

## SAMPLE PREP PROTOCOLS: DRUGS OF ABUSE

Instrument	LC/MS/MS	GC/MS	LC/MS/MS (with solvent exchange)	LC/MS/MS	LC/MS/MS	GC/MS
<b>Advantages</b>	• Low upfront cost	• Easy	• Automation possible	• Fast and easy approach	• Automation possible	• Automation possible
<b>Disadvantages</b>	• Filtration may not remove endogenous compounds such as lipids and proteins, leading to matrix interferences	• High polarity compounds may experience poor recovery	• High polarity compounds may be affected by matrix interferences	• High polarity compounds may be affected by matrix interferences	• High polarity compounds may be affected by matrix interferences	• High polarity compounds may be affected by matrix interferences
<b>Protocols</b>	• Filter	• Inject	• Add water-immiscible solvents (e.g. methylene chloride) and biological sample in a container prior to filtration. Dilute biological samples – mix with water for aqueous or centrifuge samples – and wait for phase separation	• Apply water-immiscible extraction plate or cartridge (such as acetonitrile) in a centrifugation vial removing plate or cartridge	• Vortex mix, followed by centrifugation	• Filter by applying vacuum or positive pressure
<b>Sample filtration/dilute-and-shoot</b>	• Add water-immiscible solvents (e.g. methylene chloride) and biological sample in a container prior to filtration. Dilute biological samples – mix with water for aqueous or centrifuge samples – and wait for phase separation	• Transfer water-immiscible solvent layer to an autosampler vial (filter if needed)	• Inject sample needed for LC/MS/MS	• Inject collected filtrate	• Inject	• Inject
<b>Liquid-Liquid extraction</b>	• Add biological sample and organic solvent (such as acetonitrile) in a centrifugation vial removing plate or cartridge	• Vortex mix, followed by centrifugation	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure
<b>Protein precipitation</b>	• Add biological sample and organic solvent (such as acetonitrile) in a centrifugation vial removing plate or cartridge	• Vortex mix, followed by centrifugation	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure
<b>Solid-supported liquid extraction</b>	• Add biological sample and organic solvent (such as acetonitrile) in a centrifugation vial removing plate or cartridge	• Vortex mix, followed by centrifugation	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure
<b>Phospholipid removing cartridges/plates</b>	• Add biological sample and organic solvent (such as acetonitrile) in a centrifugation vial removing plate or cartridge	• Vortex mix, followed by centrifugation	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure
<b>Solid phase extraction</b>	• Add biological sample and organic solvent (such as acetonitrile) in a centrifugation vial removing plate or cartridge	• Vortex mix, followed by centrifugation	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure	• Filter by applying vacuum or positive pressure

## BIOANALYSIS/DRUGS OF ABUSE

# QUICK-REFERENCE METHOD GUIDE

The Measure of Confidence

LC/MS methods are rapidly becoming an important tool for drug screening and confirmation because they typically don't require a derivatization step, and are often less complicated than GC/MS methods. You can simplify and optimize method development even further using new technologies from Agilent.

### Key workflow considerations:

- Depending on the analytical instruments used, sample matrices – such as blood, plasma, and urine – can adversely affect your system and columns. Accordingly, reliable sample prep is an essential part of the workflow.
- Columns and sample prep techniques should be selected with an eye toward return on investment – specifically, time and cost when compared to workflow performance and results.
- When using an electrospray source, ion suppression caused by sample matrices can negatively impact your MS results.
- Many drugs of abuse are challenging to analyze because of factors, such as volatility, hydrophobicity, and “stickiness” to glass and plastic.

