

Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Theresa Starpoli, Director of Technical Services
Date: September 21, 2026
RE: Validation Plan
 Validation Plan for Qualitative Basic Drug Screen by GC-MS

[Signature] 9/22/26

Validation Plan for the Qualitative Basic Drug Screen by GC-MS

The Toxicology Procedures Manual Section 9 (Qualtrax Revision 34) provides direction for the sample preparation and screening parameters for basic compounds using gas chromatography-mass spectrometry (GC-MS). The procedure provides a solid phase extraction (SPE) and a basic liquid-liquid extraction (LLE) option for sample preparation. Given these options, the Virginia Department of Forensic Science Scientific Advisory Committee (SAC) Toxicology Subcommittee recommended an investigation into the sample preparation procedures as well as establishing limits of detection for the compounds within the method.

It is proposed to validate a method for the qualitative analysis of basic drugs using gas chromatography-mass spectrometry (GC-MS). The target compounds are listed in Table 1. The internal standard selected for the method is methapyrilene. The target compounds listed are for the initial validation. The addition of other target compounds will follow the same validation experiments and will have an independent validation plan, as appropriate.

Table 1 Target compounds

Basic Compounds Evaluated			
Amitriptyline	Dextromethorphan	Methadone	Quetiapine
Amphetamine	Diphenhydramine	Methamphetamine	Sertraline
Buprenorphine	Doxylamine	Mirtazapine	Tramadol
Bupropion	Fentanyl	Mitragynine	Trazodone
Chlorpheniramine	Fluoxetine	Nortriptyline	Venlafaxine
Citalopram	Hydroxyzine	Oxycodone	Verapamil
Cocaine	Ketamine	Phencyclidine	Zolpidem
Cyclobenzaprine	Lidocaine	Promethazine	

An Agilent Technologies 8890 GC coupled to a 5977C MS with helium carrier gas will be utilized for validation. An HP-SMS-UI analytical column will be employed for chromatographic separation. The instrumental parameters are listed in Table 2.

Table 2 Instrumental parameters

	New Method
Initial Oven Temp (°C)	110°C
Hold Time (min)	2 min
Ramp 1	17°C/min to 200°C (hold 0 min)
Ramp 2	30°C/min to 300°C (hold 8 min)
Solvent Delay (min)	2.5 min
Syringe Size (µL)	10 µL
Injection Volume (µL)	2 µL
Column Type	HP-5MS UI

The sample preparation procedure includes a liquid-liquid extraction with 1.0 mL of biological matrix. The validation will include the assessment of blank blood, antemortem blood, postmortem blood, liver, and urine. When assessing liver, additional sample preparation steps will be employed. The sample preparation procedure is listed in Table 3.

Table 3 Sample preparation

Optimized Liquid-Liquid Extraction Procedure
Prepare controls
Pipette 1.0 mL of biological sample into test tubes
Pipette 25 µL of internal standard
If liver,
Add 2:2:1.8 mL of hexane:isoamyl alcohol:sulfuric acid
Cap and rotate test tubes for 10 minutes
Centrifuge for 5 minutes at approximately 3500 rpm
Transfer bottom layer to clean test tube
Add 100 µL concentrated ammonium hydroxide
Add 2.5 mL THIA
Cap and rotate test tubes for 10 minutes
Centrifuge for 5 minutes at approximately 3500 rpm
Transfer top organic layer and discard lower aqueous layer
Add 1.0 mL of 0.5 N sulfuric acid
Cap and rotate test tubes for 10 minutes
Centrifuge for 5 minutes at approximately 3500 rpm
Aspirate top organic layer and discard
Add 100 µL concentrated ammonium hydroxide
Add 100 µL THIA
Vortex 10-15 seconds
Centrifuge for 5 minutes at approximately 3500 rpm
Transfer approximately 100 µL of top organic layer to GC autosampler vial

A validation plan is outlined herein pursuant to the Quality Manual (Qualtrax Revision 37) and the Toxicology Procedures Manual (Qualtrax Revision 34). The validation plan is in accordance with ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology (First Edition, 2019).

1. Sensitivity
 - a. Estimated Limits of Detection (LOD)
2. Carryover
3. Interferences
 - a. Endogenous Compounds
 - b. Internal Standard
 - c. Commonly Encountered Analytes
4. Stability
5. References

1. Sensitivity

a. Estimated Limits of Detection (LOD)

The estimated LOD for this validation shall be the administratively defined decision points (threshold concentrations) established during method development. These defined concentrations will be established as the decision point for reporting analytes within this method although a lower estimated LOD may be analytically achievable. The concentrations to be evaluated are listed in Table 4.

