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Title: Simple and Rapid: An Optimized GC–MS Workflow for Screening Amphetamine in Urine

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Gas chromatography mass spectrometry is commonly used to analyze biological samples suspected to contain amphetamine. However, samples must often be derivatized, and a balance struck between low-cost (liquid-liquid extraction (LLE)) methods that minimize solvent and sample use (solid-phase microextraction). This study developed and validated a method that does not require sample derivatization and utilizes low-cost LLE while minimizing sample and solvent use. Amphetamine was detected consistently across methanol, artificial urine, and human urine matrices. It had a retention time of 4.1 minutes, with the total runtime being 13 minutes. The limit of detection (LOD) is 0.033 µg/mL which is well below 0.25 µg/mL federal cutoff limit confirming methods utility for forensic analysis. Calibration curves demonstrated excellent $R^2 > 0.99$. The liquid-liquid extraction was with 0.5 mL solvent and 0.5 mL sample. It is the lowest extraction solvent volume using chloroform. Selected Ion Monitoring (SIM) that we targeted were 44, 65, 118 m/z. The dominant quantifier observed under our conditions was 44 m/z, which is a divergence from commonly reported base peaks of m/z 91 or 118. However, it is consistent with our instrument specific fragmentation behavior under the optimized source temperature of 230 °C. The accompanying recovery rate is 84% under basified conditions. By

eliminating derivatization and minimizing sample and solvent volume, this method offers a streamlined, reproducible workflow appropriate for routine toxicology laboratory use.

Keywords: Gas chromatography–mass spectrometry, Amphetamine, Quantity, Sensitivity

Simple and Rapid: An Optimized GC–MS Workflow for Screening Amphetamine in Urine

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Presented to the School of Science and Mathematics

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Forensic Science

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by

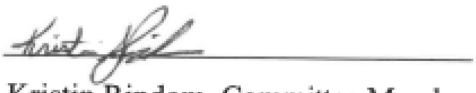
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1. INTRODUCTION

Amphetamine is one of rapidly growing drugs of abuse worldwide, creating major health and social problems (Drabińska et al., 2021; Richards & Richards, 2020; Russel & DeLucia, 2024; Sarkar et al., 2021). Given the popularity of amphetamines and the likelihood that the need to test biological samples for their presence will be encountered in a variety of circumstances, it is important to continue optimizing the confirmation and detection of amphetamine using modern instruments.

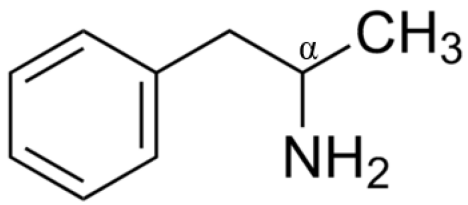


Figure 1. Amphetamine.

To understand the fundamentals of amphetamine, the molecule structure of this drug is shown in Figure 1. Amphetamine has the formula of $C_9H_{13}N$. Structurally, it has a benzene ring attached to a two-carbon chain that ends in an amine group, with a chiral carbon at the alpha position. This alpha-methyl substitution makes it more resistant to breakdown and allows it to cross the blood-brain barrier effectively. It exists as two optical isomers. D-amphetamine is more potent in the central nervous system, and L-amphetamine has stronger peripheral effects (Group, 1999). In commercial products, both isomers are combined in different ratios. For example, Adderall™ contains a 3:1 ratio of d-amphetamine to l-amphetamine salts (Child Mind Institute, n.d.; National Library of Medicine, 2024; Patel et al., 2025). In contrast, illicit amphetamine is almost always a 1:1 mixture because it is expensive and time-consuming to separate the isomers. Despite the pharmacological significance of these isomer ratios, most current research on the

detection and forensic analysis of amphetamine report total concentration (Dinis-Oliveira, 2025; Helander et al., 2025; Patel et al., 2025). Accordingly, this study focuses on the method optimization and treats the molecule as a racemic mixture.

Amphetamine is moderately lipophilic, soluble in organic solvents, and typically formulated as a salt such as amphetamine sulfate or saccharate. In humans, amphetamine is metabolized mainly through hydroxylation and deamination to form norephedrine, 4-hydroxyamphetamine, phenylacetone, and eventually benzoic or hippuric acid as end-products (Heal et al., 2013). Overall, it is a small but highly reactive molecule that acts by increasing dopamine and norepinephrine release at nerve terminals, essentially amplifying the brain's alertness and reward pathways.

This centrally acting psychostimulant is widely prescribed medically for the treatment of attention-deficit/hyperactivity disorder (ADHD) and narcolepsy; however, it also has a well-documented potential for misuse and diversion. In a 2018 study, the number of college students misusing drugs like Adderall™ was 11.1% of all enrolled students (NIDA, 2019). Between 2005 and 2010, emergency room visits involving ADHD stimulant medications rose from 13,379 to 31,244 (DAWN, 2013). From 2003 to 2015, there were 1,292,300 amphetamine-related hospital stays in the United States (Winkelman et al., 2018). Furthermore, the age-adjusted overdose death rate involving psychostimulants with abuse potential, including amphetamine, was relatively stable from 2003 to 2010 before rising sharply to 10.0 per 100000 standard population in 2021, and increasing again to 10.6 in 2023 (Garnett, 2024).

Amphetamine use spans legitimate medical indications and illicit consumption; therefore, reliable detection and quantification in biological matrices, particularly urine, remain essential for clinical monitoring, workplace testing, pain management compliance, and forensic

casework. Oral-fluid tests can detect amphetamines for 24 - 48 hours after ingestion, while blood tests can detect amphetamines for only four to six hours (FHE Health, 2026). Amphetamines may be detected in urine tests for one to five days post-ingestion in irregular users and up to seven days post-ingestion in regular users, making it a convenient matrix for estimating historical use (Verstraete, 2004). Our goal was to improve a common confirmatory testing method using Gas Chromatography Mass Spectrometer (GC–MS), making it faster and more cost effective.

In routine settings, urine drug screening is typically performed using immunoassay-based methods that are rapid, sensitive and inexpensive; however, these tests lack specificity. Structurally related compounds such as methamphetamine, MDMA, and certain over-the-counter sympathomimetics (e.g., pseudoephedrine, phenylephrine) can cause false positive results in amphetamine-class assays (Crosby & Moore, 2020). Likewise, amphetamine itself is both a parent compound and a metabolite of methamphetamine, complicating interpretation of "amphetamine positive" findings without confirmatory testing (Broussard, 2008). For these reasons, current guidelines emphasize that immunoassay results must be confirmed by a more specific analytical technique, GC–MS or liquid chromatography tandem mass spectrometry (LC–MS/MS) in clinical and forensic toxicology (Bonchev et al., 2017; Moeller et al., 2008; Pirnay et al., 2006). Although LC–MS-based techniques are selective, accurate, and precise for separation and detection in biological samples, they also require a larger volume of solvent for chromatographic separation (Vaiano et al., 2016; Woźniak et al., 2018). GC–MS methods are regarded as attractive because they can be more cost-effective and rely on a gaseous carrier phase rather than large volumes of liquid mobile-

phase solvents, thereby reducing solvent waste and the associated environmental burden (Tobiszewski et al., 2009).

Amphetamine is a small, relatively polar primary amine (Sandtner et al., 2014). Because of these properties, it can interact with active sites in the GC inlet or column, leading to peak tailing or reduced sensitivity. Derivatization produces a compound which is chemically modified from the parent compound (J. Y. Kim et al., 2010). It is commonly used in GC–MS analysis of amphetamine to reduce polarity of the compound and reactivity with the column, improve volatility, and results in chromatograms with sharper, more symmetrical peaks (Nair et al., 2013).