## TIPS AND TOOLS: DRUGS OF ABUSE SEPARATIONS

ANALYTE	CHARACTERISTICS	SAMPLE PREP	COLUMN SELECTION/SEPARATION
<b>Amphetamines</b>	• Non-polar and volatile • Can evaporate at the sample prep extraction solvent evaporation step unless precipitated as salts by adding hydrochloric acid • Add HCl toward the end of evaporation to avoid formation of ammonium chloride salts, which causes ion suppression • Recovery of longer-chain non-polar amphetamines with Log P > 2 can be optimized with two-component organic eluent (50% methanol/50% ethyl acetate); add 20% NH <sub>4</sub> OH to eluent for best results	• A simple generic Plexa PCX method works well • Elution can be optimized with 10% NH <sub>4</sub> OH added to methanol eluent (vs. commonly used 5% NH <sub>4</sub> OH) for better recovery	• Poroshell 120 EC-C18, 3 x 50 mm provides excellent separation of amphetamines and structurally similar drugs, such as ephedrine and its stereoisomer pseudoephedrine (as required by SAMHSA) • Mobile phase: A - 0.1% formic acid; B - 100% methanol • A very flat gradient (i.e. start with 15% B, hold for 1.5 min, then increase to 30% B at 3.5 min) • Flow rate of 0.8 mL/min recommended for short analyses
<b>6-Acetylmorphine (6-AM)</b>	• Non-polar heroin metabolite • Quickly hydrolyzed at room temperature • Low LOD - 1 ng/mL is required by SAMHSA	• A simple generic Plexa PCX method works well • Elution can be optimized by an eluent of 80% ethyl acetate/20% IPA with 5% NH <sub>4</sub> OH to maximize recovery	• Poroshell 120 EC-C18, 3 x 50 mm • Mobile phase: A - 0.1% formic acid; B - 1% formic acid in methanol • Flow rate of 0.8 mL/min allows for short analytical time with elution of both analytes between 2 and 2.5 min
<b>Buprenorphine/Norbuprenorphine</b>	• Highly non-polar (Log P > 5) • Very low concentrations in blood (<1 ng/mL); low LOD • Sticks to glassware, LC tubing, and injector parts	• Deactivated vials/inserts and MeOH-lined/air-dried glassware recommended • Efficient cleanup of whole blood samples with Agilent Plexa PCX provides LODs below 100 pg/mL • Optimize generic Plexa PCX method with large aqueous/organic wash volumes and acidification of organic (methanol) wash; use 70% methanol/30% of 2% formic acid as organic wash to prevent analyte breakthrough • Vial recommend an elution of 80% ethyl acetate/20% IPA with 5% NH <sub>4</sub> OH with a soaking procedure; add eluent with a closed valve to soak sorbent bed for 1-2 min before letting eluate drip through SPE bed	• Poroshell 120 EC-C18, 3 x 50 mm • Mobile phase: A - 0.1% formic acid; B - 1% formic acid in methanol • Separation from other analytes not required by SAMHSA • A moderately steep gradient with 0.8 mL/min flow rate can be used (i.e. 10% B at 0.5 min to 70% B at 2.5 min)
<b>THC-COOH (THCA THA-acid)</b>	• Highly non-polar (Log P > 5) • Sticks to glassware, LC tubing, and injector parts • Phospholipids remain in the final sample, which can cause ion-suppression • Recovery may be affected when physical/chemical similarity exists between analytes and phospholipids	• Deactivated vials/inserts and MeOH-lined/air-dried glassware recommended • SAMHSA requires converting glucuronides to THCA (most reliable conversion method is base hydrolysis – incubation with KOH at 60 °C) • 100% organic solvent wash will lead to a partial loss of THCA from the sorbent; use 10% and 30% acetonitrile washes with 2% acetic acid followed by a small pulse of hexane (200 µL) • To maximize recovery, apply a combination eluent of 80% ethyl acetate/20% IPA in 2 aliquots with a soaking procedure as described above • Negative polarity should be used for detection	• Poroshell 120 EC-C18, 3 x 50 mm • Mobile phase: A - 5 mM ammonium formate in water; B - 100% methanol • A very steep gradient (30% to 95% B in 1 minute), with 0.8 mL/min flow rate shortens analytical time (RT around 2 min)
<b>Benzyloecgonine (BE)</b>	• Cocaine metabolite, relatively stable, non-polar	• Simple generic Plexa PCX sample prep works well • Elution can be optimized by adding up to 20% NH <sub>4</sub> OH to methanol for better recovery	• Poroshell 120 EC-C18, 3 x 50 mm • Mobile phase: A - 0.1% formic acid; B - 1% formic acid in methanol • Same gradient as for PCP
<b>Opiates (morphine, codeine)</b>	• Polar (morphine) to non-polar (codeine) • Found in urine as form of glucuronide conjugates • After pre-treatment, use simple generic Plexa PCX sample prep • SAMHSA requires converting glucuronides to original compounds (most reliable conversion method is acid hydrolysis-incubation with HCl at 95 °C) • Elution can be optimized by adding up to 20% NH <sub>4</sub> OH to methanol for maximum recovery	• Start with 5% B (because of morphine polarity), hold for 0.5 min, then increase to 25% B at 1.5 min and to 55% B at 2.5 min • Mobile phase: A - 0.1% formic acid; B - 1% formic acid in methanol • hycomorphone, norcodeine, and others (as required by SAMHSA) when using a very flat gradient	

### METHOD DEVELOPMENT CONSIDERATIONS

## WHAT YOU'LL FIND IN THIS GUIDE

### WORKFLOW



Although both liquid chromatography and gas chromatography can be used for bioanalytical and forensics applications, this guide will focus on the LC/MS workflow. Depending upon the stage of testing you perform – and your ability to modify methods – the insights you find in this guide may help you improve throughput and results.