Table 4 Estimated LOD concentrations

	LOD (mg/L)	
	Blood and Urine	Liver
Amitriptyline	0.08	0.20
Amphetamine	0.30	0.30
Buprenorphine	0.60	0.40
Bupropion	0.04	0.07
Chlorpheniramine	0.02	0.03
Citalopram	0.07	0.06
Cocaine	0.02	0.04
Cyclobenzaprine	0.07	0.20
Dextromethorphan	0.05	0.04
Diphenhydramine	0.05	0.20
Doxylamine	0.04	-
Fentanyl	0.08	0.06
Fluoxetine	0.10	0.20
Hydroxyzine	0.70	0.60
Ketamine	0.02	0.01
Lidocaine	0.03	0.04
Methadone	0.06	0.09
Methamphetamine	0.08	0.20
Mirtazapine	0.02	0.03
Mitragynine	0.30	0.40
Nortriptyline	0.30	0.20
Oxycodone	0.30	0.06
Phencyclidine	0.01	0.01
Promethazine	0.09	0.04
Quetiapine	1.00	0.50
Sertraline	0.05	0.09
Tramadol	0.05	0.50
Trazodone	0.50	0.40
Venlafaxine	0.08	0.30
Verapamil	0.30	0.30
Zolpidem	0.30	0.07

The decision point shall be evaluated by fortifying, at minimum, three different blank matrix sources per matrix type (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine). The three different blank matrix sources shall be analyzed over a minimum of three

analyses to demonstrate that all predetermined detection and identification criteria are met. The predetermined identification criteria include Gaussian peak shape, retention time within $\pm 2\%$, and signal-to-noise ≥ 3.3 .

The predetermined acceptance criteria shall be met for $\geq 95\%$ of the samples to establish the estimated lower limit of detection. If the administratively defined decision point does not meet the predetermined acceptance criteria for $\geq 95\%$ of the samples, more samples shall be evaluated to establish the criteria. If the criteria cannot be met with the concentrations listed in Table 4, the following concentrations listed in Table 5 will be evaluated for the estimated limit of detection.

Table 5 Adjusted estimated limit of detection

	LOD (mg/L)	
	Blood and Urine	Liver
Amitriptyline	0.10	0.50
Amphetamine	0.50	0.50
Buprenorphine	0.70	0.50
Bupropion	0.10	0.10
Chlorpheniramine	0.05	0.10
Citalopram	0.10	0.10
Cocaine	0.05	0.10
Cyclobenzaprine	0.10	0.50
Dextromethorphan	0.10	0.10
Diphenhydramine	0.10	0.50
Doxylamine	0.10	-
Fentanyl	0.10	0.10
Fluoxetine	0.20	0.50
Hydroxyzine	0.80	1.0
Ketamine	0.05	0.05
Lidocaine	0.05	0.50
Methadone	0.10	0.10
Methamphetamine	0.10	0.50
Mirtazapine	0.05	0.05
Mitragynine	0.50	0.50
Nortriptyline	0.50	0.50
Oxycodone	0.50	0.10
Phencyclidine	0.02	0.05
Promethazine	0.10	0.10
Quetiapine	1.20	1.0
Sertraline	0.10	0.10
Tramadol	0.10	1.0
Trazodone	0.60	0.50
Venlafaxine	0.10	0.50
Verapamil	0.50	0.50
Zolpidem	0.50	0.10

2. Carryover

Carryover will be evaluated by analyzing blank matrix samples immediately following progressively higher concentrations (up to 10-20 mg/L) of fortified matrix within the injection sequence. The highest analyte concentration at which no analyte carryover is observed, in the blank matrix, is determined to be the concentration at which the method is free from carryover. Analyte carryover is indicated by a response greater than 10% of the estimated limit of detection (threshold control), retention time within $\pm 2\%$ of the standard retention time, presence of major diagnostic ions, and signal to noise ratio ≥ 3.3 . This concentration shall be confirmed using triplicate analysis with a minimum of three sources per matrix type.

3. Interferences

To assess interference, the instrumental response and identification criteria for the target compounds and internal standard within the method will be monitored. If present, the impact on identification shall be evaluated. The impact is deemed insignificant if the instrumental response is less than 10% of the estimated limit of detection response, the retention time is outside of $\pm 2\%$ of the standard retention time, the major diagnostic ions are not identified, the signal to noise ratio is < 3.3 , or the library match score is $< 80\%$.

a. Endogenous Compounds

Where possible, a minimum of ten negative matrix samples, from different sources and without the addition of an internal standard, shall be analyzed for possible endogenous interferences. A minimum of ten matrix samples for each matrix type (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine) within the validation should be evaluated, whenever possible.

b. Internal Standard

To evaluate potential interferences of the internal standard by a high concentration of analyte, samples shall be fortified without internal standard and analyzed for the absence of response for the internal standard. A single blank matrix sample, per matrix type (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine), shall be evaluated.