However, avoiding derivatization simplifies the analytical workflow, reduces sample preparation time, minimizes reagent cost and chemical waste, and decreases variability introduced during chemical modification (J. Y. Kim et al., 2010; Lin et al., 2024). The aim of this study was to develop and validate a simpler, faster, low-cost, ‘greener’ GC–MS-based method for underivatized amphetamine that was comparable in sensitivity and specificity to more complex, costly methods that could be utilized for routine confirmatory testing in clinical and forensic toxicology laboratories. This was accomplished by optimizing extraction solvents, column and ion choice, and instrument parameters. The result was a method that achieves limits of detection/quantitation (LOD/LOQ) that are similar to currently used, more labour-intensive methods.

2. METHODS AND MATERIALS

2.1. Standards and Reagents

All chemical reagents were of analytical grade or higher. Amphetamine reference standards (d-amphetamine), the isotopically labeled internal standard, synthetic urine, phenylacetone, benzoic acid, 4-hydroxyamphetamine, and caffeine were purchased from Sigma-Aldrich in Saint Louis, MO, USA. HPLC-grade methanol, hydrochloric acid (HCl), norephedrine, sodium hydroxide, chloroform, sodium acetate, and other reagents were obtained from Fisher Scientific in Pittsburgh, PA, USA. Diphenhydramine in Benadryl™ obtained as over-the-counter medication from CVS pharmacy in Emporia, KS, USA.

2.2. Preparation of Standards and Samples

Throughout the experimental process, urine samples and standard solutions were stored at 4 °C in a refrigerator when not actively being processed to reduce degradation of the amphetamine in spiked synthetic urine samples and to minimize bacterial growth. Prior to GC–MS injection, samples were allowed to equilibrate to room temperature after removal from the refrigerator to ensure consistent injection conditions.

2.2.1. Standard Preparation

A series of amphetamine standards were prepared in methanol at concentrations of 5, 10, 50, 100, and 200 µg/mL. These values were chosen to cover the expected concentration range in forensic urine testing, with a lower limit near the cutoff value for screening tests and an upper limit within toxic exposure levels. A fixed volume (10 µL) of 100 µg/mL amphetamine-d_5 internal standard (final concentration = 5 µg/mL) was added to each calibration standard.

The following amphetamine-related metabolites were included in the interference study: norephedrine, diphenhydramine, 4-hydroxyamphetamine, phenylacetone (amphetamine synthesis precursor), caffeine and benzoic acid (end-products of toluene metabolism, chromatographic

co-elution factor). The interference mixture was prepared at 100 µg/mL for each interference compound with 100 µg/mL of amphetamine, and then similarly extracted for analyses.

2.2.2. Artificial Urine Preparation and Extraction

Synthetic urine was used as a model matrix for amphetamine quantification via GC–MS. Each sample contained 500 µL of artificial urine with 20 µL of amphetamine stock (concentration varied from 5 to 80 µg/mL final) and sodium acetate. The pH was adjusted with 50 µL of 1 M NaOH. If the pH remained less than 10, an additional 50 µL of NaOH was added to achieve a target pH range of 10 - 12, confirmed using pH indicator paper.

To each pH-adjusted sample, 500 µL of chloroform was added and the samples were vortexed for 30 seconds. The organic chloroform layer, which contained amphetamine, was carefully extracted and placed into a new screw-cap tube. This procedure was repeated five times for each sample concentration. The combined organic layers were evaporated using helium gas until dry.

Dried residues were reconstituted in 200 µL of methanol, vortexed thoroughly, and transferred to GC–MS vial inserts. A 5 µL aliquot of internal standard (amphetamine-d_5 at 100 µg/mL) was added to each reconstituted sample. A blank sample (no amphetamine added, only extraction) and a positive control (50 µg/mL amphetamine + internal standard) were processed in parallel.

2.2.3. Human Urine Preparation and Extraction

Human urine samples were obtained from volunteers and anonymized (e.g., labeled 1 - 9) before and after amphetamine intake. All specimen that contained amphetamine were from discarded specimen of donors with previously prescribed amphetamine-based medications as

part of their normal medical treatment. No medication was administered, altered, or supervised by the researchers as part of this study. Both positive and negative control samples were prepared according to Section 2.2.2. To adjust the pH of the urine sample, 50 µL of 1 M NaOH were used per sample. A second 50 µL of NaOH was added as needed to ensure pH > 10.

Liquid-liquid extraction (LLE) was conducted with 500 µL of chloroform, followed by 10 - 15 seconds of vortexing. Sodium acetate was not used in later runs to avoid cloudiness. After centrifugation, the bottom chloroform layer was separated and pooled across five repetitions for each sample. The pooled extract was dried under helium gas until all solvents were evaporated.

Dried residues were reconstituted in 200 µL methanol and vortexed. 5 µL of amphetamine-d_5 (100 µg/mL) internal standard was added. Care was taken in pipetting order to avoid cross-contamination between samples. Urine samples from anonymized specimens, those that allegedly did not have amphetamine, were extracted identically.

2.3. Instrumentation and Instrument Parameters

An Agilent system 7820A GC–MS was utilized for this study. The GC–MS was operated in both Total Ion Count (TIC) and Selected Ion Monitoring (SIM) modes. An autotune was performed each day before sample runs. Instrumental parameters were as follows:

- Column: HP-5Q(19091s-433Q) ultra inert, ultra-low-bleed (30 m × 0.25 mm × 0.25 µm)
- Carrier gas: Helium
- Injection volume: 1 µL, split ratio 10:1
- Injection temperature: 250°C

- Oven program: Initial temperature at 100°C (hold for 1 min), ramp to 250°C at 15°C/min, hold for 5 min
- MS source: Electron Ionization (EI) at 70 eV
- Source temperature: 230°C
- Quadrupole temperature: 150°C
- Mass scan range: 40 - 300 m/z

2.4. Analysis

Each standard was analyzed in triplicate. Blank (methanol-only) and positive controls (methanol + 50 µg/mL amphetamine) were included in each run. The calibration curve was generated by plotting the ratio of analyte to internal standard peak areas against the known concentrations. To assess reproducibility, the relative standard deviation (RSD) of the positive and negative control peak areas was calculated across replicates.

2.4.1. Calibration Using Internal Standard

The calibration curve was constructed by plotting peak areas against known amphetamine concentrations to confirm linearity and response accuracy.

$$\frac{Peak\ area\ of\ analyte}{Concentration\ of\ analyte} = F\left(\frac{Peak\ area\ of\ internal\ standard}{Concentration\ of\ internal\ standard}\right) \quad \text{(Equation 1)}$$

Where peak area of analyte is the peak area of amphetamine at a selected m/z fragment, area of internal standard is the corresponding peak area of the internal standard, and concentrations are in µg/ml, F is the response factor.

When there were known concentrations of amphetamine and internal standard used, Eq. 1 was used to calculate the response factor (F) based on the peak areas of amphetamine and the internal standard. Then the response factor can be used to calculate the unknown concentration of amphetamine in human urine samples, based on the peak areas of amphetamine and internal standard. This equation allows quantification of the amphetamine concentration relative to the internal standard concentration, regardless of sample injection volume variation between runs.

Calibration curves were constructed using Microsoft Excel™. The linear equation and the R^2 value were displayed with the calibration curve.

2.4.2. Ion Selection

Quantifier and qualifier ions were selected through empirical evaluation of candidate fragments identified in full-scan mass spectra, with final selection based on signal intensity, signal-to-noise ratio, reproducibility, and minimal susceptibility to matrix interference under the established analytical conditions. For section 2.2, the summed response of the selected ions was used as the quantifier to improve sensitivity, while individual ions were retained as qualifiers for confirmatory identification.

