**Validation of a Rapid GC-MS Method for Forensic Seized Drug Screening Applications**

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**Highlights**

* Validation of in-house system completed; capabilities and limitations identified.
* Precision, robustness, and ruggedness studies completed; % RSDs generally ≤ 10 %.
* Validation template designed for forensic applications of rapid GC-MS.
* Validation plan and workbook developed for seized drug screening.

**Abstract**

With the lack of standardized validation protocols across the forensic chemistry community, validation of instrumentation can be a challenging and time-consuming task. However, this process is crucial to understanding the associated capabilities and limitations, especially for nascent technologies. Rapid GC-MS is one such emerging analytical technique being increasingly implemented in forensic laboratories due to its fast and informative screening capabilities. However, a full validation for forensic samples has yet to be published since its debut. This work presents the results of a comprehensive validation of a rapid GC-MS system for seized drug screening through the assessment of nine components: selectivity, matrix effects, precision, accuracy, range, carryover/contamination, robustness, ruggedness, and stability. Single- and/or multi-compound test solutions of commonly encountered seized drug compounds were used to assess method and system performance. Results met the designated acceptance criteria for a majority of components. For example, retention time and mass spectral search score % RSDs were ≤ 10 % for precision and robustness studies. Limitations were identified for components that did not meet the acceptance criteria (*e.g.*, inability to differentiate some isomers). The study design is part of a larger validation package developed for rapid GC-MS that includes a validation plan and automated workbook. The template, available for adoption by laboratories, ultimately aims to reduce the barrier of implementation for rapid GC-MS technology.

***Keywords:*** GC-MS, Validation, Seized drugs, Screening

1. **Introduction**

Validation of new methods and technologies implemented in forensic laboratories is crucial for understanding a technique’s capabilities and limitations, as well as verifying the generation of consistent and reliable results. Performing a comprehensive validation can take several months, and this process is often lengthier if forensic chemists need to design and conduct the validation in conjunction with their current workload. Though validation protocols exist for disciplines like forensic toxicology [1], similar standards do not exist for seized drug analysis. While prior resources have been available that contain broad guidelines for validation studies, such as ASTM E2549 (since withdrawn) [2] and others from the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) [3] and the United Nations Office on Drugs and Crime (UNODC) [4], recent standards have yet to be published. To address this gap, research efforts are currently underway to develop similar documentation for various forensic disciplines (*e.g.*, seized drugs, explosives, and ignitable liquids) [5, 6]; however, these resources are not available yet. As such, many laboratories rely on in-house validation procedures developed for existing instrumentation and/or analytical methods. The process of designing and developing new protocols is time-consuming and can serve as a barrier for implementing new techniques for which established plans do not yet exist. Thus, there is a need for the development of validation templates that can be made available to laboratories for such technologies.

For many laboratories conducting seized drug analysis, gas chromatography-mass spectrometry (GC-MS) is the standard confirmatory analytical technique. Prior to GC-MS analysis, a screening technique, such as color tests or thin layer chromatography [7], is often performed to first obtain chemical and/or class information about a sample. Many existing screening techniques suffer from inherent disadvantages, such as the lack of specificity and/or sensitivity, which can lead to false positives or inconclusive results [7]. Recently, the development of a rapid GC-MS system that configures directly to benchtop GC-MS instruments has been a promising alternative and/or complement to current screening methods [8]. The technique requires minimal sample preparation and enables screening with rapid chromatography (less than two minutes per injection) followed by traditional electron ionization (EI) mass spectrometric detection [9]. Rapid GC-MS was initially developed for forensic applications [10, 11] and has been demonstrated for the screening of multiple sample types, specifically controlled substances [12, 13] and ignitable liquids [14, 15]. Such work has shown that informative sample screening can be achieved prior to confirmatory GC-MS analysis for both simple and complex samples in a matter of minutes.

In order for rapid GC-MS to be adopted in practicing forensic laboratories, validation is required to demonstrate its suitability for the intended purpose(s). Because it is a chromatography-based technique and utilizes the same detector as its parent benchtop GC-MS instrument, existing GC-MS validation protocols could be adapted to the rapid GC-MS system. However, this approach still requires time for development of such a plan, which can delay or even prevent implementation. To lessen the burden, this work presents the complete validation of rapid GC-MS for seized drug screening according to a template developed specifically for the system. During this study, capabilities and limitations of the technique were identified.

The validation package was modeled after a previously developed validation template for direct analysis in real-time mass spectrometry (DART-MS) analysis of seized drugs [16, 17], and it includes validation procedures for nine components (selectivity, matrix effects, precision, accuracy, range, carryover/contamination, robustness, ruggedness, and stability) to assess analytical performance. All materials included in the package can be accessed and downloaded for immediate use [18]. The plan is designed such that validation can be performed given the provided descriptions and details or can be modified to fit a laboratory’s specific need. In total, the work presented here includes the study design and results of the full validation conducted on an in-house rapid GC-MS system.

# Materials

## Reagents and Materials

All reagents were used as received. For solutions prepared in-house, methanol (HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) or acetonitrile (≥99.9 %, Sigma-Aldrich) were used as solvents. For the precision, robustness, ruggedness, and stability studies, a custom 14-compound test solution (0.25 mg/mL per compound) in isopropanol (**Table 1**) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Unless otherwise specified, all compounds utilized were analytical reference materials.

Selectivity studies were performed using multi-compound test solutions of commonly encountered isomers listed in **Table 2**, the contents of which were designed to mitigate potential coelution of isospectral compounds. Test solutions were prepared at a low (0.05 mg/mL per compound) and high (0.5 mg/mL per compound) concentration in methanol. Matrix effects studies were performed using solutions of common seized drug matrices consisting of adulterants and diluents, along with multi-compound test solutions. All matrix and test solutions were analyzed individually to establish the instrumental response, and then the matrix solutions were spiked with the respective test solution and analyzed. Two matrix solutions were prepared to mitigate coelution of adulterants, with each compound present at a concentration of 0.25 mg/mL (**Table 3**). The test solution was prepared using the following compounds: cocaine, fentanyl (HCl), heroin, and methamphetamine (HCl), purchased from Cayman Chemical. The matrix solutions were then spiked with the test solution by evaporating aliquots of the test solution to dryness and reconstituting in each matrix solution to achieve low (0.05 mg/mL) and high (0.5 mg/mL) final test solution concentrations.