To evaluate potential interferences from the method's internal standard concentration to a low concentration of analyte, matrix shall be fortified with an appropriate concentration of internal standard without the analyte of interest and analyzed for the absence of response for the analyte. A single blank matrix sample, per matrix type (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine), shall be evaluated.

c. Commonly Encountered Analytes

Analytes which may be expected to be present in case samples shall be evaluated for their potential to interfere with the method's analytes. Matrix samples shall be fortified with commonly encountered drugs, metabolites, and other structurally similar compounds at high concentrations (i.e., highest calibrator concentration from current method).

Potential interferents to be evaluated:

- Barbiturates (40 mg/L)
- Amphetamines (2.0 mg/L)
- Benzodiazepines (2.0 mg/L)
- Carisoprodol and meprobamate (100 mg/L)
- Anti-epileptic drugs (40 mg/L)
- Basic drugs from previously made mixes (6.0 mg/L)
- Acid/neutral drugs from previously made mixes (6.0 mg/L)
- Opioids and cocaine (0.2/2.0/1.0 mg/L)
- Fentanyl derivatives (0.05/0.1 mg/L)
- Novel psychoactive substance (1.0 mg/L)
- Cannabinoids (0.1/0.5 mg/L)

In addition to commonly encountered analytes, each drug within the method will be evaluated individually to evaluate the potential impact of structural similarity or degradation. The identification of a potential interferent does not negate the target analyte from the method. If present, the interferent will be evaluated and may be noted as a limitation of the methodology.

4. Stability

During the validation period, the stability of extracted samples that are not analyzed immediately shall be addressed. Extracted samples shall be stored in autosampler vials on the instrument throughout the stability evaluation process. This enables the simulation of an abrupt abortion, delay, or interruption during instrumental analysis.

At minimum, a single blank matrix source, per matrix type (i.e., blank blood, antemortem blood, postmortem blood, liver, and urine), will be extracted at two concentrations (low [3x LOD concentration] and high [10x LOD concentration]) and analyzed at minimum every twenty-four hours for a seven-day period (or until no sample remains) with triplicate injections at each time point. For day one instrumental response, samples will be extracted and immediately analyzed. The ratioed response (ratio of peak area of analyte to internal standard) will be averaged and all other ratioed responses from subsequent time points will be evaluated against the average ratioed response. The average ratioed responses for each time point will be compared to the day one ratioed response and plotted. Compounds are considered stable if the average ratioed response of the triplicate injections for a time point falls within the method's predefined acceptable change (i.e., $\pm 20\%$) and meets identification criteria of a retention time within $\pm 2\%$ of

the standard retention time, presence of major diagnostic ions, signal to noise ratio ≥ 3.3 . For example, if the ratioed response increases above 120% or decreases below 80% of the original ratioed response the compound is no longer deemed stable.

Stability should be carried out by injecting samples from the same autosampler vial throughout the stability experiments.

5. References

Virginia Department of Forensic Science Quality Manual, Qualtrax Revision 37, 2026.

Virginia Department of Forensic Science Toxicology Procedures Manual, Qualtrax Revision 34, 2026.

ANSI/ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology. 1st Edition. 2019.

Washington State Patrol Toxicology Laboratory Division, Confirmation Method: Basic Drug, Revision 6, 2024.

Illinois State Police Toxicology Procedures Manual, IID-1 Drug Screen for Blood, Version 2024.01.22, 2024.

Arkansas Department of Public Safety Forensic Toxicology Quality Manual, 9.4 Base Screen, Revision 36, 2024.

Schember, I. et al. Validation of a GC/MS method for the determination of alkaline drugs in whole blood. Unpublished manuscript. Marshall University. Retrieved from https://www.marshall.edu/forensics/files/Schember_Isabella_Research_Paper_0805.pdf

Breil, C. et al (2017). "Blight and Dyer" and Folch methods for solid-liquid-liquid extraction of lipids from microorganisms. Comprehension of solvation mechanisms and towards substitution with alternative solvents. Int. J. Mol. Sci, 18(4), 708, <https://doi.org/10.3390/ijms18040708>.