2.4.3. Limit of Detection and Relative Standard Deviation

Limit of Detection (LOD) and the relative standard deviation (RSD) values were calculated using the following equations.

$$LOD = 3 \times (SD / slope) \quad \text{(Equation 2)}$$

$$RSD\ (\%) = (SD / Mean) \times 100 \quad \text{(Equation 3)}$$

Where SD is the standard deviation, Mean is the arithmetic mean, calculated by Microsoft Excel™. Slope is calculated using linear regression function in Excel™.

2.4.4. Recovery Rate

During the liquid-liquid extraction, samples were prepared by adding base (NaOH). The liquid-liquid extraction followed Section 2.2. Then the recovery rate was calculated using Equation 4.

$$\text{Recovery (\%)} = (\text{Calculated Conc.} / \text{True Conc.}) \times 100 \quad \text{(Equation 4)}$$

Where the calculated concentration is based on the quantification of analyte, true concentration is known as concentration or actual added amount of analyte.

3. RESULTS

3.1. Preliminary Test of AMP in Methanol with Calibration Curve

3.1.1. Retention and Chromatographic Behavior

To establish the method for detection of amphetamine, standards were prepared and run on the GC–MS to investigate the retention time, LOD, oven temperature, and other important parameters for the method. Amphetamine was consistently eluted at a retention time of 4.1 minutes as shown in Figure 2, which shows the stacked Total Ion Count (TIC) chromatograms for amphetamine at 5, 10, 50, 100, and 200 μg/mL. The amphetamine peak has a stable retention time and confirmed method reproducibility.

This range was selected based on values reported in clinical and forensic toxicology literature, where therapeutic concentrations in urine typically range from 50 to 1000 ng/mL and

toxic levels can exceed 2000 ng/mL (SAMHSA Certified Labs, 2025). The upper limit of 200 µg/mL ensures sensitivity across relevant biological ranges.

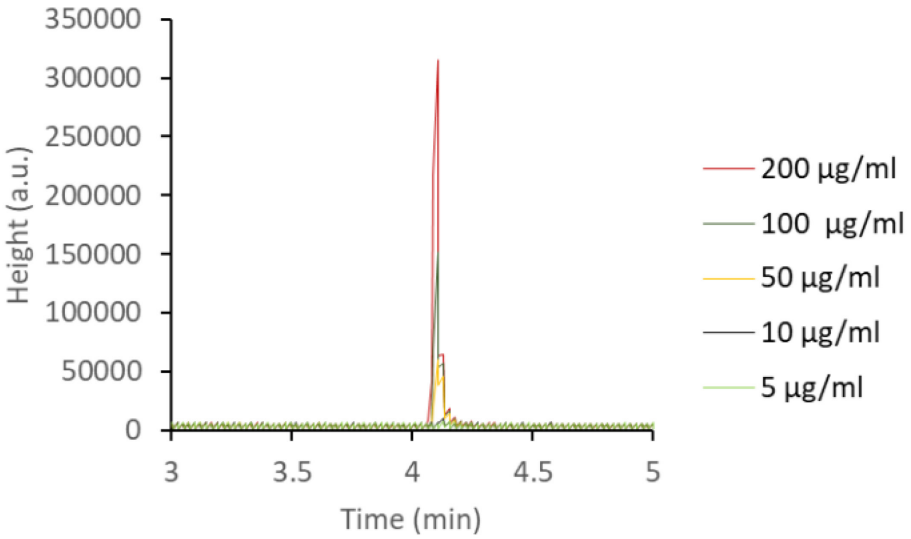


Figure 2. Stacked TIC chromatogram of amphetamine samples at 5 concentrations of 5, 10, 50, 100 and 200 µg/mL.

3.1.2. SIM Quantification and Peak Integration

Selected Ion Monitoring (SIM) was used to enhance specificity by targeting key amphetamine fragments. Using SIM mode has multiple benefits, including focusing the detector on target m/z fragments and eliminating other interference peaks and signals. Shown in Figure 3, the chromatograph of SIM has a sharper peak and a more stable baseline. Thus, SIM provides a better signal-to-noise ratio, which can lead to lower detection limits and high sensitivity and selectivity.

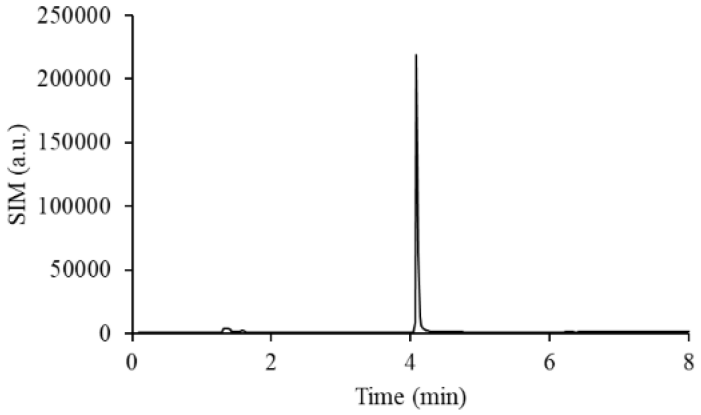


Figure 3. Typical SIM chromatogram of amphetamine at 5 µg/mL.

Initially, the fragments of m/z = 44, 65, 91, and 118 were chosen as key fragments (also called quantifier ions) for amphetamine. Figure 4 also shows key fragments of internal standard (amphetamine-d_5), which has 5 unit of mass higher than amphetamine. And fragments of m/z = 49, 70, 96, and 123 were selected as the quantifier ions for amphetamine-d_5, which are also aligned with previous published studies (Moreno et al., 2015).

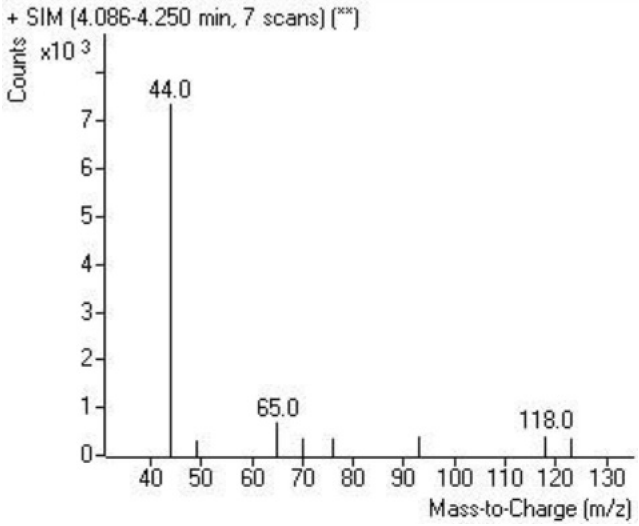


Figure 4. Mass spectra of amphetamine and amphetamine-d_5 generated using Agilent MassHunter.

Once the three key fragments were set up in the SIM mode of the MS, results of the GC–MS were analyzed using Agilent Mass Hunter software, which allowed for peak area integration and baseline correction.

By employing the described method, standards of 5, 10, 50, 100, and 200 µg/mL amphetamine were run. Each standard also contained 5 µg/mL internal standard (amphetamine-d_5). The solvent of all standards was pure methanol. All samples were injected and run in triplicate. Figure 5 shows an example of the SIM chromatograms of amphetamine peak with the selected m/z of key fragments of amphetamine and amphetamine-d_5 peak area. From top to bottom, five chromatograms were made from standards with concentrations of 5, 10, 50, 100, and 200 µg/mL.

