National Laboratory Certification Program
DRUG TESTING MATTERS

2023

Validating Liquid Chromatography–Mass Spectrometry for Urine and Oral Fluid Drug Testing

*This is the updated fourth part of a five-part **Drug Testing Matters** series on urine and oral fluid drug testing method validation, originally published in 2019. This part covers validating liquid chromatography–mass spectrometry testing methods. The first part covered validating immunoassay methods; the second part covered validating specimen validity tests (SVTs); the third part covered gas chromatography–mass spectrometry methods; and the fifth part will cover validation of oral fluid collection devices.*

Liquid Chromatography–Mass Spectrometry Overview

In the third part of *Drug Testing Matters* "Validating Gas Chromatography–Mass Spectrometry for Urine and Oral Fluid Drug Testing," we introduced mass spectrometry (MS) as an analytical technique used to identify and quantify compounds based on measuring the mass-to-electric charge ratio (*m/z*) of their ionized molecules (1, 2). Many instrument options have evolved for mass analyzers, including quadrupole (single or multiple), ion trap, tandem, and time of flight. Single-quadrupole system operating modes include either full scan or selected ion monitoring (SIM). Multiple-quadrupole MS (MS/MS, tandem) operating modes include full scan and multiple reaction monitoring (MRM). In full-scan mode, data are acquired for all ion particles, whereas in SIM and MRM monitoring only user-defined ions and product ion transitions are acquired, respectively.

Tandem mass spectrometers (MS/MS) commonly include three quadrupoles (Q_1, Q_2, and Q_3) and support MRM whereby Q_1 acts as a filter, allowing only *precursor* ions of user-defined *m/z* values to pass; Q_2 is a collision cell that fragments the *precursor* ions; and Q_3 scans or selects for the user-defined product ion *m/z* transitions.

When using single-quadrupole methods, mass detectors require chromatography to separate measurands from non-target interferences prior to full-scan or SIM mass filtering by the quadrupole. Tandem MS methods use both chromatography and the Q_1 quadrupole precursor selection to separate the measurands from interferences prior to the Q_2 quadrupole collision and subsequent Q_3 MRM transition mass filtering and mass detection.

Coupling chromatographic measurand separation with MS dates to the 1950s (3). Gas chromatography was the first separation technique combined with MS (GC-MS) by coupling the gas phase chromatographic elution to the mass spectrometer ion source. Limitations of GC separation techniques include sample preparation to remove aqueous phase and, depending on the measurand, measurand loss to thermal degradation or derivatization to increase measurand volatility. Liquid chromatography (LC) provides an alternative separation technique that does not require high-temperature volatilization or derivatization of measurands but does support analysis of aqueous samples.

As GC-MS instrumentation became commercialized in the 1970s, researchers worked to develop ionizing interfaces to couple LC and MS instrumentation (LC-MS). Some of the developments, which were later abandoned, included the moving belt interface (MBI) and the direct liquid introduction (DLI) (4, 5). MBI-evaporated LC eluates onto a moving belt from which measurands were desorbed into the MS ion source. DLI split a fraction of the LC effluent into a microbore capillary transfer tube coupled to the MS ion source. Because of mechanical complexity, belt renewal issues, frequent capillary clogging, and sensitivity limitations, these interface techniques were abandoned. Today, there are many options available for ionizing interfaces for LC-MS methods. Applications and interfaces using atmospheric pressure ionization include the following:

Analysis of small, neutral, relatively non-polar molecules

- Atmospheric Pressure Chemical Ionization (APCI)(6)
- Atmospheric Pressure Photo Ionization (APPI)(7)

Analysis of moderately polar molecules to polar molecules

- Electrospray Ionization (ESI)(8)

Using APCI as an example of how an ionizing LC-MS interface works, LC column effluent passes into a heated nebulizer that creates a fine droplet mist that is mixed with a stream of nitrogen gas. At atmospheric pressure, a corona electrode discharge ionizes the molecules in the gas stream, and the charged ions pass through a small orifice skimmer into the MS analyzer.

The APCI positive ionization mode is a common method used for drug testing. By selecting the positive ionization mode, reactant ions allow proton transfers that create charged molecular ions $[M+H]^+$. The negative ionization mode results in proton subtraction and creates the negatively charged molecular ion $[M-H]^-$.

The combination of LC column retention times and mass spectrometric measurements (*m/z* and abundance), together with quantitative cutoffs, provides a robust and definitive tool that can be used to

identity and quantify sample measurands. For drug testing, concentrations of drug(s) or drug metabolite(s) in the sample are the measurands.

Drug testing laboratories use initial testing to eliminate "negative" specimens from further consideration and to identify specimens that require confirmation or further testing. Initial testing may be performed using immunoassay or alternate technology initial tests (e.g., chromatographic mass spectrometric methods) for accurate and reliable identification of drugs of abuse or their metabolites. Positive initial tests are confirmed using chromatographic mass spectrometric identification tests to report a specimen as positive. Examples of chromatographic and mass spectrometric methods of analysis that may be used for alternate technology initial tests and confirmatory tests include GC-MS, GC-MS/MS, LC-MS, and LC-MS/MS.

The remainder of this publication focuses on validating LC-MS and LC-MS/MS methods for alternate technology initial tests and confirmatory tests.

LC-MS and LC-MS/MS Validation[a]

Industry Standards

The method validation requirements described in this article are defined by the Department of Health and Human Services (HHS) and the National Laboratory Certification Program (NLCP) (9-12) for HHS-certified laboratories that test donor specimens in compliance with the *Mandatory Guidelines for Federal Workplace Drug Testing Programs using Urine* (UrMG) and the *Mandatory Guidelines for Federal Workplace Drug Testing Programs using Oral Fluid* (OFMG). HHS-certified laboratories conduct forensic drug testing for federal agencies under Executive Order 12564 and Public Law 100-71 and for specific federally regulated industries. The HHS Mandatory Guidelines for Federal Workplace Drug Testing Programs affect all federal employees in a testing designated position, which is defined by each agency's Drug-Free Workplace Program.

Additional standards and guidance for forensic drug testing applications are published by the American National Standards Institute and the American Academy of Forensic Sciences Standards Board (ANSI/ASB).(13) The ANSI/ASB Standard 036 "Standard Practices for Method Validation in Forensic Toxicology" publication defines forensic toxicology validation practices, such as consensus standards, practice, and protocols, including quality assurance and quality control.