### Key information is organized as follows:

- **Outer panels:** General overview, plus tips for working with analytes commonly targeted in drugs of abuse testing.
- **Center panels:** Workflow recommendations for sample prep, separation, and detection.
- **Back panel:** Ordering information for products needed to perform the analyses discussed in this guide.

## ORDERING INFORMATION

### Recommended columns for Drugs of Abuse separations

We recommend you start your method development with Poroshell 120 EC-C18 as it will provide the best results across a broad range of analytes.

Size (mm)	Poroshell 120 EC-C18	Poroshell 120 EC-C8
3.0 x 150	683975-302	683975-306
3.0 x 100	686975-302	686975-306
3.0 x 75	687975-302	687975-306
3.0 x 50	689975-302	689975-306
3.0 x 30	691975-302	691975-306
2.1 x 150	683775-902	683775-906
2.1 x 100	695775-902	695775-906
2.1 x 75	697775-902	697775-906
2.1 x 50	699775-902	699775-906
2.1 x 30	691775-902	691775-906
Fast Guard 3.0 x 5	823750-911	823750-913
Fast Guard 2.1 x 5	821725-911	

There are nine different phases in the Poroshell 120 family, and 4.6 mm id columns are available as well. For a full list of part numbers, visit [agilent.com/chem/poroshell120](http://agilent.com/chem/poroshell120)

### Agilent services let you focus on what you do best

Whether you need support for a single instrument or multiple labs, Agilent can help you solve problems quickly, increase uptime, and optimize your resources.

### For more information

Learn more at [agilent.com/chem/forensics](http://agilent.com/chem/forensics)  
 U.S. and Canada **1-800-227-9770**  
[agilent\\_inquiries@agilent.com](mailto:agilent_inquiries@agilent.com)  
 Europe [info\\_agilent@agilent.com](mailto:info_agilent@agilent.com)

Asia Pacific [inquiry\\_lsca@agilent.com](mailto:inquiry_lsca@agilent.com)

In other countries, please call your local Agilent Representative or Agilent Authorized Distributor – visit [agilent.com/chem/contactus](http://agilent.com/chem/contactus)

This information is subject to change without notice.

© Agilent Technologies, Inc. 2014  
 Printed in the USA February 3, 2014  
 5991-3883EN

## HOW DO I CHOOSE THE RIGHT SAMPLE PREP TECHNIQUE?

Select your sample prep method with your analytical goals and requirements in mind – including your lab affiliation/profile, regulatory environment, matrix, target analytes, cost considerations, and detection method.

There is no standard procedure for defining a comprehensive sample preparation method, and various published procedures are often complementary, because they focus on different targeted analytes. Selecting a flexible sample preparation approach such as mixed-mode solid phase extraction (SPE) gives you a versatile tool for sample cleanup, suitable for both general screening and targeted confirmation methods.

### Common forensic sample prep challenges

- Pressure to increase throughput, without compromising data integrity. Cost-efficiency matters, so the more analytes you can combine into one analysis, the better.
- Non-group-specific methods are needed for screening, while methods with maximum selectivity are required for confirmatory testing.
- High recoveries/reproducibilities are essential for confirmatory analysis to avoid false positives.
- Blood/plasma concentrations of drug samples can be quite low when they show up as positive (i.e. analysis of buprenorphine in whole blood). An effective sample cleanup procedure that concentrates analytes can help meet the required LODs.

### Agilent Bond Elut Plexa PCX: a good first choice for forensic applications



Plexa PCX uses a mixed-mode polymeric sorbent with reversed-phase and cation-exchange properties. Plexa PCX retains positively charged (basic) analytes by both reversed-phase mechanism and cation-exchange; it retains acids and neutrals by reversed-phase only. Advantages include:

- **Flexibility:** Accommodates basic, neutral, and acidic analytes with a broad range of polarities.
- **Sensitivity:** Contains an amide-free hydroxylated particle surface that excludes protein binding, minimizing ion suppression.
- **Narrow particle distribution:** Ensures fast flow and reproducible performance.