LODs were determined using multi-compound test solutions designed to mitigate coelution and the number of injections. LODs were determined for a subset of compounds (indicated by (‡) in **Table 1**). For compounds belonging to an isomeric pair, one compound of the pair was chosen, as isomers of a respective series were expected to behave similarly. All test solutions were prepared, gravimetrically, from neat standards or stock solutions, at nominal concentrations of 0.5 µg/mL, 1 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 25 µg/mL, and 50 µg/mL in methanol, as well as a methanol blank solvent solution (0 µg/mL). For carryover studies, single-compound test solutions were prepared at low (1 mg/mL) and high (5 mg/mL) concentrations. The test compounds consisted of caffeine (prepared in methanol), acetaminophen (prepared in methanol), cocaine (prepared in acetonitrile), and heroin (prepared in acetonitrile).

## Rapid GC-MS and Traditional GC-MS Instrumentation

All rapid GC-MS analyses were conducted using an Agilent 3971 QuickProbe GC-MS system. The system is attached to a GC-mass selective detector (MSD) system consisting of an 8890 gas chromatograph and 5977B mass spectrometer, equipped with a 7693 autosampler (Agilent Technologies, Santa Clara, CA, USA). For rapid GC-MS analyses, separation was achieved *via* a DB-1ht QuickProbe GC column (2 m length × 0.25 mm outer diameter × 0.10 μm inner diameter, Agilent Technologies). The system utilizes a two-way splitter configuration [8], to which both the rapid and traditional GC columns connect, enabling both types of chromatography to be performed (separately) on the same instrument. A DB-1ms Ultra Inert QuickProbe GC column (1 m length × 0.18 mm outer diameter × 0.18 μm inner diameter, Agilent Technologies) connects the chromatography columns to the MSD transfer line. Additional details regarding system configuration can be found elsewhere [14]. Rapid GC-MS analysis was conducted using the temperature program in **Table 4**. Ultra high-purity helium (99.999 %) was used as the carrier gas for all analyses at a flow rate of 1 mL/min. The rapid GC inlet was equipped with an ultra-inert fritted liner (Agilent Technologies). For samples analyzed using traditional GC-MS (*i.e.*, samples for accuracy studies), the corresponding method parameters and temperature program are provided in **Supplementary Data A, Table SA1.**

Rapid GC-MS injections were completed using the system-compatible probe holder fitted with the glass round probes (Agilent Technologies) [9]. Initial probe cleaning was performed by sonicating the probes in hexane (≥95 %, Sigma-Aldrich) for five minutes. Following sonication, the probes were removed from the hexane solution and allowed to dry fully. Before the first use of a probe, a probe blank was analyzed using the rapid GC-MS analytical method, keeping the probe inserted in the inlet for the duration of the analysis. This process was repeated until a constant chromatographic baseline was achieved. Liquid solutions were sampled by inserting the tip of the glass probe approximately two to three millimeters below the meniscus of the solution and immediately removing. The solvent was allowed to evaporate for approximately ten seconds, followed by injection in the rapid GC inlet. Probes were cleaned between injections by rinsing with methanol and drying with a Kimwipe. Because the rapid GC inlet is exposed to the atmosphere, a system blank, in which the temperature program was initiated without injection of sample or probe, was performed at the start of each day or round of instrument use. Unless otherwise specified, the sampling order was as follows: system blank, probe blank(s), sample(s) (including same-sample replicates), probe blank, additional samples/blanks, probe blank, system blank.

## Data Processing

All data were collected using MassHunter GC-MS Data Acquisition software (version 10.0, MassHunter Workstation, Agilent Technologies) and initial background subtraction was performed using MassHunter Qualitative Analysis (version 10.0, MassHunter Workstation Agilent Technologies). Data processing, including retention time extractions and mass spectral comparisons, was performed using AMDIS (version 2.73, NIST). Retention times were extracted at the apex of a given peak in a chromatogram. Additional mass spectral searches were conducted as necessary using the NIST Mass Spectral Search Program (version 3.0, NIST) [19]. All mass spectral comparisons were performed using the NIST/EPA/NIH 2023 EI Mass Spectral Library and SWGDRUG MS Library (version 3.9), as applicable. Deconvolution was performed in AMDIS as necessary using the parameters detailed in **Supplementary Data A, Table SA2**.

# Validation Study Design

The associated validation plan, workbook (for automated data processing), and other relevant documentation to accompany this work is available on the NIST Data Repository: <https://doi.org/10.18434/mds2-3189>. For components that utilize percent relative standard deviations (% RSDs) as part of the acceptance criteria, a threshold of 10 % was chosen to align with the criteria utilized by many accredited forensic laboratories [20].

## Selectivity

Selectivity was evaluated by assessing the capability of the method to detect commonly encountered isomers in the presence of other compounds and/or isomers in solution. The three-compound test solution sets in **Table 2** were prepared at low (0.05 mg/mL) and high (0.5 mg/mL) concentrations and were analyzed five times over the course of one day. In the event of coelution, deconvolution was performed. For each test solution compound, the corresponding retention time and mass spectral search score was documented, as were the mass spectral search scores for comparisons to the library spectra of other isomeric species within a given series. Student’s t-tests were performed (alpha = 0.05) to determine if the average retention times and mass spectral search scores for a compound of interest could be differentiated from those of the remaining isomers in the series. Statistical difference was defined by a *p* value of less than 0.05. To evaluate whether data variability was present for the same compound across concentration, these tests were also performed for the low and high concentration data for each compound. For tests of mass spectral search scores, t-tests were performed only if the average search score for comparison of the test compound to its own library spectrum was greater than the average search score(s) for comparison(s) to the mass spectra for the isomer(s) in the series. If the standard deviation across replicate measurements was zero for one or more isomers in a series, a t-test was not able to be performed.