For Clinical Laboratory Improvement Amendments (CLIA) certification of clinical laboratories, the Centers for Medicare & Medicaid Services (CMS) requires laboratories to verify or establish performance specifications for any test system used by the laboratory on or after April 24, 2003.(14)

The goal of validating alternate technology initial test or confirmatory test methods is to provide objective data that (1) demonstrate that the method performs according to its intended use and (2) establish the method limitations under normal operating conditions.

---

[a] *For clarity, this series of articles uses the term "validation" to cover all aspects of laboratory methods performance assessments including verification of unmodified Food and Drug Administration (FDA)-cleared and FDA-approved assays and the validation of laboratory-developed assays.*

Before implementing new or modified methods or instrumentation for use with testing donor specimens, laboratories are required to validate their performance. Some laboratories perform an alternate LC-MS or LC-MS/MS assay (e.g., to eliminate interferences when the desired specificity cannot be obtained using the primary method). These alternate assays are subject to the same method validation requirements as the primary assays. However, if the difference between the primary and alternate methods is relatively minor, studies must focus on those assay characteristics that could be affected by the modifications to the primary method. For example, if the laboratory develops an alternate method by modifying the gradient of the mobile phase to resolve an interfering compound, interference studies would be required to document the ability of the alternate assay to resolve the interference, but a precision/accuracy study would not be necessary.

Note: If a laboratory chooses to use an alternate technology initial test, the laboratory must contact the NLCP to arrange for NLCP review of the validation records. The laboratory may be required to submit the validation study data and summaries to the NLCP for review or may be required to provide validation records for review by the NLCP inspectors prior to implementing the method.

Initial tests, including alternate technology initial tests, are used to discriminate between positive and negative samples, and confirmatory drug tests are used to identify and quantify positive measurands. Performance characteristic measurements for LC-MS and LC-MS/MS methods include the following:

- The effective linearity of the test
- The limit of detection and limit of quantification
- The precision and accuracy of the test around the cutoff and 40% of the cutoff
- The potential for carryover
- The specificity and potential for interfering substances
- The selection of the method parameters including ion/transition selection
- Comparison of results using existing and new/revised procedures (i.e., parallel study)
- The potential for matrix effects

Documentation

Validation records must include sufficient information to facilitate third-party comprehensive review of studies performed. The study summary and the laboratory's standard operating procedures (SOPs) must describe acceptance criteria for validation study data, agreement of replicate study samples, and defining or excluding true outlier values. Alternate technology initial test and confirmatory test study sample analysis must meet the same qualitative criteria (e.g., retention time, mass ratio, internal standard abundance, chromatography criteria) used for specimen analysis.

At a minimum, validation study records must include:

- A stated purpose for the validation
- Description of test methods
- Identity of the instrument(s) used for the study
- A listing of the instrument parameters used for the study
- A description of the study samples

- A summary of the statistical data collected to characterize the assay
- A discussion
- A summary with conclusions
- All raw analytical data from the samples analyzed in the study

Laboratories must maintain the LC-MS and LC-MS/MS validation study records for an indefinite period. Records for validation studies performed within the last 12 months must be available for review during NLCP inspections.

Each "end-user laboratory" must perform the validation and periodic re-verification studies for its assays and instruments. Off-site validations performed by other entities (e.g., manufacturer, other laboratory) may be used only to provide additional documentation.

Types of Validations

The types of validation studies to be used depends upon whether the laboratory is implementing a new test, a new instrument model, or an additional instrument of the same model. Examples of validations include assay, full instrument, abbreviated instrument, and re-verification.

Note: Where multiple instrument models are used for an LC-MS or LC-MS/MS assay, using the most conservative performance limits that can be determined for all models is acceptable, provided that this approach is described in the SOP and the validation study summaries.

Assay

Assay validation studies must be performed prior to use with regulated specimens for:

- A new primary or alternate method
- A revised method*
- Method calibration scheme changes
- Method chromatographic column selection

**It is usually necessary to perform complete validation studies for revised assays. However, if the modification is relatively minor, the validation studies may focus on those parameters that may have been affected.*

The following studies are required for assay validation for alternate technology initial tests and confirmatory tests using LC-MS or LC-MS/MS:

1. For confirmatory drug tests:
   - Linearity and determination of the limit of quantification (LOQ) and upper limit of linearity (ULOL)
   - Determination of the limit of detection (LOD)*
   - Precision and accuracy around each cutoff
   - Precision and accuracy around 40%
   - Carryover
   - Specificity and interference

- Parallel study of NLCP Performance Testing (PT) samples and donor specimens using existing and new/revised methods
- Method parameters (including appropriate ion and MRM transition selection)
- Matrix effects
- Dilution integrity
- Hydrolysis (if performed)

Notes:
- *Dilution integrity performed only for confirmatory drug tests with routine specimen dilution of all samples*
- *Parallel studies are not necessary if the laboratory does not have an existing assay for the analyte(s).*

2. Studies in addition to confirmatory testing studies for alternate technology initial drug tests only include positive and negative sample differentiation versus confirmatory testing.

Notes:
- *Positive/negative differentiation studies are not necessary if the laboratory uses the same method for initial and confirmatory drugs tests (i.e., all parameters are the same).*
- *Determining LOD is not required.*

Full Instrument

Full Instrument validation must be performed for at least one instrument prior to implementing:

- A new model of an instrument for use with a validated LC-MS, LC-MS/MS assay
- A new instrument component that may affect the analysis (e.g., a chromatographic column with a different phase or **from a different manufacturer**)

The following studies are required for Full Instrument validation for alternate technology initial tests and confirmatory drug tests using LC-MS or LC-MS/MS:

1. For confirmatory drug tests:
   - Linearity and determination of the LOQ and ULOL
   - Determination of the LOD
   - Precision and accuracy around each cutoff
   - Precision and accuracy around 40%
   - Carryover
   - Specificity and interference
   - Parallel study of NLCP PT samples and donor specimens using existing and new/revised methods
   - Matrix effects
   - Parameter optimization
   - Dilution integrity

Notes:
- *Dilution integrity performed only for confirmatory drug tests with routine specimen dilution of all samples.*
- *Parallel studies are not necessary if the laboratory does not have an existing assay for the analyte(s).*

2. In addition to confirmatory testing studies for alternate technology initial drug methods only studies include positive and negative sample differentiation versus confirmatory testing.