Method Development Summary: Base Screen

Memo To: James Hutchings, Ph.D., Toxicology Program Manager
From: Rebecca Wagner, Ph.D., Chemistry Research Section Supervisor
CC: Theresa Starnoli, Director of Technical Services
Date: September 21, 2026
RE: Method Development Summary
Method Development for the Basic Drug Screen by GC-MS



9/22/26

Method Development Summary for the Basic Drug Screen by GC-MS

The Toxicology Procedures Manual Section 9 (Qualtrax Revision 34) provides direction for the sample preparation and screening parameters for basic compounds using gas chromatography-mass spectrometry (GC-MS). The procedure provides a solid phase extraction (SPE) and a basic liquid-liquid extraction (LLE) option for sample preparation. Given these options, the Virginia Department of Forensic Science Scientific Advisory Committee (SAC) Toxicology Subcommittee recommended an investigation into the sample preparation procedures as well as establishing limits of detection (LODs) for the potential compounds within the method.

The evaluation of the solid phase extraction method was outside of the scope of this method development. The SPE method currently uses methylene chloride as part of the elution solvent. Given the recent restrictions placed on the utilization of methylene chloride within the laboratory, the method development focused on the development and evaluation of the basic liquid-liquid extraction.

Table 1 lists the basic compounds that were established for the initial evaluation of the basic drug screen method. These compounds were identified based on the reporting trends of basic drugs within the Toxicology Section and the input provided by the Toxicology Program Manager and the Toxicology Technical Resource Team (TRT).

Table 1: List of basic compounds used during method development

Basic Compounds Evaluated			
Amitriptyline	Dextromethorphan	Methadone	Promethazine
Amphetamine	Diphenhydramine	Methamphetamine	Quetiapine
Buprenorphine	Doxylamine	Mirtazapine	Sertraline
Bupropion	Fentanyl	Mitragynine	Tramadol
Chlorpheniramine	Fluoxetine	Morphine	Trazodone
Citalopram	Hydroxyzine	Nortriptyline	Venlafaxine
Cocaine	Ketamine	Oxycodone	Verapamil
Cyclobenzaprine	Lidocaine	Phencyclidine	Zolpidem

Instrumental Method Development

During method development, a comparison of instrumental methods was performed to develop a fundamental understanding of the current differences between laboratories. Table 2 lists the GC-MS instrumental parameters with noted differences.

Table 2: Instrumental parameters for current methods

	Laboratory A	Laboratory B	Laboratory C	Laboratory D
Initial Oven Temp (°C)	90°C	110°C	80°C	110°C
Hold Time (min)	2 min	2 min	1 min	0.5 min
Ramp 1	15°C/min to 245°C (hold 0 min)	17°C/min to 200°C (hold 0 min)	30°C/min to 250°C (hold 0 min)	12°C/min to 300°C (hold 7 min)
Ramp 2	30°C/min to 300°C (hold 8 min)	30°C/min to 300°C (hold 8 min)	25°C/min to 280°C (hold 17.5 min)	N/A
Solvent Delay (min)	3.0 min	4.0 min	3.6 min	2.5 min
Syringe Size (µL)	10 µL	10 µL	10 µL	5 µL
Injection Volume (µL)	1 µL	2 µL	1 µL	1 µL
Column Type	RTX-1	HP-5MS	HP-5MS	RXI-5MS

Each instrumental method was evaluated using an Agilent Technologies 8890 GC coupled to a 5977C MS with helium carrier gas. An ultra inert double taper splitless liner (Agilent Technologies Part # 5190-3983) was employed and the mass range scanned was 40-550 amu. An Agilent Technologies HP-5MS-UI (30 m x 250 µm x 0.25 µm) column and an Agilent Technologies HP-1MS (30 m x 250 µm x 0.25 µm) column were both evaluated. The compounds listed in Table 1 were evaluated at a concentration of 1 mg/L in THIA (78:20:2 toluene:hexane:isoamyl alcohol), ethyl acetate, and chloroform. The evaluation of ethyl acetate and chloroform minimized background peaks but also reduced the sensitivity for the later eluting compounds making the solvents less than ideal for analysis.

The instrumental parameters were evaluated using each analytical column. The chromatographic resolution, as well as the number of compounds identified with each instrumental method, were evaluated. Each compound was evaluated at a concentration of 1.0 µg/mL. Table 3 describes the results of each instrumental method using the Agilent Technologies HP-5MS-UI column with THIA as the solvent. Table 4 describes the results obtained from each instrumental method using the Agilent Technologies HP-1MS column with THIA as the solvent.