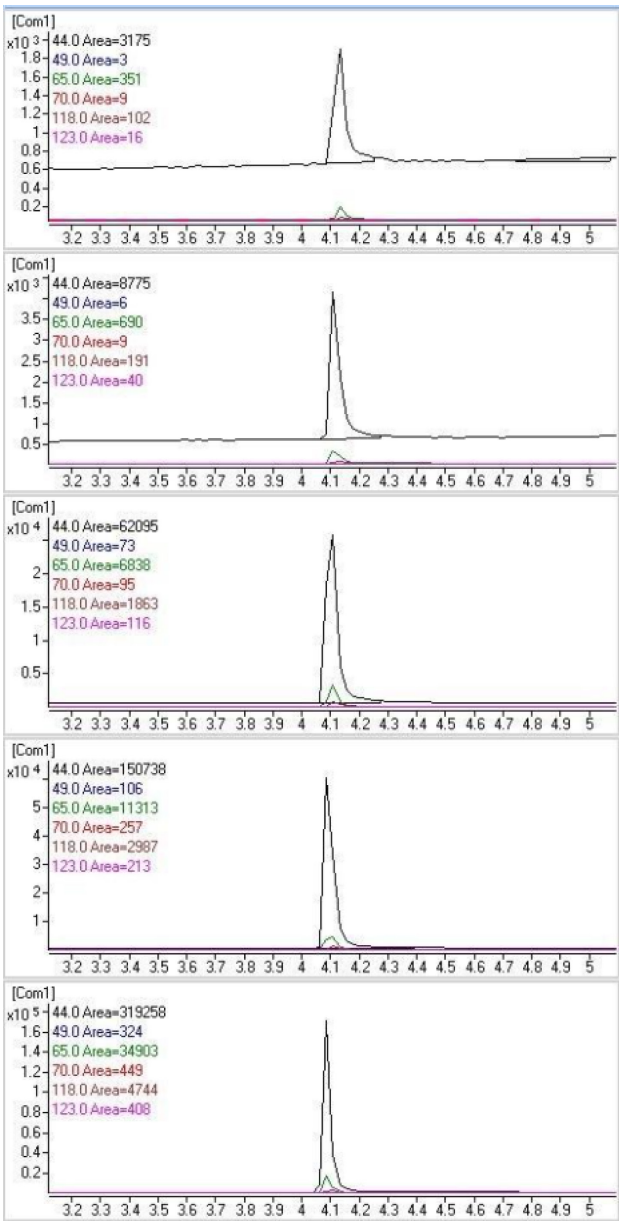


Figure 5. Integrated SIM chromatograms for amphetamine from top to bottom at concentrations of 5, 10, 50, 100, and 200 µg/mL.

Standards of 5, 10, 50, 100, and 200 µg/mL amphetamine and one blank were run using the method described in section 2.2.2. The reported values were averaged across the three runs. The area under the graph in figure 5 is summarized in Table 1.

Table 1. Average peak area from three replicate runs at selected m/z values.

	Average Peak area at each m/z							
Conc. (µg/mL)	44	49	65	70	91	96	118	123
5	5683	120	393	111	46	106	122	138
10	8814	143	952	123	84	116	190	157
50	61860	103	7389	150	805	131	1850	128
100	150715	108	14001	132	1290	127	3082	127
200	319100	132	34968	128	3015	135	5377	136

Low abundance of 91 m/z was observed and excluded from all subsequent trials. For the rest of this paper, 44, 49, 65, 70, 118, 123 m/z values were assigned to the software (Agilent MassHunter) for the integration of the peak area of amphetamine and internal standard.

3.1.3. Response Calibration and Internal Standard Normalization

By using the results from Table 1, the response factor(F) from equation 1, section 2.4.1 was calculated for calibration. The average peak area of the internal standards was then used to calibrate the peak area of amphetamine. The calibrated peak areas for m/z = 44, 65, and 118 are displayed in Table 2, which was used to construct the calibration curves.

Table 2. Calibrated peak area using Equation 1 for amphetamine in methanol.

Conc. (µg/mL)	44	65	118
5	6437	1057	142
10	15342	2303	323
50	55251	13006	1318
100	116296	25218	2610
200	283123	53613	5589

3.1.4. Calibration Curves of Amphetamine in Methanol

Amphetamine showed a consistent retention time of 4.1 minutes, demonstrating method efficiency. Using an internal standard, response factors were calculated for each run based on four major fragments. These response factors were used to calibrate the concentrations of amphetamine (AMP) standards. Figures 6, 7 and 8 present the calibration curves for m/z = 44, 65, and 118, respectively. The summed ion of all three, in Figure 9, acts as the quantifier. All calibration curves demonstrated excellent linearity, with R^2 values close to 1.0, validating the method's robustness and quantification reliability for mid to high level AMP concentrations.

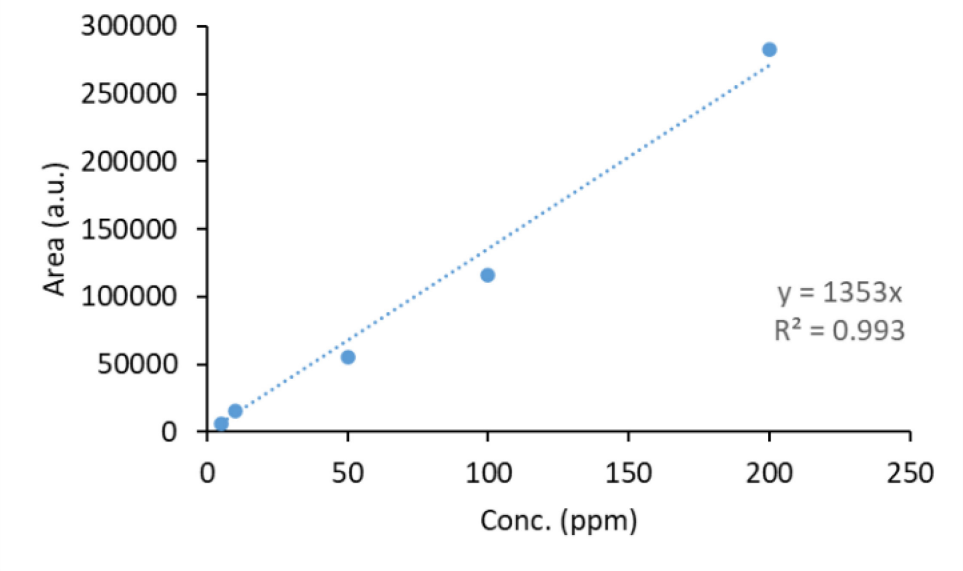


Figure 6. Calibration curve based on m/z =44.

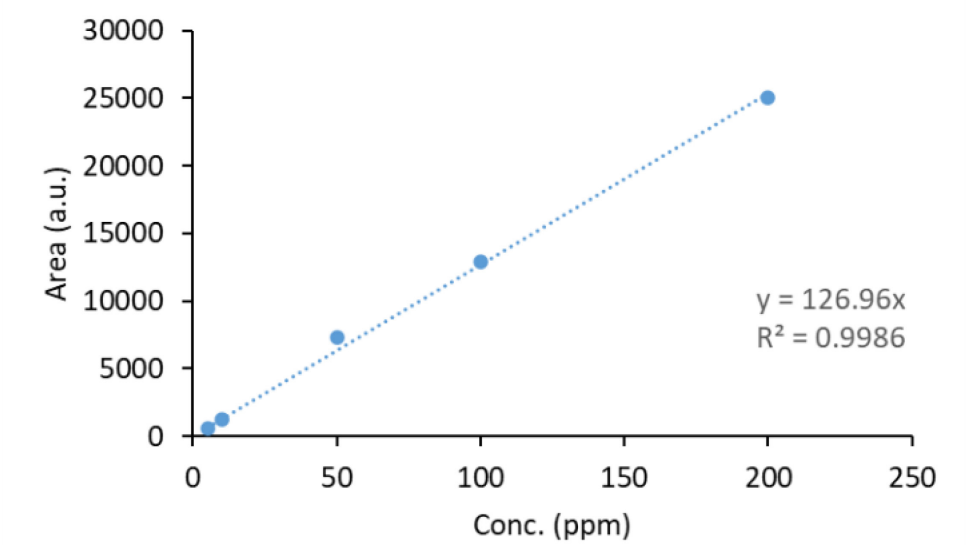


Figure 7. Calibration curve based on m/z = 65.

