N.; Flynn, C.; Ratcliffe, N. A Literature Survey of All Volatiles from Healthy Human Breath and Bodily Fluids: The Human Volatilome. *J. Breath* **2021**.
4. Sarkar, S.; Chatterjee, R.; Mukherjee, A. Mechanochemical Synthesis and Antimicrobial Studies of 4-Hydroxy-3-thiomethylcoumarins Using Imidazolium Zwitterionic Molten Salt as an Organocatalyst. *ACS Sustain. Chem. Eng.* **2021**.
5. Sun, Y.; Tang, H.; Wang, Y. Progress and Challenges in Quantifying Carbonyl-Metabolomic Phenomes with LC-MS/MS. *Molecules* **2021**.
6. Liu, Y.; Li, L.; Yue, M.; Yang, L.; Sun, F.; Xu, G.; Fu, Y. A Switch-On Fluorescent Probe for Detection of Mesotrione Based on the Straightforward Cleavage of Carbon–Nitrogen Double Bond of Schiff Base. *Chem. Eng. J.* **2022**.
7. Chiu, H. H.; Kuo, C. H. Gas Chromatography–Mass Spectrometry-Based Analytical Strategies for Fatty Acid Analysis in Biological Samples. *J. Food Drug Anal.* **2020**.
8. Putri, S. P.; Ikram, M. M. M.; Sato, A.; Dahlan, H. A. Application of Gas Chromatography–Mass Spectrometry-Based Metabolomics in Food Science and Technology. *J. Biosci.* **2022**.
9. Brown, H. M.; McDaniel, T. J.; Fedick, P. W.; Mulligan, C. C. The Current Role of Mass Spectrometry in Forensics and Future Prospects. *Anal. Methods* **2020**.
10. Kabir, A.; Furton, K. G. Applications of Gas Chromatography in Forensic Science. *Gas Chromatogr.* **2021**.
11. Woźniak, M. K.; Banaszkiewicz, L.; Wiergowski, M. Development and Validation of a GC–MS/MS Method for the Determination of 11 Amphetamines and 34 Synthetic Cathinones in Whole Blood. *Forensic Toxicol.* **2020**.
12. Żubrycka, A.; Kwaśnica, A.; Haczkiewicz, M.; Sipa, K. Illicit Drugs Street Samples and Their Cutting Agents: The Result of the GC-MS Based Profiling Define the Guidelines for Sensors Development. *Talanta* **2022**.
13. Zeki, Ö. C.; Eylem, C. C.; Reçber, T.; Kır, S. Integration of GC-MS and LC-MS for Untargeted Metabolomics Profiling. *J. Pharm. Biomed. Anal.* **2020**.
14. Luo, L.; Dong, L.; Huang, Q.; Ma, S.; Fantke, P.; Li, J.; Jiang, J. Detection and Risk Assessments of Multipesticides in 1771 Cultivated Herbal Medicines by LC/MS-MS and GC/MS-MS. *Chemosphere* **2021**.
15. Jaiswal, S.; Dutta, P. K.; Kumar, S.; Koh, J.;

Lee, M. C. Synthesis, Characterization and Application of Chitosan-N-(4-hydroxyphenyl)-methacrylamide Derivative as a Drug and Gene Carrier. *Int. J. Biol. Macromol.* **2022**.

16. Li, C.; Gao, X.; Zhu, B.; Yuan, X.; Qiu, Z. Improve the Interfacial Properties of Carbon Fiber Reinforced Epoxy Resin Composites by Maleimide-Modified Waterborne Polyurethane Sizing Agent. *J. Appl. Polym. Sci.* **2023**.

17. Zango, M. S.; Auta, T.; Ibrahim, Y. Organic Pollutants in Water, Sediment, and Muscle Tissue of African Catfish (*Clarias gariepinus* Burchell 1822) in Ajiwa Lake. *FUDMA J. Sci.* **2024**.

18. Joseph, A.; Kumar, G. J.; Pawar, S. D. Analytical Developments of p-Hydroxy Prenylamine Reference Material for Dope Control Research: Characterization and Purity Assessment. *Drug Test. Anal.* **2022**.

19. Garedew, M.; Lin, F.; Song, B.; DeWinter, T. M. Greener Routes to Biomass Waste Valorization: Lignin Transformation through Electrocatalysis for Renewable Chemicals and Fuels Production. *ChemSusChem* **2020**.

20. David, V.; Moldoveanu, S. C. Derivatization Procedures and Their Analytical Performances for HPLC Determination in Bioanalysis. *Biomed. Chromatogr.* **2021**.

21. Hammad, S. F.; et al. Homogeneous Liquid–Liquid Extraction as an Alternative Sample Preparation Technique for Biomedical Analysis. *J. Sep. Sci.* **2022**, 45 (1), 185–209.

22. Mazzola, P. G.; et al. Liquid–Liquid Extraction of Biomolecules: An Overview and Update of the Main Techniques. *J. Chem. Technol. Biotechnol.* **2008**, 83 (2), 143–157.