Table 3: Agilent Technologies HP-5MS-UI column with THIA as the solvent

	Laboratory A	Laboratory B	Laboratory C	Laboratory D
Number of Compounds Detected	28	29	26	25
Compounds Not Detected	Trazodone Hydroxyzine Buprenorphine Quetiapine	Amphetamine Methamphetamine Quetiapine	Trazodone Amphetamine Hydroxyzine Mitragnine Buprenorphine Quetiapine	Trazodone Amphetamine Morphine Hydroxyzine Mitragnine Buprenorphine Quetiapine
Co-Eluting or Poorly Resolved Compounds	Fluoxetine co-eluting with ketamine	Fluoxetine poorly resolved from ketamine Dextromethorphan poorly resolved from methadone	Diphenhydramine co-eluting with ketamine Dextromethorphan co-eluting with methadone	Fluoxetine co-eluting with ketamine

Table 4: Agilent Technologies HP-1MS column with THIA as the solvent

	Laboratory A	Laboratory B	Laboratory C	Laboratory D
Number of Compounds Detected	25	29	25	25
Compounds Not Detected	Trazodone Morphine Oxycodone Hydroxyzine Mitragnine Buprenorphine Quetiapine	Amphetamine Methamphetamine Quetiapine	Trazodone Amphetamine Morphine Hydroxyzine Mitragnine Buprenorphine Quetiapine	Trazodone Amphetamine Morphine Oxycodone Hydroxyzine Buprenorphine Quetiapine
Co-Eluting or Poorly Resolved Compounds	Cyclobenzaprine co-eluting with mirtazapine Fentanyl co-eluting with background peak	Fentanyl co-eluting with background peak	Ketamine co-eluting with fluoxetine PCP co-eluting with doxylamine Dextromethorphan co-eluting with methadone Fentanyl co-eluting with background peak	Doxylamine co-eluting with background peak

Given the number of interferences identified for each instrumental method when using the Agilent Technologies HP-1MS column, the focus of method development shifted to the HP-5MS-UI column. Ultimately, Laboratory B method using a HP-5MS-UI column was further investigated to assess the potential to enhance the chromatographic separation between the closely eluting compounds as well as gain detection of the early eluting compounds (amphetamine and methamphetamine). By decreasing the solvent delay to 2.5 minutes, amphetamine and methamphetamine were detected using the method. When assessing changes to oven

temperature ramps, to enhance chromatographic separation and shorten the analytical run time, negative impacts were observed regarding peak resolution. Therefore, the oven temperature ramps of Laboratory B method were maintained.

The optimized method was then evaluated using a 10 μ L and 5 μ L syringe with both a 2 μ L and 1 μ L injection volume. All other instrumental parameters were maintained with the exception of the necessary adjustments to injection speed and wash volumes/speeds. To assess the impact of the syringe volume and injection volume, samples containing compounds were injected in triplicate for each analytical condition. The relative response factor (RRF) and coefficient of variation (CV) were calculated for each compound using methapyrilene as the internal standard.

When assessing the results, the RRF was greater for the 2 μ L injection volumes as compared to the 1 μ L, except for the 1 μ L injection volume with the 10 μ L syringe, which had a higher RRF when compared to the 2 μ L injection volume with the 5 μ L syringe. The RRF was greater for the 2 μ L injection with the 10 μ L syringe as compared to the 2 μ L injection with the 5 μ L syringe. Overall, the CVs were lower for the 1 μ L injection volume (with both syringes) when compared to the 2 μ L injection volume using a 5 μ L syringe. When evaluating the statistical significance, no statistically significant difference was noted between the two injection volumes when using the 10 μ L syringe. As expected, the 2 μ L injections produced larger peak areas overall. Given the larger RRF compared to all other testing parameters and the increased area counts from the 2 μ L injection volume, the 2 μ L injection volume using a 10 μ L syringe was selected for this application. The optimized instrumental parameters are listed in Table 5.

Table 5: Optimized instrumental parameters

New Method	
Initial Oven Temp (°C)	110°C
Hold Time (min)	2 min
Ramp 1	17°C/min to 200°C (hold 0 min)
Ramp 2	30°C/min to 300°C (hold 8 min)
Solvent Delay (min)	2.5 min
Syringe Size (μ L)	10 μ L
Injection Volume (μ L)	2 μ L
Column Type	HP-5MS UI

The optimized parameters were utilized for the remainder of the method development, including the evaluation of internal standard and sample preparation.

Internal Standard Evaluation

The Toxicology Procedures Manual has three options for internal standard when evaluating basic drugs in the Acid/Neutral/Base Drug Screen and Quantitation by GC and GC-MS. The internal standards are Sertis, methapyrilene, and mepivacaine. The concentration is also not delineated in the Toxicology Procedures Manual. After surveying each laboratory, it was noted that each lab is using primarily methapyrilene, which can only be purchased in powder form. A literature

review was performed as well as investigation into the internal standards that other laboratories are currently using. The internal standards evaluated included mepivacaine, methaqualone, methapyrilene, proadifen (SKF-525A), and phenacetin. Table 6 lists the retention time of each internal standard evaluated.