Notes:
- *Positive/negative differentiation studies are not necessary if the laboratory uses the same method for initial and confirmatory drugs tests (i.e., all parameters are the same).*
- *Determination of LOD is not required.*

Abbreviated Instrument

Abbreviated Instrument validation must be performed prior to implementing each additional instrument of the same model that has been previously validated by the laboratory. For an additional confirmatory drug test instrument, the same model will have:

- Same chromatographic and mass spectrometric instrument models
- Same model components (e.g., same model pump and autosampler)
- Same phase and manufacturer column

The following studies are required for abbreviated instrument validation for alternate technology initial tests and confirmatory drug tests using LC-MS or LC-MS/MS:

1. For confirmatory drug tests:
   - Determination of the LOD, LOQ, and ULOL
   - Carryover
   - Specificity and interference
   - Matrix effects
   - Parameter optimization
2. For alternate technology initial drug methods only, studies in addition to confirmatory testing studies:
   - Positive and negative sample differentiation versus confirmatory testing

Notes:
- *Positive/negative differentiation studies are not necessary if the laboratory uses the same method for initial and confirmatory drugs tests (i.e., all parameters are the same).*
- *Determining LOD is not required.*

Re-Verification

Re-verification must be performed at least annually. Studies must be performed for each instrument model. For confirmatory drug test instrument, the same model will have the following:

- The same chromatographic and mass spectrometric instrument models
- The same model components (e.g., same model pump and autosampler)
- The same phase and manufacturer of column

The following studies are required for re-verification for alternate technology initial tests and confirmatory drug tests using LC-MS or LC-MS/MS:

- For confirmatory and alternate technology initial drug testing:
  - Determination of the LOD, LOQ, and ULOL
  - Carryover
  - Specificity/interference
  - Matrix effects
  - Hydrolysis (if performed)

Note: LOD studies are not required for alternate technology initial drug methods

Minimum General Study Requirements for Assay Validation, Full Instrument Validation, and Abbreviated Instrument Validation

LOD Studies for Confirmatory Drug Tests

The laboratory must characterize the LOD of the confirmatory assay using **at least five replicates** for decreasing concentrations of the measurand. It is not acceptable to analyze the same sample five times. The LOD is the lowest concentration at which the identity (but not concentration) of the measurand can be accurately established. The LOD must be below 40% of the cutoff. The LOD may be assigned a more conservative value than supported by the validation studies. However, this value must reflect the concentration of a sample analyzed in the linearity studies. Values extrapolated from data or those that do not meet all the acceptance criteria may not be used as an LOD.

Note: The LOD validation is not required for alternate technology initial tests; however, laboratories must be able to quantify drug analytes at or below 40% of the cutoff.

Linearity Studies (LOQ, ULOL)

The laboratory must characterize the linearity of the assay using at least five replicates for *at least seven concentrations* of the measurand. It is not acceptable to analyze the same sample five times. The LOQ and ULOL are the lowest and highest concentrations, respectively, at which the identity and concentration of the measurand can be accurately established. The LOQ must be below 40% of the cutoff. Quantitative acceptance criteria for study samples include an acceptance range of ±20% from the target concentration. The laboratory may establish a ULOL or LOQ at a more conservative value than supported by the validation studies. However, these values must reflect the concentration of a sample analyzed in the linearity studies. Values extrapolated from data or those that do not meet all acceptance criteria may not be used as an LOQ or ULOL.

It is **not** acceptable to use batch calibrators as linearity study samples and to use the linearity of the resulting calibration curve as a demonstration of linearity. All study samples must be analyzed as if they

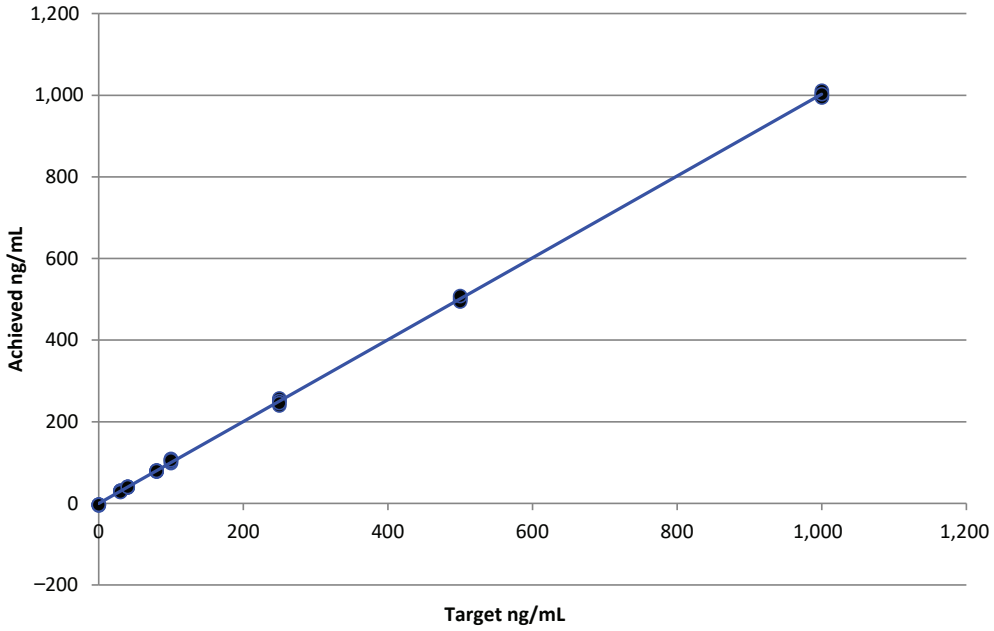
were routine undiluted specimens using the same qualifier criteria (e.g., the same retention time, mass ratio, and chromatography criteria) used for donor specimens.

The concentrations should be distributed as follows:

- A minimum of three below the cutoff (at least one concentration below 40% of cutoff)
- One equivalent to the cutoff
- A minimum of three above the cutoff

The linearity results must be plotted for review (e.g., achieved concentration units on the Y axis against target concentration on the X axis)

Exhibit 1. Example of LC-MS/MS Linearity Study



Target ng/mL	Linearity 100 ng/mL Cutoff					Results			LOD LOQ ULOL
	1	2	3	4	5	Mean	−2SD	+2SD	
0	−3.0	−3.4	−2.7	−5.2	−2.9	−3.4	−5.5	-1.4	
30	31.3	29.1	30.2	30.5	27.7	29.8	27.0	32.6	LOD, LOQ
40	38.9	39.9	40.7	41.2	39.8	40.1	38.3	41.9	
80	79.9	79.5	78	80.7	79.8	79.6	77.6	81.6	
100	109	99	107	102	106	105	97	113	
250	253	240	257	251	246	249	236	263	
500	502	499	506	495	508	502	492	512	
1,000	1,005	998	995	1,011	1,003	1,002	990	1,015	ULOL

Precision and Accuracy Studies

The laboratory must characterize the precision of the assay using **at least five replicates** for **at least five concentrations** of the measurand at critical concentrations relative to the cutoff. It is not acceptable to analyze the same sample five times. The precision data is evaluated by calculating the mean, standard deviation (SD), and coefficient of variation (CV). The study samples must exhibit the appropriate response relative to one another (i.e., study samples should yield appropriate responses versus the cutoff and relative to the other study samples). The laboratory must also characterize the accuracy (expressed as bias) of the assay by calculating the percentage difference between each analyzed sample result and the target concentration. It is acceptable to use the data from the precision study analysis for the accuracy study. The precision and accuracy studies may be performed using separate batches on multiple days to assess the intra-batch and inter-batch variability.