## Matrix Effects

Matrix effects were evaluated by assessing the capability of the method to detect test solution compound(s) in the presence of relevant matrix compounds. Each matrix, test, and spiked matrix solution was analyzed by rapid GC-MS three times over the course of one day, on the same day. For each test solution compound, the corresponding retention time and mass spectral search score was extracted. Criteria for successful test solution compound identifications were: (1) chromatographic signal-to-noise (S/N) greater than 3:1; (2) mass spectral search score (for correct compound identity) > 80 a.u. (on a scale of 0 a.u. to 100 a.u.); *and* (3) minimal retention time shifting across replicates (percent relative standard deviation [% RSD] ≤ 10 %).

## Precision (Repeatability & Reproducibility)

Repeatability was evaluated by assessing the capability of the method to produce consistent results using the same method, solution, and analyst, on the *same* day, across replicate measurements. The 14-compound test solution (**Table 1**) was analyzed ten times over the course of one day. Reproducibility was evaluated by assessing the capability of the method to produce consistent results using the same method, test solution, and analyst, on *different* days, across replicate measurements. The 14-compound test solution (**Table 1**) was analyzed five times over the course of each day, on seven non-consecutive days, over the span of at least four weeks. For both repeatability and reproducibility studies, blank injections were analyzed between replicate sample injections. Retention times and mass spectral similarity scores for test solution compounds were recorded for all replicate measurements. Repeatable and reproducible measurements were defined by % RSDs ≤ 10 % for both metrics within each study.

## Accuracy (Correctness)

Accuracy, or correctness, was evaluated by assessing the ability of rapid GC-MS and traditional GC-MS to detect the same compounds within a set of 25 authentic seized drugs samples. Comparisons to traditional GC-MS were made to mimic the likely combination of analytical techniques that would be used in a forensic laboratory. Traditional GC-MS method parameters are listed in **Supplementary Data, Table SA1**.Previously adjudicated (non-probative) seized drug case samples were obtained from the Maryland State Police Forensic Sciences Division. To establish accuracy of the rapid GC-MS method, agreements in compound identifications between rapid GC-MS and GC-MS results were identified, and any inconsistencies were explained. Deconvolution was performed if coelution was observed.

## LOD

LODs were determined in accordance with ASTM E2677 and calculated using the NIST web-based LOD calculator [21, 22]. For each compound studied, ten replicates of each concentration were analyzed over the course of one day. The order of analysis within the given concentration range was randomized using a random number generator in Microsoft Excel (version 2308, Microsoft Office 365, Microsoft Corporation, Redmond, WA, USA). LODs were calculated at a confidence limit of 0.10 (90 % confidence interval) using the chromatographic peak areas for each compound from the total ion chromatogram.

## Carryover / Contamination

Carryover was evaluated by determining if the presence of a compound signal reappeared in later analyses, as this occurrence could lead to inaccurate or false positive qualitative results. For each test solution compound, a single injection of a low- and high-concentration single-compound test solution was analyzed immediately following and preceding a blank injection. Blank injections, referred to as probe blanks, consisted of a clean probe (first washed with methanol and dried) injected into the inlet.

The chromatogram of the probe blank injection following the sample injection was visually assessed to determine if a detectable peak was present at the respective sample compound retention time. For a positive determination where carryover was confirmed, consecutive probe blanks were analyzed until the absence of carryover was observed. Regardless of the number of probe blank injections required after sample analysis, the analysis concluded with a final probe blank injection to ensure no carryover. The chromatographic intensity of each test compound was normalized (see Section 4.6 for a detailed description of normalization) and compared to that in the subsequent probe blank injection(s). A normalized intensity of 3 a.u. in subsequent probe blank injections was used as the upper threshold for acceptable intensities to confirm the absence of carryover. This value was chosen based on S/N ratios used to determine the presence of a chromatographic peak. Relative carryover (RC), or the ratio of compound peak abundance in the probe blank injection to compound peak abundance in the sample injection total ion chromatogram, was calculated for each compound and concentration analyzed.

Contamination was evaluated by identifying the presence of substances external to the test solution compound(s) that may have originated from solvents, reagents, or other materials. Three different clean probes were analyzed in triplicate (*i.e.*, probe blanks) on a single day, prior to any sample injections. The presence of any unintentional, extraneous compounds visually identified in the chromatograms was documented, including the associated retention times, identities (if possible), and *m/z* values of major mass spectral ions (abundance > 25 %).

## Robustness

Robustness was evaluated by determining if the results of the rapid GC-MS methods were unaffected by small, intentional modifications made to internal method parameters. Five parameters (factors) were modified. A low and high modification level (low, high) were designated for inlet temperature (240 °C, 260 °C), injection time (3 s, 7 s), pressure (96.527 kPa [14 psi], 110.316 kPa [16 psi]) and MSD filament (filament 1, filament 2). The fifth parameter was injection apparatus, for which different probes from the same pack were utilized (probe 1, probe 2). The combination of parameter modifications was determined according to a fractional factorial design of experiments, and the order of combinations was randomized prior to analysis (**Supplementary Data A, Table SA3**). The 14-compound test solution (**Table 1**) was used for this component of the validation study. For each combination, the test solution was analyzed two times over the course of one day. A third test solution injection was analyzed if inconsistencies in chromatographic abundances (*i.e.*, relative intensities) were visually observed in the first two injections. For each injection replicate, the retention time and mass spectral search score for each compound was extracted and recorded. The method was determined to be robust if the percent difference between the average response of the low and high levels for a given factor was ≤ 10 % for each metric.