Table 6 Base screen internal standards

Internal Standard	Retention Time (min)
Mepivacaine	9.285
Methaqualone	9.623
Methapyrilene	8.935
Proadifen (SKF-525A)	10.129
Phenacetin	7.338

When evaluating the internal standards, mepivacaine had poor chromatographic separation from venlafaxine. Other than this noted potential interferent, all other internal standards had no indication of interferences. Both methaqualone and phenacetin performed well as internal standards, but these compounds have been identified in the illicit drug market and therefore were no longer considered as viable internal standards. Methapyrilene was selected as the internal standard for the method. Proadifen was recognized as a viable substitute.

Sample Preparation Development

The Toxicology Procedures Manual provides a liquid-liquid extraction for basic extraction in Section 9.6.2. This extraction procedure was used to evaluate the compounds listed in Table 1 at a concentration of 0.2 mg/L. Only amphetamine, methamphetamine, and morphine were not detected. Given the chemical properties of morphine and the solvents used in the liquid-liquid extraction, it was not anticipated that morphine would extract.

Several method modifications were evaluated to improve extraction efficiency. These modifications were from other published basic drug liquid-liquid extractions. In addition to these evaluations, the effect of working solution dry down was investigated. When preparing control samples, drying down working solution solvent prior to the addition of biological specimen was evaluated against no dry down. A statistically significant difference between the two scenarios was noted with working solution dry down performing superior to no dry down.

Upon establishment of the modified extraction procedure, the duration of sample rotation and centrifugation steps were evaluated. These experiments were performed to evaluate and optimize the total duration of sample preparation.

During the initial evaluation, the sample volume was maintained at 2.0 mL of biological specimen. The sample rotation times were evaluated at 5, 10, 15, and 20 minutes. Additionally, centrifugation time and speed were evaluated at 15 minutes/2500 rpm, 10 minutes/3100 rpm, and 5 minutes/3500 rpm. There was no statistically significant difference between the methods

(i.e., rotation time, centrifuge time and speed) when evaluating the compounds within 20% of the highest RRF.

After initial evaluation, the sample volume and extraction solvent volumes were decreased by half. Therefore 1.0 mL of sample was used for extraction. With this change, a selection of the abovementioned times and speeds were reassessed including 10 minute rotation with 10 minutes/3100 rpm centrifugation, 10 minute rotation with 5 minutes/3500 rpm centrifugation, and 15 minute rotation with 5 minutes/3500 rpm centrifugation. As shown in the 2.0 mL sample volume experiments, there was no statistically significant difference between rotation times and centrifugation times/speed. This was further confirmed by assessing the 10 minute rotation with 10 minutes/3100 rpm centrifugation and 10 minute rotation with 5 minutes/3500 rpm centrifugation in triplicate. Therefore, a rotation of 10 minutes with centrifugation at approximately 3500 rpm for 5 minutes was selected for the optimized method.

When assessing matrix types with the optimized method, it was noted that liver samples produced inconsistent instrumental responses and did not meet the estimated limits of detection when comparing other matrix types. This was due to the significant background associated with liver. Multiple fatty acid peaks interfered with doxylamine, oxycodone, and venlafaxine. Additional sample clean-up was evaluated for liver samples including acetonitrile protein precipitation. The Bligh and Dyer method was evaluated, which employed chloroform/methanol/water (2:2:1.8) and produced promising results. This technique was further optimized by evaluating the use of sulfuric acid, isoamyl alcohol, chloroform, hexane, and toluene. Table 7 lists the solvents combinations that were evaluated.

Table 7 Bligh and Dyer method alterations

Solvents	Number of Compounds Identified
Chloroform/Methanol/Sulfuric Acid	22/27
Toluene/Isoamyl Alcohol/Sulfuric Acid	10/27
Hexane/Isoamyl Alcohol/Sulfuric Acid	19/27

The three methods were compared and there was a statistically significant difference between the three methods. The chloroform/methanol/sulfuric acid and hexane/isoamyl alcohol/sulfuric acid methods were compared to each other, and no statistically significant difference was observed. Therefore, hexane/isoamyl alcohol/sulfuric acid (2:2:1.8) was utilized for liver sample clean-up prior to extraction. After the addition of the solvent, samples are capped and rotated for 10 minutes. Samples are then centrifuged for 5 minutes at approximately 3500 rpm. The bottom layer is then transferred for extraction with the liquid-liquid extraction procedure.

Figure 1 provides an example of a liver background spectra using the current liquid-liquid extraction procedure in the Toxicology Procedure Manual Section 9 (Qualtrax Revision 34). Figure 2 is an example of the liver background spectra using the optimized sample liquid-liquid

extraction procedure including liver clean-up step. It should be noted that this liver does contain both methamphetamine and amphetamine.