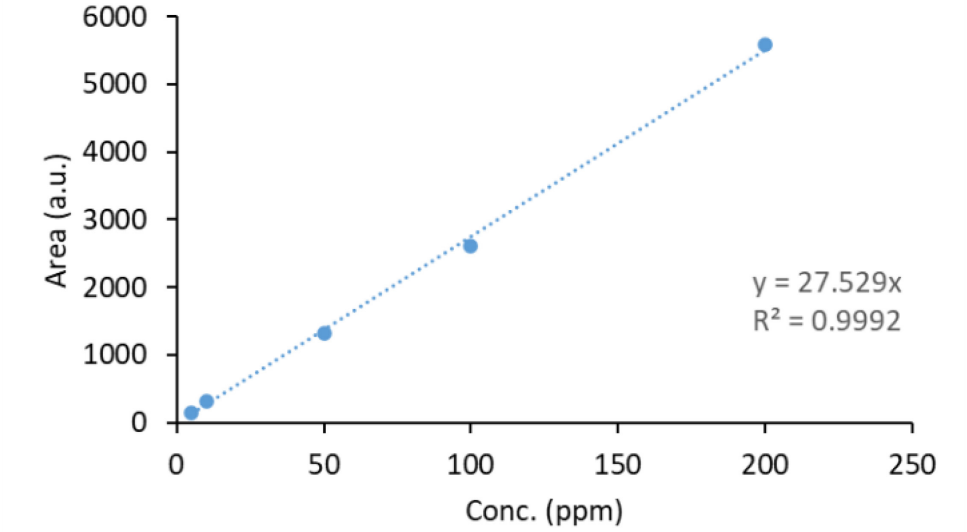


Figure 8. Calibration Curve based on m/z= 118.

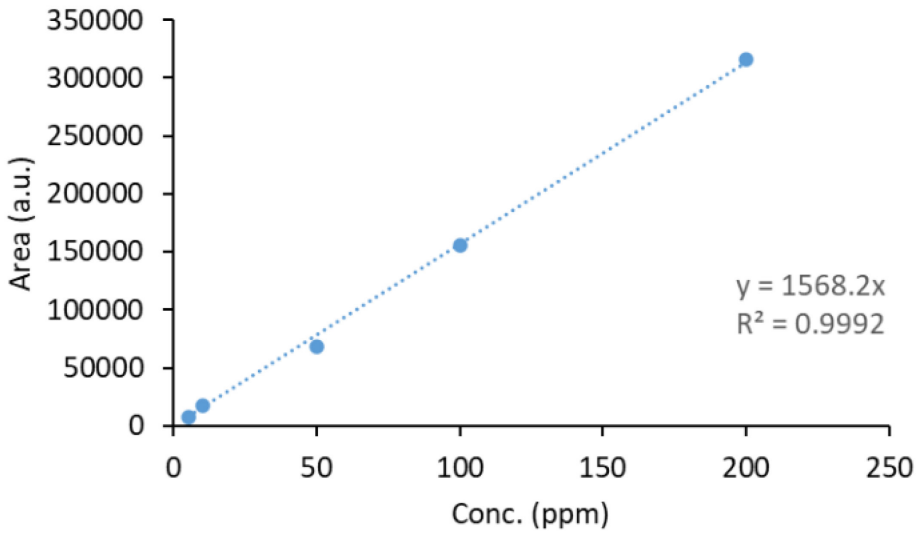


Figure 9. Calibration curve using summed ion quantification in methanol.

The calculated LOD was 28 ng/mL by using Table 2 and Equation 2, which meets forensic cutoff requirements. The Relative Standard Deviation (RSD) was calculated to be less than 5%. The method developed in this section (Section 3.1) was used in the following 3.2 and 3.3 Sections.

3.2. Standards in Artificial Urine

3.2.1. Calibration Curves of Amphetamine in Artificial Urine

Amphetamine consistently eluted at ≈ 4.1 minutes. Calibration curves showed better reproducibility for peak areas of m/z = 65 and 118, while lower fragments (e.g., m/z = 44, 49) often had lower reproducibility (lower R^2 value). Figure 2 presents an example TIC overlay, and Table 3 lists peak areas across a range of concentrations from 5 - 200 µg/mL, and Figure 9 is an example of the calibration curves for summed ion quantification similar to that in artificial urine.

Table 3. Calibrated peak area using Equation 1 for amphetamine in artificial urine.

	Conc. (µg/mL)							
m/z	5	10	20	50	80	100	150	200
44	6	11	18	48	88	111	173	253
65	155	473	742	2223	3136	3998	6077	8753
118	115	207	540	1490	2327	2917	4495	6211

Calibration curves were constructed with R^2 values were > 0.99, confirming linearity (Figure 10). The Relative Standard Deviation (RSD) for positive control replicates (50 µg/mL of amphetamine standard) was within acceptable limits (<10%), indicating method precision.

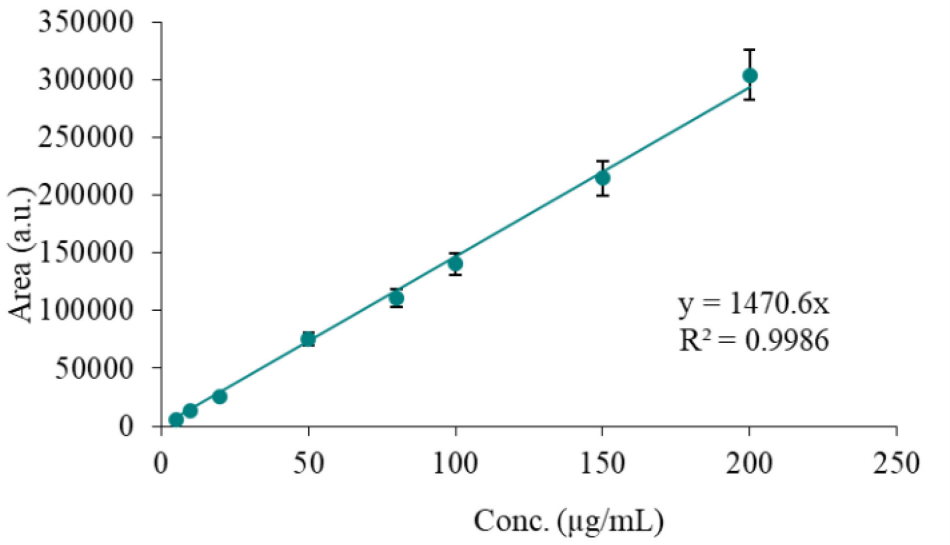


Figure 10. Calibration curve using summed ion quantification in artificial urine.

The summed ion quantification shown in Figure 10 increases total signal intensity, reduces random noise and improves LOD precision at low concentrations. The calculated LOD was 0.033 µg/mL by using Eq. 2, which meets forensic cutoff requirements. The Relative

Standard Deviation (RSD) was calculated to be less than 5. The recovery rate was calculated to be 84% using Eq. 4.

3.3. Interference Study Results

The interference compounds used were norephedrine, 4-hydroxyamphetamine, phenylacetone (amphetamine synthesis precursor), diphenhydramine, caffeine, and benzoic acid (shared aromatic ring structure). The peak at 1.5 minutes was the solvent front peak, with other small mass compounds. In Figure 11, the amphetamine peak was still at 4.1 minutes and may have partially co-eluted with matrix components as the peak was not very symmetrical. Peaks at 7.2 minutes were phenylacetone, at 9 minutes benzoic acid, then at 10.3 minutes was 4-hydroxyamphetamine which overlapped with norephedrine. Caffeine and diphenhydramine resulted in a cluster of peaks around 12 minutes.