## Ruggedness (Intermediate Precision)

Ruggedness was evaluated by determining if the results of the rapid GC-MS method were unaffected by modifications made to external method parameters. Similar to the robustness studies, the 14-compound test solution (**Table 1**) was used for this component of the validation study. Four external parameters were modified: analyst, carrier gas cylinder batch, probe pack (probes from a different probe pack). For the gas cylinder and inlet liner, the existing consumables were replaced with a new cylinder and liner, respectively. Of the parameters studied, one parameter was modified at a time, rather than simultaneous modifications, to enable direct correlation in instrument response to differences with the respective parameter. Furthermore, as the parameters were external to the method, interaction effects should be minimal, at most. Following each parameter modification, the test solution was analyzed five times over the course of one day. Retention times and mass spectral search scores for each test solution compound were extracted and recorded. The method was determined to be rugged if both metrics had % RSDs ≤ 10 %.

## Stability

Stability studies were performed to assess the response of the rapid GC-MS method to any changes in sample stability over time, based on the agreement of results after analysis of samples prepared at various conditions. To assess stability effects, the reproducibility study was repeated using the 14-compound test solution (**Table 1**), prepared under two conditions. The first solution (referred to as “old solution”) was prepared once at the beginning of the study and used for all seven days of analysis. The second solution (referred to as “fresh solution”) was prepared fresh on the day of analysis for each of the seven days in the study. Because the test solution was received pre-prepared in a 1-mL ampoule, the fresh solution was prepared by cracking one ampoule and dividing evenly across seven GC vials, as this is most realistic to what would be performed in a forensic laboratory. The vials were capped and stored at -13 °C until the designated day of analysis. For each test solution, the retention times and mass spectral search scores for all compounds were extracted on each day of analysis to determine if the reproducibility of the method was affected by changes in sample stability (if any). Stability was defined as a cumulative % RSD ≤ 10 % (across old and fresh test solutions) for both metrics.

# Results and Discussion

## Selectivity

All data for the selectivity studies, including retention times and mass spectral search scores, are shown in **Supplementary Data B, Table SB1**. Selectivity was generally unaffected by concentration for retention time differentiation. At low and high concentrations, methamphetamine, *m*-fluorofentanyl, 2,3-pentylone isomer, and pentylone could be differentiated from one or more isomeric species in the corresponding series. For the fluorofentanyl series, the retention time of *m*-fluorofentanyl was statistically different than that of *o*-fluorofentanyl (but not of *p*-fluorofentanyl). For the pentylone series, the retention time for 2,3-pentylone isomer was statistically different than pentylone, but it was not statistically different than the retention times for the remainder of isomers in the series. Alternatively, the retention time of pentylone was statistically different than those of all compounds in its isomer series. For cases in which statistically different retention times were observed between the test compound and respective isomers of a given series, the test compound retention time was later than that of the isomeric species.

For the concentration comparison of each individual compound, retention times for methamphetamine and *m*-fluorofentanyl were statistically different across concentrations. Of the 15 compounds studied, methamphetamine was the earliest eluting compound, likely to be most affected by manual injection variability compared to the remaining compounds. Retention of methamphetamine, a relatively polar compound, on a nonpolar column (100 % polydimethylsiloxane) was likely less than that of the other test solution compounds, leading to its early elution. For this reason, it was more likely to be prone to retention time shifting. In the case of *m*-fluorofentanyl, a mid-eluting compound, retention times at low and high concentration were statistically different, with a *p*-value of ≈0.04, just below the 0.05 threshold. Given that this occurrence was not observed for *p*- and *o­*-fluorofentanyl, it was attributed to random error inherent in the manual injection process. In further support of this result, *m*-fluorofentanyl was in solution with methamphetamine and 2,3-pentylone isomer (**Table 2**). While the retention times for 2,3-pentylone isomer were not statistically different across concentrations, the *p*-value was ≈0.07, just above 0.05 (**Supplementary Data B, Table SB1**). Retention times for the remainder of the compounds were not statistically different across concentrations, demonstrating overall consistency of the rapid GC-MS system when exposed to varying concentrations.

Conversely, concentration did affect selectivity based on mass spectral search scores. Lower scores overall were observed at low concentration, which was not unexpected given that mass spectral quality is proportional to analyte concentration [23]. At low concentration, the relative intensities of spectral peaks were likely affected, leading to lower search scores in general. Of the t-tests that were performed, methamphetamine was differentiated from phentermine at low concentration (standard deviation of zero at high concentration), and phentermine was differentiated from methamphetamine at low and high concentrations. For the fluorofentanyls, two instances of differentiation were observed, in which *o*-fluorofentanyl was differentiated from *m*-fluorofentanyl at both concentrations and *o*-fluorofentanyl was differentiated from *p*-fluorofentanyl at high concentration. For the pentylone series, mass spectral search scores for a majority of test compounds were statistically different than those of the isomers in the series. At low concentration, differentiation was not observed for the following compound pairs: 2,3-pentylone and pentylone, *n*-methylethylone and eutylone, *bk*-DMBDB and 2,3-pentylone, and *bk*-DMBDB and pentylone. At high concentration, differentiation was not observed for *n*-methylethylone and eutylone. For the cannabinoid and synthetic cannabinoid series at both concentrations, statistical differences between spectral search scores were observed for all instances in which t-tests were completed. Spectral variability due to similar concentration effects also affected cross-concentration comparisons of spectra from the same compound. For 11 of the 15 compounds, mass spectral search scores at the high concentration were statistically different than scores for the same compound at the low concentration (**Supplementary Data B, Table SB1**). However, given similar results with the inter-compound comparisons, this outcome was anticipated for intra-compound spectral comparisons.