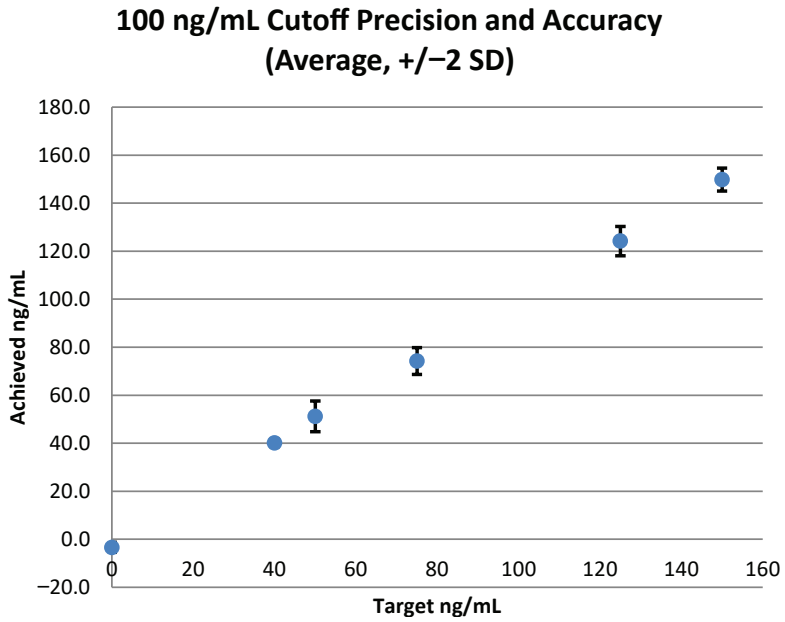
The concentrations relative to the cutoff should be distributed as critical points that may include the following:

- 0% of cutoff
- 50% of cutoff
- 75% of cutoff
- 125% of cutoff
- 150% of cutoff

Notes:

- *Precision and accuracy around 40% of the cutoff of the assay is also required.*
- *The laboratory must establish criteria for evaluating the statistical analysis. These must be described in the validation study summary.*

Exhibit 2. Example of LC-MS/MS Precision and Accuracy Study



Precision and Accuracy 100 ng/mL Cutoff

Target ng/mL	Reps	Result ng/mL	Accuracy %	Precision
0 ng/mL *0% of cutoff*	1	−2.7	N/A	SD: 1.1 ng/mL
	2	−3.4		
	3	−2.7		
	4	−5.2		
	5	−2.9		
40 ng/mL *40% of cutoff*	1	39.3	−1.8%	SD: 0.8 ng/mL CV: 1.9%
	2	39.7	−0.7%	
	3	40.6	+1.5%	
	4	41.2	+3.0%	
	5	39.8	−0.5%	
50 ng/mL *50% of cutoff*	1	51.1	+2.2%	SD: 3.2 ng/mL CV: 6.3%
	2	52.7	+5.4%	
	3	54.9	+9.8%	
	4	48.8	−2.4%	
	5	47.6	−4.8%	

Precision and Accuracy 200 µg/mL Cutoff

Target µg/mL	Reps	Result ng/mL	Accuracy %	Precision
75 ng/mL *75% of cutoff*	1	72.5	−3.3%	SD: 2.8 ng/mL CV: 3.8%
	2	78.5	+4.7%	
	3	71.2	−5.1%	
	4	74.0	−1.3%	
	5	75.2	+0.3%	
125 ng/mL *125% of cutoff*	1	124	−0.8%	SD: 3.0 ng/mL CV: 2.4%
	2	126	+0.8%	
	3	123	−1.6%	
	4	120	−4.0%	
	5	128	+2.4%	
150 ng/mL *150% of cutoff*	1	151	+0.7%	SD: 2.4 ng/mL CV: 1.6%
	2	153	+2.0%	
	3	148	−1.3%	
	4	150	±0.0%	
	5	147	−2.0%	

Carryover Studies

The laboratory must characterize the potential for one sample to carry over to another during testing. The laboratory should perform these carryover studies by analyzing highly concentrated samples followed by negative samples (i.e., without the measurand of interest) and evaluating the negative samples for carryover. The measurand concentrations in the high samples should be realistic (i.e., high concentrations that may be found in the testing population) and at least as high as the established ULOL. The ULOL is the highest value at which carryover may be assigned.

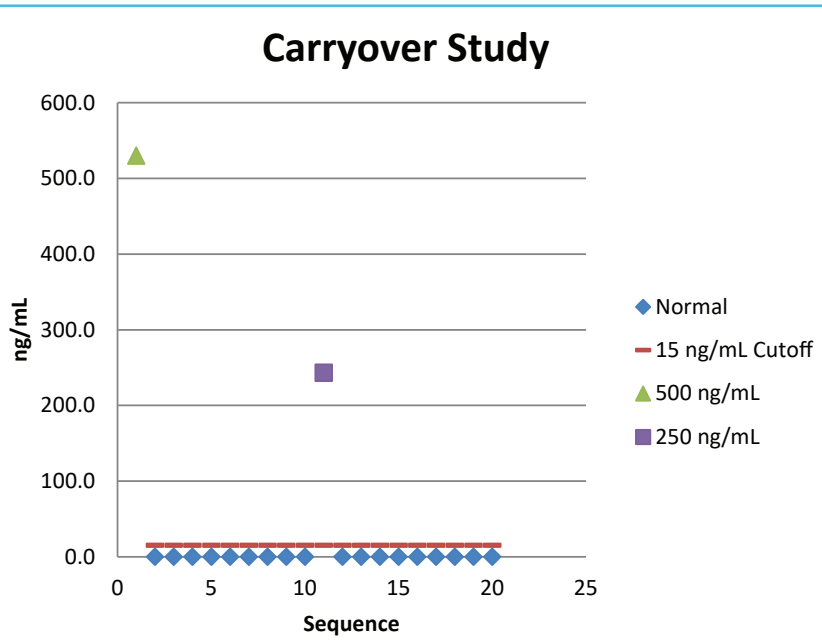
In some cases, carryover may not be demonstrated until the concentration exceeds the ULOL of the assay. When this occurs, the observed concentration is no longer an accurate quantitative value and is not an appropriate value to use as a prompt for corrective action. In such cases, **the laboratory must assign the carryover limit to be the same value as the ULOL**.