Figure 1 Liver background with current method

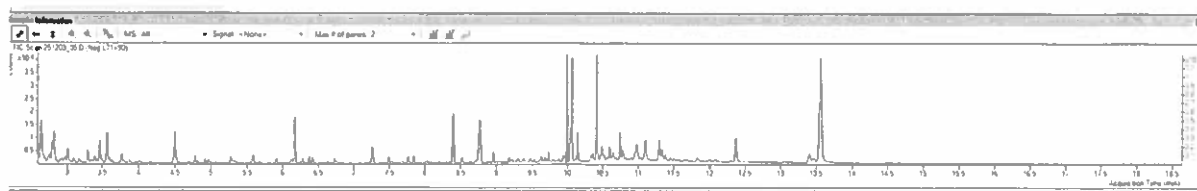
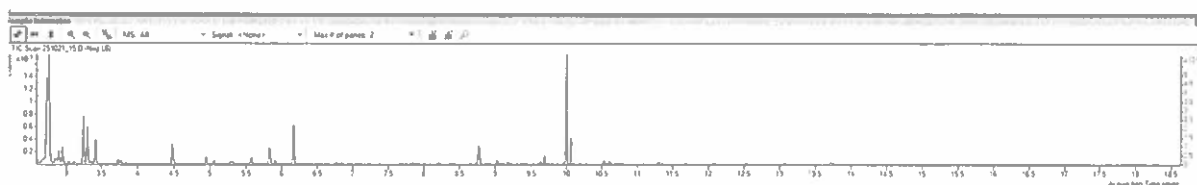


Figure 2 Liver background with optimized procedure



The optimized sample preparation procedure is listed in Table 8.

Table 8 Optimized liquid-liquid extraction

Optimized Liquid-Liquid Extraction Procedure
Prepare controls
Pipette 1.0 mL of biological sample into test tubes
Pipette 25 µL of internal standard, vortex
If liver,
Add 2:2:1.8 mL of hexane:isoamyl alcohol:sulfuric acid
Cap and rotate test tubes for 10 minutes
Centrifuge for 5 minutes at approximately 3500 rpm
Transfer bottom layer to clean test tube
Add 100 µL concentrated ammonium hydroxide
Add 2.5 mL THIA
Cap and rotate test tubes for 10 minutes
Centrifuge for 5 minutes at approximately 3500 rpm
Transfer top organic layer and discard lower aqueous layer
Add 1.0 mL of 0.5 N sulfuric acid
Cap and rotate test tubes for 10 minutes
Centrifuge for 5 minutes at approximately 3500 rpm
Aspirate top organic layer and discard
Add 100 µL concentrated ammonium hydroxide
Add 100 µL THIA
Vortex 10-15 seconds
Centrifuge for 5 minutes at approximately 3500 rpm
Transfer approximately 100 µL of top organic layer to GC autosampler vial

Estimated Limit of Detection

Once optimized, the method was evaluated to assess the estimated LOD in blank blood, antemortem blood, postmortem blood, urine, and liver. The blank blood consists of human packed blood cells that are diluted in 100 mL of water and approximately 100 mL of 1% sodium fluoride. Three sources per matrix type were fortified in duplicate with the target compounds at decreasing concentrations (0.002 µg/mL to 1.0 µg/mL). The lowest concentration at which a compound was identified was considered the estimated limit of detection. The peak had to have a retention time within $\pm 2\%$ of the retention time of the controls, a library match score of $\geq 80\%$, a signal-to-noise ratio of ≥ 3.3 , and major diagnostic ions identified. The data was collected and evaluated in the Agilent Technologies MassHunter Quantitative Analysis Software.

Upon identification of the presumptive LOD for the optimized method, the matrix types were also evaluated using the existing Toxicology Procedure Manual Section 9 (Qualtrax Revision 34) method. Initial evaluation included one matrix source per matrix type. If the presumptive LOD of the optimized and existing methods were within $\pm 30\%$ agreement, no further evaluations were performed. If the current method's results produced an LOD lower than the optimized method, testing was expanded to a total of three matrix sources per matrix type evaluated in duplicate. The highest LOD for each matrix type is listed in Table 9. The highest LOD observed across all matrices is also listed in the table.