The ability to differentiate between some of the isomers in a given series was promising for rapid GC-MS; however, inability to differentiate between all isomers points to an expected limitation of the technique. Isomer differentiation is a known challenge in traditional GC-MS analyses, one being addressed by many ongoing research efforts [24-28]. Thus, it was anticipated that it would also be a limitation for rapid GC-MS, especially considering the technique’s condensed chromatographic timescale. This challenge is important to identify and be cognizant of for future use of rapid GC-MS for seized drug applications. Despite this limitation, given that the technology is intended as a screening technique, isomer differentiation is not necessarily a requirement. At the very least, identifying the presence of a compound in a broader controlled substance class (*e.g.*, stimulant, opioid, cathinone), not necessarily the specific compound identity, is still useful information.

## Matrix Effects

All test solution compounds were able to be identified in the presence of matrix compounds. Data for this study, including retention times, signal-to-noise ratios, and mass spectral search scores, are presented in **Supplementary Data, Table SB2**. When both matrix solutions (**Table 3**) were spiked with the test solution, retention time % RSDs were below the 10 % threshold for acceptance. For the matrix solutions containing a high concentration of the test solution, no major retention time variations were observed for cocaine, heroin, and fentanyl (% RSDs < 1 %). However, for the high concentration of methamphetamine in both matrix solutions, increased retention time variability was observed. The higher % RSDs were likely due to the lack of retention for methamphetamine in the column (**Supplementary Data, Figure SA1**). Despite the wider retention time range, % RSDs for methamphetamine were still below 10 %, meeting the acceptance criteria. Signal-to-noise ratios were greater than the threshold (3 a.u.) for all compounds and were proportional to concentration, as expected. Mass spectral search scores for all compounds were greater than 90 a.u., above the 80 a.u. threshold, demonstrating successful identification of the four test solution compounds as a function of concentration and matrix identity.

## Precision (Repeatability & Reproducibility)

Data for the repeatability study are presented in **Supplementary Data B, Table SB3**. Results of the repeatability measurements using the 14-compound test solution demonstrated retention time % RSDs below the 10 % acceptance criteria for all compounds. More specifically, all retention time % RSDs were less than 5 %, with the majority below 1 %. For 13 of the 14 compounds, mass spectral search score % RSDs were also below the 10 % threshold, with many below 5 %. Larger variation was observed for stanozolol, for which scores ranged from 64 a.u. to 86 a.u., resulting in a % RSD of 11 %. Comparatively, the scores for stanozolol were among the lowest observed for this component of the validation. Such results were attributed to compound- and instrument-specific factors. Steroids, if underivatized, are difficult to analyze using GC-MS due to their high boiling points and low thermal stability, resulting in decreased chromatographic signal and detector response [29]. Analysis of stanozolol in its underivatized form as part of the test solution could explain the lower scores. More notably, however, based on the results of the entire validation, it is possible that steroids are sensitive to the cleanliness of the MS source. Instrument maintenance was performed after the precision studies were completed and included EI source cleaning and filament replacement. Enhancements in mass spectral search scores were observed for stanozolol in studies completed after the maintenance (*i.e.*, robustness component). As such, it was determined that instrument maintenance helped improve the detection of stanozolol (see the Robustness section for more details) and the low scores observed for the precision studies were likely due to the dirty source.

Reproducibility results are also included in **Supplementary Data B, Table SB3**. % RSDs for replicate retention times were below 10 % for all compounds, meeting the acceptance criteria. Retention times from individual measurements, normalized to the global average retention time, are shown in **Figure 1**. Excluding methamphetamine, retention time variations for all individual daily measurements were within the 10 % accepted range. Due to lack of retention, methamphetamine had the widest variation in retention times across all compounds, where one replicate injection fell outside the acceptable range. However, the retention time % RSD for methamphetamine still met the acceptance criteria and was less than 10 %. For mass spectral search scores, % RSDs for 13 of the 14 compounds were 5 % or below (**Supplementary Data B, Table SB3**). Scores for stanozolol fell just outside the acceptable range, with a % RSD of 11 %. Similar to the repeatability study, these lower scores were attributed to the EI source dirtiness prior to source cleaning, made evident by the data from the robustness studies.

## Accuracy (Correctness)

Complete results for all 25 case samples (non-probative) analyzed are shown in **Supplementary Data B, Table SB4**, including the specific compound(s) detected in each sample and with what analytical method the compound(s) were identified. Chromatograms of all samples from both rapid GC-MS and GC-MS analyses are shown in **Supplementary Data A, Figures SA2 – SA26.** It is important to note that the chromatographic quality of rapid GC-MS is not ideal (*e.g.*, asymmetrical peaks, wide peak widths, lack of resolution), nor is it comparable to that of traditional GC-MS. However, when translated to a practical analytical workflow, rapid GC-MS is not intended to replace traditional GC-MS. Rather, the technique would be implemented as a screening tool to obtain chemical information about a sample, equivalent to a Category B technique according to SWGDRUG recommendations, with reliance on the resulting mass spectra for preliminary compound identification [7].

For most of the case samples analyzed, at least one controlled substance was identified in addition to cutting agents or diluents. For 18 of the 25 samples, rapid GC-MS detected all compounds detected by traditional GC-MS. For the remaining seven samples, more than 50 % agreement was observed in compounds identified by both rapid GC-MS and traditional GC-MS. However, additional compounds were present in each sample that were only detected by one of the analytical techniques. **Table 5** shows the identities of the compounds detected by a single analytical technique in each of these samples and the likely explanation for why they were not detected by either rapid or traditional GC-MS.

As with any screening technique, the potential for false positives or negatives is an important concern and should be minimized. Four of the seven samples in **Table 5** (case samples 8, 10, 23, and 25) contained compounds that were identified by traditional GC-MS but not by rapid GC-MS (**Table 5**). In these instances, coelution and/or insufficient abundances prevented identification using rapid GC-MS. However, in each of these samples, at least one controlled substance was identified by both techniques. Because rapid GC-MS is intended as a screening technique, identification of every compound present in a sample may not be feasible given the short analysis timescale, nor is it the goal of the technique. In practice, all samples with a presumptive positive identification would still be analyzed with traditional GC-MS or another confirmatory technique. While these case samples demonstrate the realistic possibility that identification of every sample component is not always possible with rapid GC-MS, identification of one or more controlled substances in real-world samples was possible and would be sufficient to warrant further confirmatory analysis.