If the laboratory plans to use multi-well plates, the SOP must describe measures to prevent carryover in the well plate during pipetting. If evaporators are used with multi-well plates the laboratory must perform a carryover study to ensure no cross-contamination occurs (e.g., fluorescence test). If the laboratory uses multiplexing, the laboratory must have procedures to evaluate carryover on both streams (i.e., when carryover is suspected, both streams must be evaluated).

Notes:

- *If solvent blanks are routinely injected between donor samples and the laboratory has objective criteria for reviewing the blank data, a carryover study is not required. If this approach is used, it must be described in the SOP.*
- *The laboratory must establish criteria (i.e., allowable response or concentration) for evaluating the negative sample tested after a high sample in carryover studies. These must be described in the validation study summary and the SOP.*
- *For use of multi-well plates, it is not acceptable to remove plate caps or plate mats.*

Exhibit 3. Example of LC-MS/MS Carryover Study



Carryover Check Samples		ng/mL	
		Sample Result	Cutoff Calibrator
1	500 ng/mL THCA	530.0	15.0
2	Normal Urine	0.0	15.0
3	Normal Urine	0.0	15.0
4	Normal Urine	0.0	15.0
5	Normal Urine	0.0	15.0
6	Normal Urine	0.0	15.0
7	Normal Urine	0.0	15.0
8	Normal Urine	0.0	15.0
9	Normal Urine	0.0	15.0
10	Normal Urine	0.0	15.0
11	250 ng/mL THCA	243.0	15.0
12	Normal Urine	0.0	15.0
13	Normal Urine	0.0	15.0
14	Normal Urine	0.0	15.0
15	Normal Urine	0.0	15.0
16	Normal Urine	0.0	15.0
17	Normal Urine	0.0	15.0
18	Normal Urine	0.0	15.0
19	Normal Urine	0.0	15.0
20	Normal Urine	0.0	15.0

Specificity and Interference Studies

The laboratory must characterize the assay performance when challenged with compounds commonly encountered in the testing population. Whether the presence of a compound would reduce or increase the concentration of the measurand is of concern. Assays should include appropriate ions and transitions that prevent measurand misidentification and chromatographic column selection and gradient programming that prevents interference from co-eluting compounds. It may be necessary to validate an alternate method for specimens exhibiting interference that cannot be resolved using the primary method. In addition to selecting appropriate ions and transitions to prevent analyte misidentification, assay modifications such as gradient program changes may be needed to prevent interference from co-eluting compounds.

Compounds to be evaluated include illicit drugs and over-the-counter and prescription drugs at concentrations exceeding those encountered with therapeutic doses. Interference studies must be performed by analyzing samples containing interferents in the presence of the measurands at 40%* of the cutoff and without the measurand (see below for separate urine and oral fluid criteria).

** Enantiomer (amphetamine and methamphetamine) interference studies must be performed by analyzing samples containing interferents in the presence of the measurands (d-methamphetamine, l-methamphetamine, d-amphetamine, and l-amphetamine) at a defined ng/mL and without the measurand (see below for separate urine and oral fluid criteria).*

For alternate technology initial drug tests with no hydrolysis, the laboratory must monitor the free drug and the drug as the glucuronide in the interference study for analytes that may be in the conjugated form (i.e., THCA, codeine, morphine, oxymorphone, and hydromorphone).

The **minimum** requirements for multi-analyte assays interference studies include the following concentrations and drugs to test:

Urine

- 6-acetylmorphine assays
  - 40,000 ng/mL: free morphine, codeine
  - 5,000 ng/mL: hydrocodone, hydromorphone, oxycodone, oxymorphone, norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone
- Amphetamines (amphetamine, methamphetamine, MDMA, and MDA) assays
  - 50,000 ng/mL: phentermine
  - 1 mg/mL: phenylpropanolamine, ephedrine, pseudoephedrine
  - Structurally similar compounds such as substituted phenethylamines in amphetamines assay interference studies to evaluate and document the effects of such compounds on amphetamines analysis
- Amphetamine and methamphetamine assays
  - 50,000 ng/mL: phentermine
  - 1 mg/mL: phenylpropanolamine, ephedrine, pseudoephedrine
  - 5,000 ng/mL: MDA, MDMA

  - Structurally similar compounds such as substituted phenethylamines in amphetamines assay interference studies to evaluate and document the effects of such compounds on analysis for the analytes of interest
- Enantiomer (amphetamine, methamphetamine) assays
  - Samples with 50 ng/mL d-methamphetamine, l-methamphetamine, d-amphetamine, l-amphetamine, and samples without amphetamine or methamphetamine
  - 50,000 ng/mL: phentermine
  - 1 mg/mL: phenylpropanolamine, ephedrine, pseudoephedrine
  - 5,000 ng/mL: MDA, MDMA
  - Structurally similar compounds such as substituted phenethylamines are not required for enantiomer assays
- MDA or MDMA assays
  - 50,000 ng/mL: phentermine
  - 1 mg/mL: phenylpropanolamine, ephedrine, pseudoephedrine
  - 5,000 ng/mL: amphetamine, methamphetamine
  - Structurally similar compounds such as substituted phenethylamines in amphetamines assay interference studies to evaluate and document the effects of such compounds on analysis for the analytes of interest
- Codeine and morphine assays
  - 200 ng/mL: 6-acetylmorphine
  - 5,000 ng/mL: hydrocodone, hydromorphone, oxycodone, oxymorphone, norcodeine
- Opioid (codeine, morphine, oxycodone, oxymorphone, hydrocodone, hydromorphone, and 6-acetylmorphine) assays
  - 5,000 ng/mL: norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone
- Oxycodone, oxymorphone, hydrocodone, and hydromorphone assays
  - 200 ng/mL: 6-acetylmorphine
  - 40,000 ng/mL: free morphine, codeine
  - 5,000 ng/mL: norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone
- Oxycodone and oxymorphone assays
  - 200 ng/mL: 6-acetylmorphine
  - 40,000 ng/mL: free morphine, codeine
  - 5,000 ng/mL: hydrocodone, hydromorphone, norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone