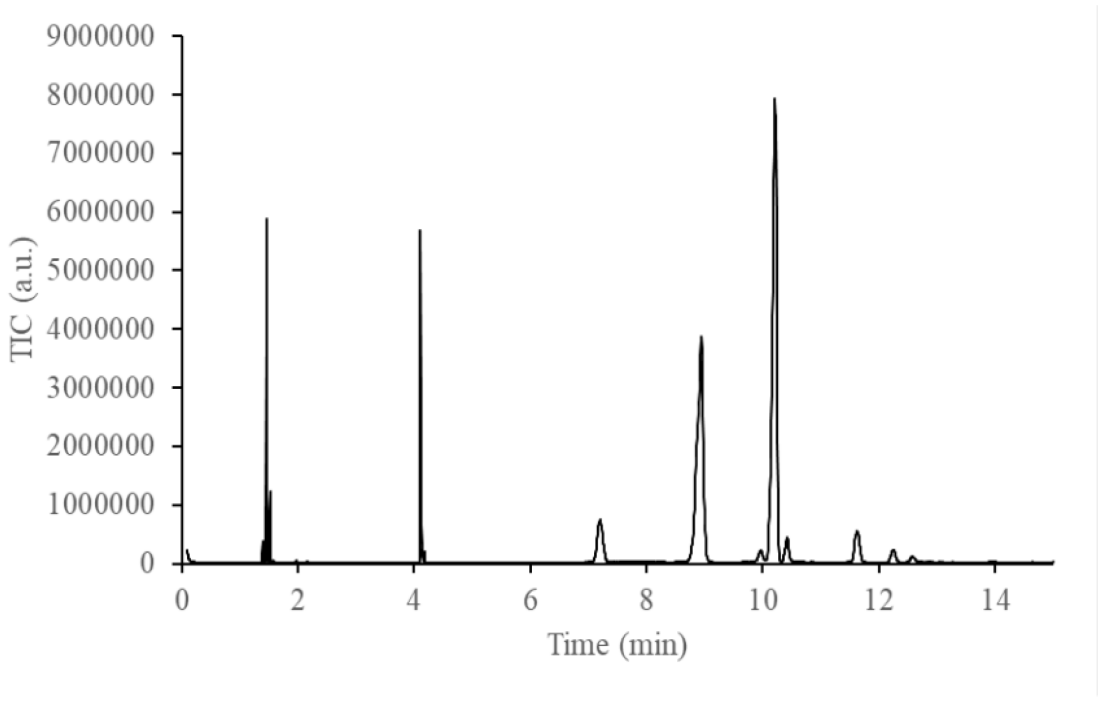


Figure 11. TIC of interference study.

Comparing Figure 11 to Figure 12 shows that with SIM, the chromatogram was clean and symmetrical for an amphetamine peak.

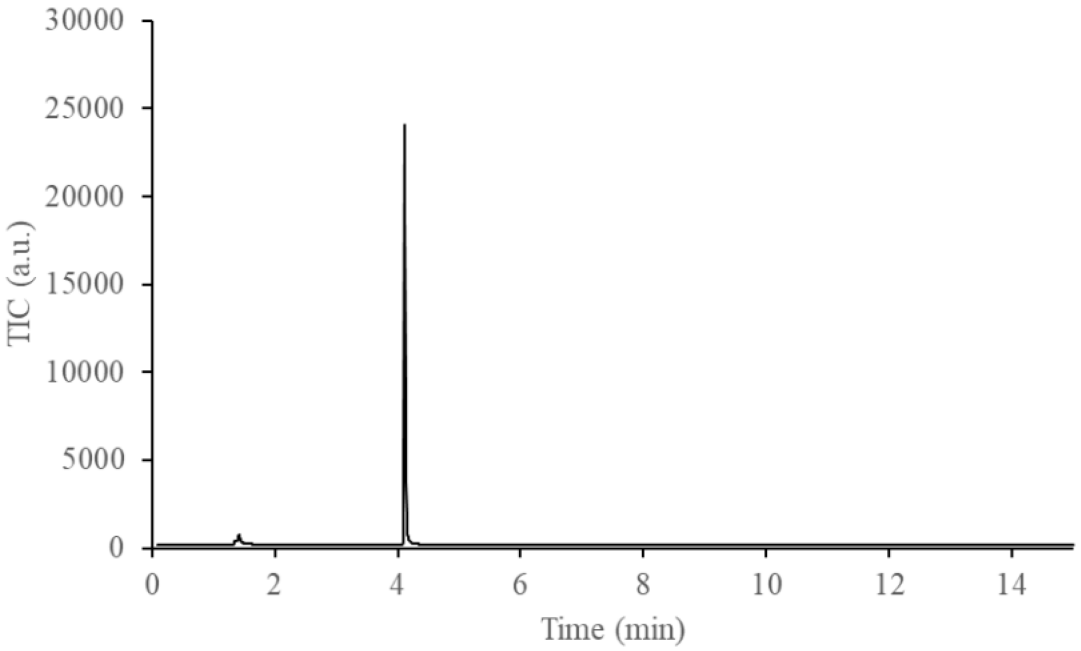


Figure 12. SIM of interference study.

In Figure 12, the results of the interference study confirmed that the amphetamine-related metabolites, such as norephedrine, 4-hydroxyamphetamine, phenylacetone, diphenhydramine, caffeine, and benzoic acid, did not produce interfering signals at the SIM ions selected for amphetamine.

3.4. Detection of Amphetamine in Human Urine Samples

By using the method developed in Section 3.3, the short (24 - 96 hrs.) and long (1 - 6 months) stability of amphetamine in human urine samples was determined.

3.4.1. Short-term Stability Observations

The concentration of amphetamine in the positive control was 0.22 µg/mL. The short-term stability study was conducted using the method developed in Section 3.1 to 3.3. Calibration curves were obtained from standards and were used to calculate the concentration of amphetamine in urine samples portrayed in Table 4.

Table 4. Amphetamine concentration of urine sample 1 - 7 in ng/mL (24 - 96 hrs.)

Sample	24 hrs.	48 hrs.	72 hrs.	96 hrs.
1	0	0	0	0
2	0	0	0	0
3	71	71	71	71
4	0	0	0	0
5	119	119	119	119
6	29	29	29	29
Pos	218	218	221	216
Neg	0	0	0	0

3.4.2. Long-term Stability Observations

During the six-month stability study, the same positive control of urine samples as the short-term stability study were used. A slight decrease (less than 15%) in amphetamine concentrations was observed, as shown in Table 5. This trend indicates potential degradation of amphetamine in the urine. Additionally, reduced peak intensities at lower concentrations suggest that further method optimization, such as using a more sensitive ion source or adjusting SIM dwell times, may be required to improve trace-level detection sensitivity.

Table 5. Amphetamine concentration of urine sample 1 - 7 in ng/mL (1 - 6 month).

Sample	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
1	1	0	0	0	0	0
2	98	97	97	89	88	85
3	0	0	0	0	0	0
4	29	27	27	26	26	26
5	71	72	71	70	70	69
6	0	0	0	0	0	0
7	70	69	64	65	66	64
Pos	219	220	219	219	219	218
Neg	0	0	0	0	0	0

4. DISCUSSION

Retention time (RT) is not an intrinsic property of the analyte alone; rather, it is determined by the interaction between the analyte and the entire chromatographic system. RT is influenced by the stationary phase chemistry, column dimensions (length, internal diameter, and film thickness), carrier-gas linear velocity, inlet conditions, and the oven temperature program (Borgsmüller et al., 2019). Published methods demonstrate that amphetamine-class separations can be compressed into short chromatographic windows using steeper temperature ramps and, in some cases, shorter or narrower-bore columns that increase efficiency and reduce analysis time (Marais & Laurens, 2009). In the present method, an initial oven temperature of 100 °C followed by a ramp of 15 °C/min to 250 °C contributed to a reduced retention time while maintaining peak symmetry. The relatively early elution of amphetamine (RT ≈ 4.1 min) reflects the combined effects of the moderate ramp rate, column dimensions, carrier-gas flow, and the non-polar 5% phenyl stationary phase, rather than any single factor alone. Notably, while underivatized amphetamine typically elutes between 2.5 and 3 minutes and derivatized forms between 4 and 6.5 minutes (Ash et al., 2018), the retention time observed in this study falls within the range

associated with derivatized amphetamine. This suggests that the combination of the 15 °C/min oven ramp and the 5% phenyl-95% dimethylpolysiloxane stationary phase of the HP-5Q column extended chromatographic retention relative to other underivatized workflows, while still achieving efficient separation.