In five of the seven samples in **Table 5** (case samples 7, 9, 10, 17, and 25), one or more compounds were identified by rapid GC-MS but not traditional GC-MS. Of the compounds that were only identified by rapid GC-MS, the main reason that they were not detected by GC-MS was due to insufficient peak abundances. While it is possible that rapid GC-MS sensitivities are better than those for traditional GC-MS for the particular compounds identified, this reasoning is unlikely because the same detector is utilized for both techniques. Rather, a more likely explanation could be that a greater volume of solution was sampled and analyzed by rapid GC-MS for these samples compared to the 1 μL fixed-volume traditional GC-MS injection. Thus, a greater amount of solution injected into the rapid GC-MS system could have led to higher abundances relative to those for traditional GC-MS analysis, resulting in detection solely by rapid GC-MS. Alternatively, differences in chromatographic behavior for rapid *versus* traditional separation could have led to detection differences. The majority of these compounds are sugars or fatty acids, which are difficult to separate and detect using relatively nonpolar columns, such as a DB-5 used for traditional GC-MS in this study [29]. For example, in case sample seven, palmitic acid and stearic acid were detected by rapid GC-MS but not traditional GC-MS. Given that these compounds are fatty acids, it is possible that they were either trapped in the traditional inlet liner or held in the traditional column due to the length of the column. As such, these compounds may not have been retained as well on the rapid column, and due to the timescale, were simply pushed through the column. Despite this occurrence, the majority of compounds in each of these samples were detected using both analytical techniques.

## LOD

LODs were determined for the ten compounds indicated in **Table 1**. Values were calculated at the 90 % confidence interval and are reported in **Table 6**, with the complete raw data shown in **Supplementary Data B, Table SB5**. Because the test solution was transferred to the glass probe by dipping the probe in solution, the corresponding detection limits are reported as concentrations (μg/mL), rather than exact masses. LODs ranged from 0.857 μg/mL (α-PBP) to 18.2 μg/mL (alprazolam). Ranges for these values were comparable to LODs determined for rapid GC-MS and traditional GC-MS analysis of similar compounds in previous work [12].

## Carryover / Contamination

To assess the possible effects of carryover, a slightly modified approach was taken for data processing. Rapid GC-MS is unique in that solvent delays are not feasible given the fast chromatographic timescale. For non-volatile samples such as seized drugs, a solvent peak is eliminated by allowing the solvent to evaporate from the probe prior to injection. However, for volatile samples that are introduced as syringe injections, solvent tailing can possibly interfere with background levels. To maintain consistency across data processing in anticipation of future samples that would require syringe injections due to sample volatility, such as ignitable liquids, abundances were normalized with respect to the blank injection prior to the respective test solution injection. For each set of injections of a given compound, extracted ion chromatograms (EICs) were generated using the respective test solution compound’s base peak mass-to-charge value. In the test solution and subsequent blank EICs, the chromatographic abundance was extracted at the retention time of the compound of interest and normalized to the abundance at the same retention time in the preceding blank injection.

At low- and high-test solution concentrations, minimal carryover was observed after a single subsequent probe blank injection (**Figure 2**). Normalized abundances were below the threshold for acceptance. For all compounds, relative carryover, or the ratio of the normalized abundance in a blank injection to the normalized abundance of the test compound, was approximately 0.01 a.u. for subsequent blank injections for all test compounds (**Supplementary Data B, Table SB6**). The relative abundances in the subsequent probe blank immediately following the test solution compound injection returned to background levels similar to those in the preceding blank injection (indicated by the red dashed line in **Figure 2**).

When assessing contamination through analysis of a clean probe, higher-than-average baseline response was observed at retention times 0.0767 min, 0.1355 min, 0.1942 min, 0.2254 min, 0.5612 min, and 0.7963 min in the probe blank injections. Due to the low abundances at these retention times (104 counts) compared to typical compound abundances (106 counts), identifications could not be made. However, ions with > 25 % relative abundance were identified at *m/z* 44, *m/z* 73, *m/z* 91, *m/z* 126, *m/z* 127, *m/z* 207, and *m/z* 281. Most of these ions are common GC-MS background ions attributed to column bleed (*m/z* 73, *m/z* 207, *m/z* 281) and residual carbon dioxide (*m/z* 44) [30]. Those not associated with the instrument (*m/z* 91, *m/z* 126, *m/z* 127) were likely associated with the probe itself (**Supplementary Data B, Table SB6**).

## Robustness

Small changes to internal rapid GC-MS method parameters did not affect the data overall. Complete results of these studies, including retention times and mass spectral search scores for each of the experiments, are shown in **Supplementary Data B, Table SB7**. For all 14 test solution compounds, minimal differences in average retention times (less than 6 % difference) and mass spectral search scores (less than 3 % difference) between the high and low levels of a given factor were observed, successfully meeting the upper criteria threshold of 10 %. Comparatively, the mass spectral search scores for stanozolol were higher for this component of the validation than for the precision component, with the majority of scores above 90 a.u. As these data were collected after instrument maintenance, the effects of source cleanliness on mass spectral quality for steroids is apparent. For this reason, these scores for stanozolol obtained prior to maintenance cannot be directly compared to those obtained post-maintenance. Nonetheless, the rapid GC-MS system was determined to be robust for the compounds studied.

## Ruggedness (Intermediate Precision)

Modifying parameters external to the instrumental method did not negatively affect retention times and mass spectral search scores. % RSDs were below 10 % for both metrics for all compounds, with the majority below 5 % for retention times and below 2 % for mass spectral search scores. **Supplementary Data B, Table SB8** shows the results of all individual analyses involving the utilization of a glass probe from a different pack, replacement of the inlet liner and helium gas cylinder, and performance of replicate injections by a different analyst. Such data show that the method and instrument is largely unaffected by these modifications. Demonstration of ruggedness is especially applicable in practicing forensic laboratory settings, in which instrumentation is utilized by a variety of analysts and frequently undergoes routine maintenance due to constant usage.