Table 9 Preliminary LOD for optimized method

	Estimated LOD for Optimized Method					
	BB	AM	PM	Urine	Liver	Combined
	LOD (mg/L)	LOD (mg/L)	LOD (mg/L)	LOD (mg/L)	LOD (mg/L)	Highest (mg/L)
Amitriptyline	0.06	0.07	0.08	0.04	0.20	0.20
Amphetamine	0.10	0.10	0.06	0.30	0.30	0.30
Buprenorphine	0.60	0.60	0.60	0.60	0.40	0.60
Bupropion	0.02	0.04	0.03	0.02	0.07	0.07
Chlorpheniramine	0.02	0.02	0.02	0.02	0.03	0.03
Citalopram	0.06	0.07	0.06	0.07	0.06	0.07
Cocaine	0.02	0.01	0.02	0.02	0.04	0.04
Cyclobenzaprine	0.04	0.07	0.07	0.04	0.20	0.20
Dextromethorphan	0.02	0.03	0.05	0.02	0.04	0.05
Diphenhydramine	0.04	0.05	0.03	0.03	0.20	0.20
Doxylamine	0.02	0.03	0.04	0.02	n/a	0.04
Fentanyl	0.05	0.07	0.05	0.08	0.06	0.08
Fluoxetine	0.05	0.10	0.05	0.06	0.20	0.20
Hydroxyzine	0.60	0.60	0.40	0.70	0.60	0.70
Ketamine	0.02	0.008	0.01	0.01	0.01	0.02
Lidocaine	0.03	0.02	0.02	0.03	0.04	0.04
Methadone	0.04	0.06	0.06	0.04	0.09	0.09
Methamphetamine	0.05	0.06	0.06	0.08	0.20	0.20
Mirtazapine	0.008	0.008	0.008	0.02	0.03	0.03
Mitragynine	0.30	0.30	0.30	0.30	0.40	0.40
Nortriptyline	0.07	0.30	0.08	0.06	0.20	0.30
Oxycodone	0.08	0.08	0.10	0.30	0.06	0.30
Phencyclidine	0.008	0.01	0.008	0.004	0.01	0.01
Promethazine	0.04	0.09	0.06	0.05	0.04	0.09
Quetiapine	0.70	0.80	0.60	1.00	0.50	1.00
Sertraline	0.04	0.04	0.05	0.03	0.09	0.09
Tramadol	0.05	0.04	0.05	0.05	0.50	0.50
Trazodone	0.30	0.30	0.20	0.50	0.40	0.50
Venlafaxine	0.05	0.03	0.06	0.08	0.30	0.30
Verapamil	0.20	0.30	0.20	0.30	0.30	0.30
Zolpidem	0.20	0.20	0.10	0.30	0.07	0.30

Doxylamine in liver produced highly variable results. For example, the same liver source was evaluated at 0.06 mg/L, 0.07 mg/L, 0.08 mg/L, and 0.09 mg/L. The predetermined identification criteria were met for the 0.06 mg/L concentration but failed at the other three concentrations evaluated. Given this variability, a reproducible LOD was not established for doxylamine in liver.

To evaluate the performance of the optimized method, the established LOD was compared to the LOD of the current method. Table 10 lists the LOD of the optimized method and the current method for the combined blank blood, antemortem blood, postmortem blood, and urine matrices.

Table 10 Preliminary LOD comparison

	LOD Comparison	
	Optimized Method LOD (mg/L)	Current Method LOD (mg/L)
Amitriptyline	0.08	0.05
Amphetamine	0.30	>0.3
Buprenorphine	0.60	>0.9
Bupropion	0.04	0.02
Chlorpheniramine	0.02	0.02
Citalopram	0.07	0.09
Cocaine	0.02	0.02
Cyclobenzaprine	0.07	0.06
Dextromethorphan	0.05	0.05
Diphenhydramine	0.05	0.04
Doxylamine	0.04	0.05
Fentanyl	0.08	0.20
Fluoxetine	0.10	0.20
Hydroxyzine	0.70	>0.9
Ketamine	0.02	0.02
Lidocaine	0.03	0.03
Methadone	0.06	>0.08
Methamphetamine	0.08	>0.09
Mirtazapine	0.02	0.02
Mitragynine	0.30	0.20
Nortriptyline	0.30	0.20
Oxycodone	0.30	0.30
Phencyclidine	0.01	0.02
Promethazine	0.09	>0.09
Quetiapine	1.00	>0.9
Sertraline	0.05	0.05
Tramadol	0.05	0.06
Trazodone	0.50	0.40
Venlafaxine	0.08	>0.3
Verapamil	0.30	>0.3
Zolpidem	0.30	0.20

When comparing the LOD from the current method to the optimized method minimal differences were observed. Overall, the optimized method had lower LODs with the most significant differences observed with amphetamine, buprenorphine, hydroxyzine, methadone, methamphetamine, promethazine, quetiapine, venlafaxine, and verapamil. These compounds had LODs with the current method significantly higher (>30%) than the optimized method.

Summary

A liquid-liquid extraction has been optimized for the evaluation of basic drugs using GC-MS. The optimized method was compared to the current method and was shown to produce LODs equivalent to or less than the current method. The optimized sample preparation procedure

significantly decreases the extraction time while providing additional clean-up steps for the analysis of liver.

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