The selection of the Agilent J&W HP-5Q ultra inert, ultra-low-bleed GC–MS column was central to the method design. Gas chromatography separates compounds using a thin capillary column (Agilent, 2024; Barnes et al., 2014). The column design parameters, such as stationary phase chemistry, length, inner diameter, and film thickness, directly determine the duration a compound remains on the column, thereby affecting the observed retention time and resolution, defined as the ability to separate amphetamine from co-eluting matrix components. (Frolova et al., 2025). The HP-5Q (30 m × 0.25 mm × 0.25 μm) is from Agilent's ultra-low-bleed (Q) GC–MS selection, which combines ultra inert performance with ultra-low-bleed technology to improve peak symmetry, reduce background noise, and minimize bleed-derived ions reaching the mass spectrometer (Gerardi & Technologies, 2024). The stationary phase is composed of low-polarity fused silica with 5% phenyl-methyl polysiloxane / 95% dimethylpolysiloxane, a bonded and cross-linked chemistry that is robust and rinseable, with a temperature range of 60°C to 350 °C (*Chromtech*, 2026; *Shop*, 2026). This thermal stability was an important consideration given the relatively high initial oven temperature and aggressive ramp rate employed in this method.

Column bleed introduces background ions that elevate the baseline and can distort ion ratios, particularly in TIC mode at low analyte concentrations, reducing calibration stability across analytical runs (Ayala & Kerrigan, 2025). By minimizing bleed, the HP-5Q column supported more stable baselines and improved integration accuracy for low signal-to-noise

analytes (Honles et al., 2022; Vaiano et al., 2016). Furthermore, the high inertness of the stationary phase is particularly relevant for the basic amphetamine, which is prone to peak tailing and adsorption in less inert systems (Smith & Technologies, 2019). In the present study, the observed peak shape was sharp and symmetrical, consistent with the expected performance of ultra inert (UI) column chemistry. The HP-5Q also retains the general selectivity class of HP-5ms UI columns, ensuring comparability with established amphetamine GC–MS workflows while providing enhanced baseline stability (Fan & Qian, 2006; Gerardi & Technologies, 2024). It is important to note that the relatively short retention time of amphetamine should not be attributed solely to the low-bleed property of the column. Although low bleed primarily improves baseline stability and signal-to-noise ratio at elevated temperatures (Honles et al., 2022), the early and clean elution observed is more accurately explained by the combined influence of the oven program, column geometry, and stationary phase chemistry, which together provided appropriate polarity balance for amphetamine and enabled efficient separation without derivatization.

The initial oven temperature controls early elution behavior and solvent focusing, while the ramp rate determines how quickly analytes transition through the column and reach the detector (Moreno et al., 2015). In this study, the oven program began at 100 °C, held for 1 minute, to allow solvent evaporation and prevent excessive broadening of early-eluting compounds. The temperature was then ramped at 15 °C/min to a final temperature of 250 °C, followed by a 5-minute hold to ensure complete elution of amphetamine and any potential late-eluting interferences. The resulting total runtime was approximately 16 - 17 minutes. This yields a throughput of approximately 15 runs per 3.25 hours, which represents improved efficiency compared to traditional 25 - 30-minute methods while preserving peak integrity.

The single-ramp program employed in this study is comparatively simple relative to more complex, multistep temperature programs reported in the literature. For example, one published method began at 90 °C and applied five sequential ramps to reach a final temperature of 300 °C for the separation of amphetamine alongside other compounds in fingernail matrices (J. Y. Kim et al., 2010). Slower ramp rates can improve separation but significantly extend total run time, whereas excessively aggressive ramps may compromise peak resolution (Czyż et al., 2025). The 15 °C/min ramp allowed amphetamine to elute efficiently while maintaining peak symmetry and acceptable separation, with the final temperature of 250 °C, which is lower than the 300 °C commonly used in broad drug screening methods, reducing unnecessary column stress and bleeding. The use of the HP-5Q ultra inert, low-bleed column supported this optimized temperature strategy, as the inert stationary phase minimizes adsorption of basic analytes such as amphetamine, contributing to sharper peak shape and improved reproducibility even under moderately aggressive ramp conditions.

Although derivatization can improve analytical performance, it adds complexity and cost to the workflow (Mohamed, 2017). Additional steps including reagent addition, incubation, and cleanup increase sample preparation time (Mohamed, 2017; Wei & Su, 2024). Derivatizing agents such as trifluoroacetic anhydride (TFAA) react with amine functional groups to form more non-polar derivatives, which typically reduce polarity, improve volatility, and produce sharper, more symmetrical peaks. This enhances chromatographic behavior and detectability in electron ionization GC–MS (Vaiano et al., 2016). However, TFAA and similar reagents can be relatively expensive, require careful handling, and generate additional chemical waste. The use of fluorinated reagents also raises environmental considerations (Woźniak et al., 2020). Unlike older methodologies that relied on derivatization to achieve these benefits, the high inertness of

the HP-5Q column enabled successful analysis of underivatized amphetamine with symmetrical peak shape in the present study, thereby reducing sample preparation complexity while maintaining analytical performance.

Mass spectrometric detection was performed using Selected Ion Monitoring (SIM). In SIM mode, the instrument monitors predefined mass-to-charge (m/z) ratios rather than scanning across the full mass range as in total ion chromatogram (TIC) full-scan mode (Pramod et al., 2021; Tőkés, 2017). This targeted approach enhances sensitivity and reduces background interference, particularly in complex biological matrices such as urine (Markway & Baker, 2011). During method development, multiple candidate ions (e.g., m/z 44, 65, 77, 91, 118, 112) were evaluated under the established chromatographic conditions. Based on the mass spectrum shown in Figure 4, m/z 44 (alkyl amine fragment), 65 (C_5H_5), 91 (benzyl), and 118 (benzyl fragment) were aligned with reports from substantial publications. These represent the most abundant and consistent fragments observed under electron ionization. Although the literature frequently reports a dominant ion near m/z 91 for amphetamine (Dragan et al., 2021; J. Y. Kim et al., 2010), ion abundance and relative ion ratios are influenced by several factors, including instrument source tuning, scan parameters, column stationary-phase chemistry, and matrix background effects (Kojić et al., 2025). Due to incomplete or low-intensity peaks at m/z = 91 and 96 (Figure 4 and Table 1), and to maximize the detection time for key fragments of amphetamine, the fragments of m/z = 91 and 96 were excluded from all subsequent trials. Thus, the m/z of 44, 65, and 118 were chosen as key fragments (also called quantifier ions) for amphetamine. Similarly, the three m/z of 49, 70, and 123 were selected as the quantifier ions for amphetamine-d_5, which are also aligned with previous published studies. SIM ions were therefore selected empirically rather than adopted directly from the literature, using criteria

including signal intensity, signal-to-noise ratio in urine, reproducibility across replicates, and minimal matrix interference. The ion demonstrating the most stable and intense response was designated as the quantifier ion, while additional characteristic ions were retained as qualifier ions to support confirmatory identification through consistent ion ratios and fragmentation patterns.