- Hydrocodone and hydromorphone assays
  - 200 ng/mL: 6-acetylmorphine
  - 40,000 ng/mL: free morphine, codeine
  - 5,000 ng/mL: oxycodone, oxymorphone, norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone

Oral Fluid

- Amphetamines (amphetamine, methamphetamine, MDMA, and MDA) assays
  - 10,000 ng/mL: ephedrine, pseudoephedrine, phentermine, 4-fluoromethamphetamine
  - Structurally similar compounds such as substituted phenethylamines in amphetamines assay interference studies to evaluate and document the effects of such compounds on amphetamines analysis
- Enantiomer (amphetamine, methamphetamine) assays
  - Samples with 5 ng/mL d-methamphetamine, l-methamphetamine, d-amphetamine, l-amphetamine and samples without amphetamine or methamphetamine
  - 10,000 ng/mL: phentermine, phenylpropanolamine, ephedrine, pseudoephedrine, MDA, MDMA
- Opioid (codeine, morphine, and 6-acetylmorphine) assays
  - 45 ng/mL: norcodeine, normorphine
  - 100 ng/mL: dextromethorphan, dextrorphan
- Oxycodone, oxymorphone, hydrocodone, and hydromorphone assays
  - 300 ng/mL: codeine, morphine, norcodeine, normorphine, norhydrocodone, norhydromorphone, noroxycodone, noroxymorphone
- Tetrahydrocannabinol (THC) assays
  - 2,000 ng/mL: cannabidiol
  - 100 ng/mL: delta 8-tetrahydrocannabinol (delta 8-THC)

Positive/Negative Differentiation Studies for Alternate Technology Initial Tests

The laboratory must characterize the ability of the assay to differentiate positive and negative samples using **at least two replicates of at least 10 positive** samples at different concentrations for each initial drug test measurand and at **least two replicates** of **at least 10 negative samples** (i.e., 40 results for each analyte). The laboratory may analyze negative donor specimens or controls, negative specimens fortified with known amounts of the assayed measurands, non-NLCP proficiency testing samples, or discarded positive donor specimens.

Note: A positive/negative differentiation study is not necessary if the laboratory uses the same method for its initial and confirmatory drugs tests (i.e., all parameters are the same).

Parallel Studies

Note: Parallel studies are not necessary if the laboratory does not have an existing assay for the analyte(s) in regulated specimens.

When the current and new methods use different technologies, the laboratory must conduct parallel studies that compare results of the new method with results from the existing procedure. The laboratory analyzes at least 10 human specimens positive for the assay and the **specimens from two PT cycles**. When donor samples are used, previously obtained values are used for the comparison (note: the laboratory may reanalyze positive donor specimens by the existing method if discrepant values could be due to drug analyte stability issues). Results obtained using the new method should be within ±20% of the results using the existing method. Any discrepancies should be investigated and explained.

Human (donor specimen) study samples include:

- 5 specimens with concentrations between the cutoff and two times the cutoff
- 5 specimens with concentrations greater than two times the cutoff

Additional notes:

- Use positive donor specimens for THC, marijuana metabolite (THCA), codeine, morphine, hydromorphone, and oxymorphone.
- Spiked samples with non-conjugated measurands may be used if positive donor specimens are not available for amphetamines, cocaine, cocaine metabolite, phencyclidine, 6-acetylmorphine, hydrocodone, and oxycodone. Spiked study samples may include:
  - 5 replicate samples prepared at one concentration that is between the cutoff and two times the cutoff
  - 5 replicate samples prepared at one concentration that is greater than two times the cutoff

PT study samples include the PT samples from the two most recent NLCP PT cycles (excluding those categorized as invalid, substituted, or adulterated).

The re-analysis of NLCP PT samples requires that the laboratory request permission to use the specimens to validate the new method. *If additional NLCP PT material is needed, submit another request to the NLCP.*

Method Parameters

Laboratories must perform a systematic evaluation of instrument parameters (e.g., mass analyzer settings, LC interface settings, ion optics settings, chromatographic conditions, mobile phase composition and gradient programming, ion source settings) that would impact the analysis. The laboratory must document the evaluation and selection of ions and transitions to be monitored for drug measurands and internal standards.

Note: Staff responsible for method development and oversight must know the chemical structure of select ions and transitions that represent the structural components that make the measurand a unique molecule.

The laboratory must use equivalent ions and transitions for the deuterated internal standard and non-deuterated measurand whenever possible. It is not appropriate to use trivial loss fragments or adduct ions (e.g., dimers). A stable isotope internal standard must be used for each analyte. It is not acceptable to use the same internal standard for multiple analytes.

Consider the following example: Laboratories using LC-MS/MS for non-derivatized analysis of amphetamine and methamphetamine should use the molecular ions ($[M+H]^+$ *m/z* 136 for amphetamine and $[M+H]^+$ *m/z* 150 for methamphetamine) as the Q1 precursors and define *m/z* 91 and *m/z* 118 as the product ion transitions. Monitoring isotopes of a fragment is only acceptable when other suitable ions are not available.

Instrument Parameter Optimization

The laboratory must objectively evaluate instrument parameters to determine the optimal values for the particular configuration and manufacturer.

If a laboratory uses a tandem mass spectrometric method for both the initial and confirmatory drug tests, the laboratory should obtain enough information during method validation to develop appropriate acceptance criteria for each test (e.g., can use less stringent ratio criteria for the initial test). The laboratory must use at least one transition for the initial test. For confirmatory tests, the laboratory must use at least two transitions (i.e., at least one quantifier and one qualifier transition for the analyte) and evaluate the ratio of the abundance of these transitions for the target analyte. The same requirements apply to the internal standard. For single MS methods, the laboratory must use at least three ions for the analytes and two ions for the internal standard. These transitions/ions should be free of interferences and matrix effects and should be specific to the target analyte (e.g., should be a transition from the target analyte or minimally be a justifiable structure relative to the target analyte). The laboratory must provide a structural justification of the selected transitions.

Matrix Effects

The laboratory must evaluate the potential for components of the sample matrix to either suppress or enhance the ionization of drug and internal standard analytes. Studies must include the evaluation of at least 10 different lots of human specimens (i.e., from 10 individuals or from 10 different specimen pools), with drug analyte at 40% of the cutoff concentration. The laboratory must perform studies to assess the entire confirmatory test processes, including sample preparation. The recommended approach is that by Matuszewski et al. (15).