### Sample prep workflow recommendations for Bond Elut Plexa PCX

#### Conditioning

- Conditioning with methanol is sufficient; equilibration with water not needed.

#### Sample loading

- Acidification (pH<6) recommended for basic and acidic analytes. Allow samples to drip by gravity, or apply low vacuum (2-3" Hg).

#### Wash 1

- Acidified aqueous wash for all analytes – use 2% formic acid.

#### Wash 2

- 5% methanol wash for most acidic and neutral analytes – test if higher proportion of organic can be used without leading to analyte breakthrough.
- 100% methanol wash for most basic analytes (at this step, acidic/neutral analytes will elute and should be collected).
- Sample drying: pull air through vacuum manifold (high vacuum) for 5 min (30 mg sorbent bed) and 10 min (60 mg sorbent bed).

#### Sample elution strategies

- **Basic analytes:** Add a strong base to the organic eluent to break the ionic interaction between the analyte and the strong cation-exchange sorbent. We recommend 5% NH<sub>4</sub>OH, but up to 20% can be used to maximize recoveries.
- **Neutral/acidic analytes:** Use 100% organic eluent. If analyzing together with bases, these analytes will be eluted by 100% organic wash, and should be collected at this step. (See Wash 2)
- **Organic Eluent:** Methanol is best for more polar analytes. Combine 50% protic and 50% aprotic solvents (methanol or IPA + ethyl acetate) for non-polars with Log P >2. Increase proportion of ethyl acetate in eluent to 80% for analytes with Log P>3.5-4.
- A soaking procedure is recommended at elution to enhance solvent-analyte interaction.
- Allow eluate to drip by gravity, then apply low vacuum to expel solvent from the sorbent pores.
- Dry under a stream of nitrogen at T<40° C, and reconstitute with initial mobile phase.

### Additional resources

- Benzylecgonine in Urine (5990-9624EN)
- 11-nor-9-carboxy- in Urine (5990-9627EN)
- Opiates (Morphine and Codeine) in Urine (5990-9625EN)
- 6-Acetylmorphine in Urine (5990-9622EN)
- Amphetamines in Urine (5990-9623EN)
- Phencyclidine in Urine (5990-9626EN)
- Fast Screening Methods for Steroids by HPLC (5991-0451EN)
- LC/MS/MS of Buprenorphine and Norbuprenorphine in Whole Blood (5990-9930EN)
- Hydrophilic Interaction Chromatography (HILIC) (5991-1242EN)
- Proven Approaches for Today's Forensic/Toxicology Challenges – Application Compendium (5991-3398EN)

To find these publications, look them up by pub number at [agilent.com/chem/library](http://agilent.com/chem/library)

## WHICH LC COLUMN IS BEST FOR MY APPLICATION?

You are likely under pressure to process more samples, faster – without compromising accuracy; therefore, you need to consider several factors during LC column selection. For example, poor column robustness can reduce column lifetime and lead to costly rework. In addition, samples with high analyte concentrations may require a column with higher capacity.

### A robust first choice for drugs of abuse: Poroshell 120 columns



We recommend Poroshell 120 columns for bioanalytical applications, because they deliver robust performance for fast LC. Their advantages include:

- **Less clogging:** Because Poroshell 120 columns have a 2 µm frit, they are less likely to clog and shorten column lifetime. This is particularly important when working with dirty biological samples in forensic applications. Fast Guards for UHPLC are also available to further extend column lifetime without reducing Fast LC performance.
- **Fast separations with very high resolution:** Unique superficially porous particles provide speed and resolution similar to sub-2 µm columns – with ~40% less backpressure.
- **Excellent column capacity** for demanding forensics applications.
- **Numerous bonded phases** that give you the flexibility to perfect your separation.

### Ensuring excellent resolution

One way to increase resolution is by using columns packed with smaller-diameter media; however, smaller particles can increase system backpressure. Superficially porous particles, such as Agilent Poroshell 120, achieve 90% of the efficiency of 1.8 µm materials with considerably lower pressure.