## Stability Studies

The reproducibility of rapid GC-MS was maintained when exposed to old and freshly prepared test solutions. Data for this study, including individual solution results and summative results, are presented in **Supplementary Data B, Table SB9**. Except for stanozolol, results across the old and fresh test solutions revealed retention time and mass spectral search score % RSDs below 10 %, meeting the acceptance criteria. Comparatively, retention times spanned a slightly narrower range across replicate measurements of the fresh solution (% RSDs < 4 %) compared to the old solution (% RSDs < 5 %). The highest variation in retention time was observed for methamphetamine, which is reasonable given the chromatographic challenges described in previous sections of this work. For analysis of the old solution, the spectral search score % RSD for stanozolol was 11 %, just outside the acceptance criteria. The scores for stanozolol in both the old and fresh solutions were generally lower than those for the remaining 13 compounds in the test solution. While the scores for stanozolol in the fresh solution did not vary as widely as those for the old solution, both sets of scores were likely lower in magnitude due to the dirty source, as discussed above. The wider variation in scores for the old solution could have been due to possible compound degradation over time, in addition to the sensitivity to source cleanliness. For all remaining compounds, the method of test solution preparation did not affect mass spectral quality, as search score % RSDs, irrespective of solution, were less than or equal to 5 %.

# Conclusions

In this work, a comprehensive validation was completed for rapid GC-MS according to a plan designed specifically for seized drug screening, and the validation was successful in identifying the various capabilities and limitations of the technique. Isomer differentiation was possible for some isomer pairs using both retention time and mass spectral search scores, but differentiation was not achieved for all isomers analyzed. This latter result was expected due to similar difficulties experienced with traditional GC-MS analyses. In totality, the results from all studies have enabled for the successful validation of the rapid GC-MS system and analysis method. In a practical setting, the technique has been demonstrated as a viable screening tool that can be used to preliminarily identify controlled substances in a sample. A complete validation package, which includes the validation plan and accompanying workbook for automated data processing, is available for practicing laboratories to utilize [18]. The plan can be adapted to fit a laboratory’s needs and translated to similar techniques. Future work involves validating the technique and developing an implementation workflow for other samples of forensic relevance, specifically ignitable liquids. While such samples are inherently more complex and require comparisons of relative chromatographic abundances across compounds of interest, the utility of rapid GC-MS is still applicable for preliminarily identifying these diagnostic compounds. In total, this work is part of a larger effort to reduce the barrier of implementation for rapid GC-MS and other novel technologies, including separation-based techniques and ambient ionization mass spectrometry techniques, for forensic and public health applications.

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# CRediT authorship contribution statement

# Briana A. Capistran (ORCID: 0000-0001-6937-0403): Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft.

# Edward Sisco (0000-0003-0252-1910): Conceptualization, Supervision, Writing – review & editing.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

# Disclaimer

Certain commercial products are identified in order to adequately specify the procedure; this does not imply endorsement or recommendation by NIST, nor does it imply that such products are necessarily the best available for the purpose.

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**Tables**

Table 1. Compounds, with respective molecular formulas and masses, present in the 14-compound seized drug test solution used for the precision, robustness, ruggedness, and stability components of the validation study.

|  |  |  |
| --- | --- | --- |
| **Compounda** | **Formula** | **Molecular Mass (Da)** |
| Methamphetamine†‡ | C10H15N | 149.23 |
| α-Pyrrolidinobutiophenone (PBP)†‡ | C14H19NO | 217.31 |
| α-Pyrrolidinopentiophenone (PVP)† | C15H21NO | 231.33 |
| bk-MDEA (Ethylone)†‡ | C12H15NO3 | 221.25 |
| bk-MBDB (Butylone)† | C12H15NO3 | 221.25 |
| Tenocyclidine (TCP)†‡ | C15H23NS | 249.44 |
| Phencyclidine (PCP)† | C17H25N | 243.44 |
| Cocaine‡ | C17H21NO4 | 303.35 |
| Nandrolone‡ | C18H26O2 | 274.40 |
| 5-Fluoro ADB‡ | C20H28FN3O3 | 377.45 |
| Heroin‡ | C21H23NO5 | 369.42 |
| Alprazolam‡ | C17H13ClN4 | 308.76 |
| Furanyl Fentanyl†‡ | C24H26N2O2 | 374.44 |
| Stanozolol | C21H32N2O | 328.49 |

aIn addition to the pre-made 14-compound solution purchased from Cayman Chemical, compounds were also purchased individually as bulk solids or stock solutions (in methanol or acetonitrile).

†Purchased as HCl salt form.

‡Indicates compounds also used for Range (LOD) component of this study.

Table 2. Compounds, with respective molecular formulas, molecular masses, and isomer series, present in the isomer sets used for the selectivity component of the validation study.

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound** | **Formula** | **Molecular Mass (Da)** | **Isomer Series** |
| **Set 1** |  |  |  |
| Methamphetamine1† | C10H15N | 149.23 | A |
| *m*-Fluorofentanyl1† | C22H27FN2O | 354.46 | B |
| 2,3-Pentylone isomer1† | C13H17NO3 | 235.27 | C |
|  |  |  |  |
| **Set 2** |  |  |  |
| Phentermine1† | C10H15N | 149.23 | A |
| *o*-Fluorofentanyl1† | C22H27FN2O | 354.46 | B |
| Pentylone1† | C13H17NO3 | 235.27 | C |
|  |  |  |  |
| **Set 3** |  |  |  |
| *p*-Fluorofentanyl1† | C22H27FN2O | 354.46 | B |
| N-Methylethylone1† | C13H17NO3 | 235.27 | C |
| Δ8-Tetrahydrocannabinol2 | C21H30O2 | 314.46 | D |
|  |  |  |  |
| **Set 4** |  |  |  |
| Δ9-Tetrahydrocannabinol2 | C21H30O2 | 314.46 | D |
| Eutylone1† | C13H17NO3 | 235.27 | C |
| AB-FUBINACA1 | C20FH21N4O2 | 368.40 | E |
|  |  |  |  |
| **Set 5** |  |  |  |
| Cannabidiol2 | C21H30O2 | 314.46 | D |
| bk-DMBDB (Dibutylone)1† | C13H17NO3 | 235.27 | C |
| AB-FUBINACA 3-fluorobenzyl isomer1 | C20FH21N4O2 | 368.40 | E |

1Purchased from Cayman Chemical, 2Purcahsed from Cerilliant Corporation.