In the observed fragmentation pattern, m/z 44 was the most intense ion, with supporting ions at m/z 65 and 118. All this represents the characteristic fragmentation behavior of amphetamine under the specific ion source tuning and acquisition parameters used in this study. While reference spectra often report m/z 91 or 118 as prominent or base peaks, relative ion abundance can vary depending on instrument conditions, tuning, scan parameters, and matrix effects; therefore, differences in base peak intensity do not indicate misidentification but instead reflect instrument-specific fragmentation behavior under consistent operating conditions. The prominence of m/z 44 ($C_2H_6N^+$), a relatively small fragment ion, suggests more aggressive fragmentation under the conditions employed, likely attributable to the high ion source temperature of 230 °C within the typical range of 200 - 300 °C (Woźniak et al., 2018). This combined with the high initial oven temperature of 100 °C, falls at the upper end of the typical 40 - 100 °C range (Raikos et al., 2003). Electron ionization at 70 eV is standard; however, relative ion abundances are known to vary between different Agilent ion source configurations even at identical electron energies (Margolin Eren et al., 2020). While fragmentation patterns in electron ionization (EI) at 70 eV are primarily governed by ionization energy and source conditions rather than oven temperature, chromatographic efficiency directly influences signal intensity and signal-to-noise ratio in SIM mode (Catarro et al., 2025). Under the selected instrument conditions, the dominant fragment ion (m/z 44) produced strong and consistent

response, supporting reliable quantification and confirmation. Collectively, these findings describe a unique, instrument-parameter-dependent fragmentation fingerprint for underivatized amphetamine on the Agilent 7820A GC–MS system using the HP-5Q (30 m × 0.25 mm × 0.25 μm) column.

Several additional instrumental parameters were optimized to support method performance. Helium at approximately 1.0 mL/min was selected as the carrier gas to balance resolution and runtime; although some rapid GC–MS methods employ higher flow rates to shorten analysis time, increased flow can compromise resolution, so the chosen flow rate prioritizes reproducibility and separation quality. A 10:1 split injection was employed because analyte concentrations permitted split introduction, which provided improved peak shape, reduced detector saturation, enhanced injector cleanliness, and mitigated matrix effects compared to split less injection (Nair et al., 2013). While split less mode is often used for trace detection, it increases the risk of column overload and matrix contamination. The moderate split ratio, therefore, supports long-term instrument stability and routine laboratory applicability. An injector temperature of 250 °C was selected to ensure complete volatilization while minimizing the risk of thermal degradation or adsorption that can occur at higher inlet temperatures such as 270 - 280 °C and may contribute to improved liner and column longevity (S. Y. Kim et al., 2013). The mass scan range of m/z 40 - 300 was selected to focus detector cycling on relevant fragment ions while excluding unnecessary higher mass regions, thereby increasing scan speed and improving data density across chromatographic peaks.

Daily autotuning was performed to maintain optimal sensitivity and mass accuracy, preventing detector sensitivity loss and mass drift. It is particularly important when monitoring subtle changes in amphetamine stability or potential degradation of products. The combined use

of TIC and SIM acquisition represents a deliberate methodological optimization. The full-scan acquisition enables detection of unexpected impurities or degradation products, whereas SIM enhances sensitivity for target amphetamine ions (Margolin Eren et al., 2020). Literature supports this dual-mode strategy, as reliance on SIM alone may overlook non-target compounds while full-scan acquisition alone can compromise detection limits. Integrating both modes therefore improves qualitative comprehensiveness and quantitative sensitivity relative to single-mode approaches.

In addition to modes, the solvent used is imperative. As shown in Table 6, the method also employs a minimal sample volume of 0.5 mL with an extraction solvent volume of 0.5 mL using chloroform, representing an efficient, resource-conscious approach to amphetamine screening in urine. While in future studies, we can further lower the sample volume; however, any lower than 0.1 mL sample volume results in inadequate solvent to sample ratio (Woźniak et al., 2020). The resulting method present is optimized for routine laboratory use, emphasizing efficiency, reproducibility, and instrument longevity rather than maximal theoretical sensitivity. Chloroform, a halogenated solvent with relatively high polarity and significant hydrogen bond accepting capability, is also less expensive at $0.91/kg (Chloroform Prices 2025) which contrast sharply with ethyl acetate, a moderately polar aprotic solvent priced at $1.37 - 1.11/kg (Ethyl Acetate Prices 2025).

Table 6. Comparing literature on LOD, sample volume, solvent volume.

Instrument	Sample volume	Solvent volume	Solvent name	LOD for amp µg/ml	Extraction	Derivatized	Retention time (min)
GC–MS	0.5 mL	0.5 mL	chlorof-orm	0.03	LLE	No	4.1
GCFID (Raikos et al., 2003)	4 mL	-	solid salt	0.03 - 0.04	SPME	no	3.5 - 4.5
GC–MS (Woźniak et al., 2018)	0.2 mL	2 ml	ethyl acetate	0.0005 - 0.17	LLE	50ul TFAA	4.68
GC–MS (S. Y. Kim et al., 2013)	0.5 ml	0.8 ml	ethyl acetate	0.0005 - 1.7	LLE	50ul TFAA	6.18
GC–MS (Meng et al., 2015)	1 ml	0.1 ml	toluene	0.002	UA-LDS-DLLME	no	4 - 4.5

The limit of detection (LOD) represents the lowest concentration at which an analyte can be confidently detected above background noise, while the limit of quantification (LOQ) represents the lowest concentration at which the analyte can be measured with acceptable accuracy and precision (Mercieca et al., 2018; Yudiana et al., 2023). In this study, the LOD was 0.033 µg/mL (equivalent to 33 ng/mL in urine) for the quantifier ion at m/z 118. This performance is comparable to published non-derivatized GC–MS approaches. For example, one rapid method from Table 6, reported an LOD of 31.25 ng/mL for amphetamine (Raikos et al., 2003). Some more sensitive workflows utilizing the more costly Ultrasound-Assisted Low-Density Solvent Dispersive Liquid-Liquid Microextraction (UA-LDS-DLLME), often involving derivatization strategies or alternative detector configurations, report LODs in the single-digit ng/mL range of approximately 1.5 - 6.25 ng/mL (Meng et al., 2015). For real-world toxicological utility, however, what matters most is that the detection capability falls comfortably

below established regulatory cutoffs. The federal workplace drug-testing cutoffs for urine are 0.5 µg/mL for the initial immunoassay screen and 0.25 µg/mL for confirmatory testing. Since the achieved LOD of 33 ng/mL is well below the 0.25 µg/mL confirmatory threshold, the method is sufficiently sensitive to support confirmatory-level decision-making while remaining aligned with the practical goal of this project: developing a robust, high-throughput workflow using the HP-5Q ultra inert column and system-specific GC–MS parameters.

With respect to validation, stability testing was conducted according to Scientific Working Group for Forensic Toxicology (SWGTOX) to evaluate method robustness for routine laboratory workload (SWGTOX, 2013). Minor degradation was observed under refrigerated storage conditions over the evaluated time periods, whereas no measurable degradation was observed during short-term stability testing at room temperature. These findings suggest that the method is capable of reliably detecting small changes in concentration while maintaining stability under typical handling conditions. A total of twenty-seven samples were analyzed in the present study. While this sample size is sufficient to demonstrate preliminary robustness and reproducibility, increasing the sample size in future work would strengthen statistical power and further support method validation for broader laboratory implementation.

Collectively, these optimizations demonstrate that underivatized amphetamine can be reliably analyzed using a modern ultra inert 5% phenyl column with a moderate split injection and a shortened oven program, without compromising peak shape or identification criteria. Whereas many older GC–MS methods rely on derivatization, slower temperature ramps, higher final oven temperatures, or split less injection to achieve sensitivity, the present configuration demonstrates that modern column chemistry and controlled parameter optimization can simplify workflow while maintaining analytical rigor.

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