### Increasing selectivity

You can improve your selectivity – and separations – by:

- Changing mobile phase composition and pH
- Changing column temperature
- Changing stationary phase composition

### Choosing the right mobile phase

A good general mobile phase for basic drugs of abuse applications is:

- Mobile Phase A: 0.1% formic acid in water
  - Mobile Phase B: 0.1% formic acid in methanol
- For THCA, use 5 mM ammonium formate in water for A, and 100% methanol for B.

### Optimizing column temperature

For maximum column lifetime, you should generally run your analyses below 40 °C.

### Selecting the column stationary phase

Poroshell 120 EC-C18 is the best place to start your method development, because the C18 phase is designed for improved selectivity in a broad range of separations. Another good choice to test is Poroshell 120 SB-C18, which can interact with silanol groups to provide an alternative C18 selectivity at neutral- to low-pH mobile phases.

Other bonded chemistries – such as Phenyl-Hexyl, polar SB-Aq, or polar-embedded Bonus-RP – may provide improved selectivities for an array of polar and aromatic compounds.

### Agilent Method Development Kits contain columns with different bonded chemistries for convenient testing

Description	Kit Contents	Size	Part Number
Poroshell 120 Selectivity	Includes one each of Poroshell 120 EC-C18, Phenyl-Hexyl and Bonus-RP	2.1 x 50 mm	5190-6155
		4.6 x 50 mm	5190-6156
Poroshell 120 Aqueous	Includes one each of Poroshell 120 SB-Aq, Phenyl-Hexyl and Bonus-RP	2.1 x 50 mm	5190-6057
		4.6 x 50 mm	5190-6158

More kits are available for other Agilent columns; see [agilent.com/chem/methoddevkits](http://agilent.com/chem/methoddevkits) for more information.

## WHAT OTHER METHOD PARAMETERS SHOULD I CONSIDER?

### Gradients can help improve speed and separation

Shorter columns can reduce analytical time; however, you should scale your gradient and injection volume according to column length and diameter. The following parameters should be used:

High polarity: Start with very low organic (5%)

Low Polarity: Start with 15-30% organic

To avoid carryover, use a 95% organic column rinse before returning to initial conditions, and allow sufficient re-equilibration time at the end of the run to stabilize column backpressure. Confirm that there is no analyte carryover by running a mobile phase blank after your high-concentration standards, and after every 10 samples.

Suggested starting gradient for drugs of abuse:

Time (min)	% B
0.0	15
2.0	70
2.1	95
5.5	95
5.51	15

It is best to start your LC analysis at a relatively low fraction of organic solvent (10-15%) to allow salts and other polar components of blood or urine to elute at the beginning of the run. A flow rate of 0.8 mL/min shortens retention and re-equilibration time.

Prior to starting your gradient, it is important to equilibrate your column by flushing for a minimum of 10 column volumes of your starting mobile phase.

### Capillary selection guidelines

For fast LC applications, always use the smallest possible id capillaries to minimize extra column volume; however, you should always take the increased backpressure into account. The 0.075 mm id tubing from Agilent's low dispersion kit p/n 5067-5189 can improve results by minimizing the volume in the capillaries.

### Tips for optimizing your MS results

- Dynamic MRM is best for analyzing a large number of compounds on QQQ LC/MS.
- Use up to ten triggered MRM (tMRM) transitions to detect, quantify, and confirm your target analyte using QQQ LC/MS.
- The **Agilent MassHunter tMRM Forensic/Toxicology database** for LC/MS QQQ provides up to ten MRM transitions per compound with fragmentor and collision energy parameters optimized for Agilent QQQ instruments.
- Start your LC analysis at a relatively low fraction of organic solvent (5-15%) to allow salts and other polar components in blood or urine to elute at the beginning of the run. In Agilent drugs of abuse methods, we recommend diverting the LC stream to waste in the initial portion of the run *before* the elution of your first peak. This will keep the source clean for longer periods of time and reduce required source cleaning.
- Low injection volumes (2-10 µL in Agilent drugs of abuse methods) prevent your detector from overloading.
- To enhance your signal, use the Delta EMV option in your acquisition method. Delta EMV ± 300 is a common setting.
- **Agilent Forensic Toxicology LC/MS Application Kits** are ready to use and allow quick, efficient method development and implementation for thousands of compounds important to forensic toxicologists. The kit includes LC columns, analytical standards, LC/MS methods, databases, libraries, and on-site application support for Agilent Triple Quadrupole, TOF, and Q-TOF instruments.



Learn more about performing fast, confident forensic/toxicology analysis at [agilent.com/chem/forensics](http://agilent.com/chem/forensics)