The Matuszewski et al. approach includes the following:

- 3 sets of sample preparations:
  a. 10 replicate solutions (not repeated injections) neat drug/metabolite and internal standards injected into LC-MS/MS
  b. 10 lots of matrix processed and then spiked with drug/metabolite and internal standards injected into LC-MS/MS
  c. 10 lots of matrix spiked with drug/metabolite and internal standards then processed and injected into LC-MS/MS

- Calculations for measure peak areas:
  - Matrix effects (%) = B/A × 100
  - Recovery (%) = C/B × 100
  - Process efficiency (%) = C/A × 100

Dilution Integrity

If the laboratory pre-dilutes all sample preparations for confirmatory testing method, a dilution integrity study must be performed to document that the dilution does not affect assay performance. These studies consist of the precision/accuracy studies using samples at the dilution specified in the SOP.

Note: The dilution integrity study must use at least five dilutions, including the highest dilution used routinely by the laboratory.

Hydrolysis Studies

If the laboratory performs hydrolysis for alternate technology initial tests, a hydrolysis study must be performed. For confirmatory drug tests, hydrolysis is required for THCA and opioid confirmatory drug tests; therefore, the hydrolysis study is required.

Laboratories must demonstrate and document acceptable hydrolysis (i.e., at least 80% recovery). For THCA hydrolysis studies, the laboratory must analyze positive THCA donor specimens with and without hydrolysis. For codeine and morphine hydrolysis studies, the laboratory must accurately quantify at least 15,000 ng/mL of codeine as the glucuronide and morphine as the glucuronide (i.e., each with at least 15,000 ng/mL of the free drug). For oxymorphone and hydromorphone hydrolysis studies, the laboratories must accurately quantify at least 1,000 ng/mL of oxymorphone as the glucuronide and 1,000 ng/mL of hydromorphone as the glucuronide (i.e., each with at least 1,000 ng/mL of the free drug).

Note: Laboratories must document acceptable hydrolysis performance at the same concentrations in re-verification studies, unless the laboratory's hydrolysis controls included in each batch are at or above the concentrations stated above.

Special Considerations Specific to Alternate Technology Initial Test Validation

- Determining an LOD is not required; however, laboratories must be able to quantify drug analytes at or below 40% of the cutoff. To meet this program requirement, the laboratory must establish an LOQ below 40% of the cutoff.
- Positive/Negative Differentiation Studies. The laboratory must analyze positive and negative samples that have been verified by the confirmatory drug test method to assess the ability of the assay to differentiate positive and negative samples. The laboratory may analyze a combination of negative donor specimens or controls, negative samples fortified with known amounts of the assayed drugs, non-NLCP proficiency testing samples, or discarded positive donor specimens. The laboratory should analyze a minimum of 10 positive samples at differing concentrations for each initial drug test analyte and 10 negative samples, in duplicate (i.e., 40 results for each analyte).

Note: A positive/negative differentiation study is not necessary if the laboratory uses the same method for its initial and confirmatory drugs tests (i.e., all parameters are the same).

Special Considerations Specific to Confirmatory Drug Test Validation

- Laboratories must be able to identify and quantify drug analytes at or below 40% of the cutoff. To meet this program requirement, the laboratory must establish an LOD and LOQ below 40% of the cutoff. NLCP PT samples challenge laboratories with drug analytes at approximately 40% of their confirmatory test cutoffs.
- For confirmatory drug test methods, laboratories are required to identify and accurately quantify at or below 40% of the cutoff. Therefore, laboratories must document the precision/accuracy around 40% of the cutoff and document the lack of interference of the assay in the presence of analyte at 40% of the cutoff.
- The amphetamine/methamphetamine enantiomer validation study depends on whether the test is quantitative (i.e., determining quantitative values for methamphetamine enantiomers and, if analyzed, amphetamine enantiomers) or semi-quantitative (using area to determine the relative percentages of methamphetamine enantiomers and, if analyzed, amphetamine enantiomers).

Quantitative Enantiomer Tests

If the enantiomer test is quantitative, the laboratory must perform the following studies for assay or full instrument validation:

- Determination of the LOD, LOQ and ULOL
- Precision/accuracy
  - For urine: around 250 ng/mL (total analyte), around 100 ng/mL (total analyte), and around 50 ng/mL (each enantiomer)
  - For oral fluid: around 25 ng/mL (total analyte), around 10 ng/mL (total analyte), and around 5 ng/mL (each enantiomer)
- Carryover
- Interference
- For an assay validation: method parameters including appropriate ion or MRM transition selection
- For a full instrument validation: instrument parameter optimization
- Matrix effects

Abbreviated instrument validation studies for quantitative enantiomer assays must include the following:

- Determination of the LOD, LOQ, and ULOL
- Carryover
- Interference
- Parameter optimization
- Matrix effects

Semi-Quantitative Enantiomer Tests

If the enantiomer test is semi-quantitative, the laboratory must perform the following studies for assay validation, full instrument validation, or abbreviated instrument validation:

- Carryover
- Interference
- For an assay validation: method parameters, including appropriate ion or MRM transition selection
- For a full instrument or abbreviated instrument validation: instrument parameter optimization
- Precision/accuracy of enantiomer ratios

- A laboratory may perform additional tests (i.e., other than the drug and specimen validity tests specified by the HHS Guidelines) at the request of a Medical Review Officer or at the direction of a federal agency. The validation and re-verification study requirements for these additional tests depend on the measurand.
  - Urine validation requirements for tetrahydrocannabivarin (THCV) and semi-synthetic opioid metabolites
    - Determination of the LOQ
    - Full linearity study (LOQ, ULOL) is required if the laboratory reports quantitative results rather than "≥ [established LOQ]"
    - Interference:
      - For THCV, the laboratory must demonstrate the ability to accurately resolve structurally similar compounds (e.g., THCA)
      - Validation for opioid metabolites has the same requirements as for opioid confirmatory drug tests (described above)
    - Carryover study and procedures to address carryover (as an alternative to a carryover study, the laboratory may choose to inject a negative control or solvent blank between specimens, and have criteria for evaluating carryover)
    - Method parameters, including appropriate ion or MRM transition selection
    - Matrix effects
    - Instrument parameter optimization
  - Re-verification requirements for THCV and semi-synthetic opioid metabolites
    - Determination of LOQ
    - ULOL (if the laboratory reports quantitative results rather than "≥ [established LOQ]")
    - Interference
    - Carryover
    - Matrix effects

Minimum Requirements for Periodic Re-verification Studies

Note: All confirmatory drug test methods (primary and alternate) must be re-verified at least annually.