†Purchased as HCl salt form.

Table 3. Cutting agents and diluents used for the matrix solutions as part of the matrix effects component of the validation study.

|  |  |  |
| --- | --- | --- |
| **Compounda** | **Formula** | **Molecular Mass (Da)** |
| **Matrix Solution 1** |  |  |
| Caffeine1 | C8H10N4O2 | 194.19 |
| Diphenhydramine2† | C17H21NO | 255.36 |
| Ibuprofen3 | C13H18O2 | 206.29 |
| Levamisole2† | C11H12N2S | 205.29 |
| Noscapine (S,R)1 | C22H23NO7 | 413.43 |
| Quinine1 | C20H24N2O2 | 324.42 |
|  |  |  |
| **Matrix Solution 2** |  |  |
| Acetaminophen1 | C8H9NO2 | 151.17 |
| Dimethyl Sulfone1 | C2H6O2S | 94.13 |
| Lidocaine1† | C14H22N2O | 234.34 |
| Metamizole3 | C13H17N3O4S | 333.30 |
| Papaverine1† | C20H21NO4 | 339.39 |

1Purchased from Sigma-Aldrich; 2Purchased from Cerilliant Corporation, 3Purchased from Cayman Chemical

aAll compounds had a purity of ≥97 %.

†Purchased as HCl salt form.

Table 4. Rapid GC-MS method parameters.

|  |  |
| --- | --- |
| **Parameter** | **Value** |
| Rapid GC-MS temperature program | 80 °C, hold 2 s  5 °C/s to 180 °C, hold 5 s  7 °C/s to 310 °C, hold 20 s *(Total run time:86 s)* |
| Rapid GC inlet split ratio | Split (10:1 fixed) |
| Rapid GC inlet temperature | 250 °C |
| Rapid GC carrier gas pressure | 103.421 kPa (15 psi) |
| Rapid GC injection time | 5 s |
| GC oven temperature | 280 °C |
| Helium conservation | On |
| MSD transfer line temperature | 280 °C |
| Ionization source | Electron ionization (EI), 70 eV |
| Source temperature | 250 °C |
| Quadrupole temperature | 150 °C |
| Gain factor | 1 arbitrary unit (a.u.) |
| Scan rate | N = 1 (5.9 scans/s) |
| Mass scan range | *m/z* 40 – *m/z* 550 |
| Threshold | 50 counts |
| Tune method | atune |

Table 5. Compounds in case samples detected either by rapid GC-MS *or* traditional GC-MS and the reasoning for why the respective compound was not detected in the second technique.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Compound(s) Detected** | **Technique that Detected Compound** | **Reason for Non-Detection in Complementary Technique** |
| 7 | Palmitic Acid | Rapid GC-MS | INS |
| Stearic Acid | Rapid GC-MS | INS |
| 8 | 4-ANPP | GC-MS | CE (coeluting with Mannitol) |
| 9 | Palmitic Acid | Rapid GC-MS | INS |
| Stearic Acid | Rapid GC-MS | INS |
| 10 | Mannitol | Rapid GC-MS | INS |
| 4-ANPP | GC-MS | CE (coeluting with Mannitol) |
| 17 | Dimethylsulfone | Rapid GC-MS | INS |
| 23 | Deschloroetizolam | GC-MS | CE (coeluting with Quinine) |
| *p*-Fluorofentanyl | Rapid GC-MS | CE (coeluting with Fentanyl) |
| 25 | Tropacocaine | GC-MS | CE (coeluting with Levamisole) |
| Norcocaine | GC-MS | CE (coeluting with Cocaine) |
| Cannabidiol | Rapid GC-MS | INS |

INS = Peak present in GC-MS result but at insufficient abundance for reporting.

CE = Unable to identify in rapid GC-MS result due to coelution.

Table 6. Limits of detections calculated at the 90 % confidence interval (LOD90) for each of the compounds studied in the Range component of the validation study.

|  |  |
| --- | --- |
| **Compound** | **LOD90 (μg/mL)** |
| Methamphetamine | 7.128 |
| α-Pyrrolidinobutiophenone (PBP) | 0.8573 |
| bk-MDEA (Ethylone) | 4.944 |
| Tenocyclidine (TCP) | 1.769 |
| Cocaine | 1.715 |
| Nandrolone | 7.190 |
| Heroin | 1.801 |
| 5-Fluoro ADB | 5.484 |
| Alprazolam | 18.16 |
| Furanyl Fentanyl | 6.945 |
|  |  |

**Figures**

Chart

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**Figure 1.** Individual retention time replicates (normalized to the global average) for the reproducibility studies. The red dashed lines represent the upper and lower bounds of the acceptance criteria (±10 %).

Chart, line chart

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**Figure 2.** Normalized abundances of each test solution compound and at the corresponding compound retention time in the subsequent blank injection(s) for (A) low concentration and (B) high concentration solutions. The “0” point in each plot represents the test solution compound injection. The red dashed line indicates the normalized background in blank injection preceding compound analysis (normalized to itself). The gray shaded area represents the acceptable abundance threshold in the subsequent blank injections (S/N ≤ 3).