Periodic re-verification studies are required for alternate technology initial tests and confirmatory tests to verify that existing limits are still valid. These studies may not be as extensive as those performed for implementing a new method. The following are minimum requirements:

- Determination of the LOQ and ULOL (LOD must be determined for confirmatory tests)
- Carryover
- Specificity/interference (for confirmatory tests)
- Matrix effects
- Hydrolysis (if performed)

- **LOD, LOQ, and ULOL Re-verification:** The laboratory must analyze a minimum of three replicates targeted at the existing limit concentration. If the limit is not re-verified, the limit must be re-established using the validation protocol.

  Notes:

  If a control with a target concentration at the stated LOQ or ULOL is analyzed in each test batch, the laboratory may not need to perform a periodic formal study to re-verify the existing limit (LOQ or ULOL). The laboratory will have data to demonstrate the validity of the existing limit(s). The SOP would need to describe this practice and the results obtained from batch QC records summarized for review.

  LOD studies are not required for confirmatory testing if the LOD is assigned the same value as the LOQ.

  LOD studies are not required for alternate technology initial drug tests.

- **Carryover Re-verification:** The laboratory must perform carryover studies unless blanks are injected between samples in each batch. Using blanks, formal studies may not be required, because the laboratory will be evaluating the potential for carryover in each batch. If this approach is used, the laboratory must have objective criteria for evaluating the blanks and have procedures to ensure that possible carryover would be identified and appropriate corrective actions taken.

  Note: For an extraction method with elution into multi-well plates, the laboratory is not required to perform a periodic formal cross-contamination study if the laboratory includes at least one contamination check sample in each batch that demonstrates the lack of cross-contamination.

- **Interference Re-verification:** The laboratory must perform re-verification of potential interferences for all confirmatory drug tests (and for amphetamine enantiomers) unless controls with high concentrations of common interferents are analyzed in test batches. These controls may be used to replace formal periodic interference studies only if the controls are equivalent to the samples required by the NLCP for a formal interference study (i.e., controls with and without the compounds of interest, with the interfering substances at the concentrations specified by the program). If this approach is used, the laboratory must have data to support its continued ability to accurately resolve the analyte from structurally similar compounds. The SOP must describe this practice and the results obtained from batch QC records must be summarized for review.

- **Amphetamine/Methamphetamine Enantiomer Tests Re-verification:** Re-verification studies depend on whether the test is quantitative or semi-quantitative.

For quantitative enantiomer tests (i.e., quantitative values for methamphetamine enantiomers and, if analyzed, amphetamine enantiomers), the laboratory must perform the following studies to evaluate each analyte:

  - Determination of the LOD, LOQ, and ULOL
  - Carryover
  - Interference
  - Matrix effects

For semi-quantitative enantiomer tests (i.e., relative percentages of methamphetamine enantiomers and, if analyzed, amphetamine enantiomers), the laboratory must perform the following studies:

  - Carryover
  - Interference
  - Matrix effects

- **Matrix Effects Re-verification:** The laboratory must perform matrix effect studies as performed for the original method validation but may evaluate fewer specimen matrix lots. The re-verification must include evaluation of at least three different lots of human specimens (i.e., urine or oral fluid from three individuals or from three different urine or oral fluid pools), with drug analyte at 40% of the cutoff concentration.
- **Hydrolysis Re-verification:** The laboratory must perform hydrolysis studies as performed for the original method validation (see ***Hydrolysis Studies***).

Note: Hydrolysis studies are not needed if the laboratory includes hydrolysis controls at or above the specified concentration in each batch.

References

1. Hedenus M. Eugen Goldenstein and his laboratory work at Berlin Observatory. Astronomical Notes (Astronomische Nachrichten). 2002;323(6):567-9.
2. Nobel Media. Wilhelm Wien-Biographical [Internet]. 2019 [Available from: http://www.nobelprize.org/nobel_prizes/physics/laureates/1911/wien-bio.html.
3. Gohlke RS, McLafferty FW. Early gas chromatography/mass spectrometry. Journal of the American Society for Mass Spectrometry. 1993;4(5):367-71.
4. Hayes MJ, Lankmayer EP, Vouros P, Karger BL, McGuire JM. Moving belt interface with spray deposition for liquid chromatography/mass spectrometry. Analytical Chemistry. 1983;55(11):1745-52.
5. Eckers C, Skrabalak DS, Henion J. On-line direct liquid introduction interface for micro-liquid chromatography/mass spectrometry: application to drug analysis. Clin Chem. 1982;28(9):1882-6.
6. Niessen WMA. Liquid Chromatography-Mass Spectrometry, Third Edition: CRC Press Taylor & Francis; 2006.
7. Robb DB, Covey TR, Bruins AP. Atmospheric pressure photoionization: an ionization method for liquid chromatography-mass spectrometry. Analytical Chemistry. 2000;55(15):3653-9.
8. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. Science. 1989;246(4926):64-71.
9. Mandatory Guidelines for Federal Workplace Drug Testing Programs using Urine (effective October 1, 2017), 82 FR. Sect. 7920-7970 (2017).
10. Mandatory Guidelines for Federal Workplace Drug Testing Programs using Oral Fluid (effective January 1, 2020), 84 FR. Sect. 57554-57600 (2019).
11. RTI International, Center for Forensic Sciences. Manual for Urine Laboratories, National Laboratory Certification Program (NLCP). Research Triangle Park, NC: RTI International, Center for Forensic Sciences,; 2017. Contract No.: Rev. 0222.
12. RTI International, Center for Forensic Sciences Manual for Oral Fluid Laboratories, National Laboratory Certification Program (NLCP). Research Triangle Park, NC: RTI International, Center for Forensic Sciences; 2020. Contract No.: Rev. 0222.
13. ANSI/ASB Standard 036. Standard Practices for Method Validation in Forensic Toxicology. 2019. Available from: https://www.aafs.org/sites/default/files/media/documents/036_Std_e1.pdf
14. Clinical Laboratory Improvement Amendments (CLIA), Standards and Certification, Laboratory Requirements, Subpart K, Standard: Establishment and verification of performance specifications, 42 CFR. Sect. Part 493.1253.
15. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC– MS/MS. Analytical Chemistry. 2003;75:3019-30.

F. Leland McClure, MSc, PhD, F-ABFT, is a recognized expert in the fields of pharmacology and toxicology, with over 30 years of toxicology experience, including testing for drugs of abuse. He is an inspector for the National Laboratory Certification Program and previously served as the Responsible Person for an HHS-certified laboratory. Dr. McClure is a Fellow of the American Board of Forensic Toxicology (ABFT). From 1989 to 2019, he was employed by Quest Diagnostics, most recently as the Corporate Medical Affairs Director for Prescription Drug Monitoring and Toxicology. He currently works as a drug testing, toxicology, and pharmacology subject matter consultant.