# Method Development of Mixed-Mode Solid Phase Extraction for Forensics Applications

Application Note

Forensics & Toxicology


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## Abstract

Mixed-mode solid phase extraction has the versatility and power to extract target compounds from biological matrixes and is one of the most widely used sample preparation methods in forensic laboratories. This application note is an overview of sample preparation method development in its entirety, prior to LC/MS/MS or GC/MS, using Agilent Bond Elut Certify mixed-mode solid phase extraction to shorten the method development process or to improve an existing method.


## Introduction

Many workplace screening tests of drugs of abuse are done by mass spectrometry with gas chromatography or liquid chromatography. Sometimes, scientists in forensic laboratories encounter the need to change existing standard operating procedures (SOPs) when there are requirements for lower detection limits, improved linearity in the lower or upper concentration range, calibration range extension, limited sample volume, or too-frequent replacement of the analytical column. When new solid phase extraction (SPE) method development is under consideration, there is no straightforward shortcut to the new method. In this study with phencyclidine (PCP) in urine, the complete workflow for solid phase extraction is reviewed, and the decision making process to the final method is highlighted.


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## Materials and Methods

Acetonitrile, methanol, and formic acid were LC/MS grade. Dichloromethane was LC grade. Ethyl acetate was GC residue analysis grade, and hexane, $NH_4OH$, and $KH_2PO_4$ were reagent grade. Water was Milli-Q filtered or LC/MS grade. Acetic acid was premium quality. PCP and PCP-D5 analytes were purchased from Sigma-Aldrich, Corp. The QC sample was Liquichek urine toxicology control, level C2, from Bio-Rad Laboratories. The PCP concentration was 19 ng/mL.

Sample preparation was accomplished using Agilent Bond Elut Certify, 130 mg, 3 mL, 50/pk (p/n 12102051).

## Instrument conditions

Instrument parameters of LC/MS/MS and GC/MS with pulsed splitless and pulsed split injections are discussed in greater detail elsewhere [1].

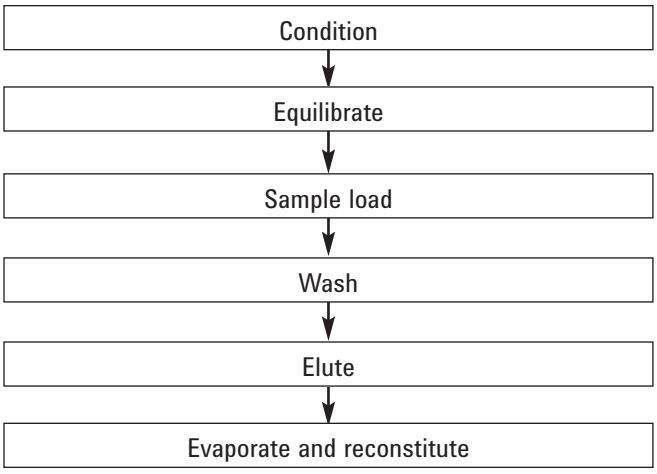
## Principles of solid phase extraction

A wide range of solid phase extraction sorbents exist, including reversed-phase, cation-exchange, anion-exchange, or mixed-mode. The principle of SPE is to let the sample, with compounds of interest or interferences, or both, bind to a solid phase sorbent (usually chemically modified silica or polymeric material packed in cartridges or well plates), followed by rinsing off interferences to waste, and finally eluting and collecting the compounds of interest. All these steps take place selectively and so interferences and compounds of interest will separate from sample matrix successfully.

The first step in SPE method development is to understand the characteristics of the target compound. PCP is relatively basic and hydrophobic, with pKa = 8.29 and log P = 4.69. Ideally, using cation-exchange interaction and hydrophobic interaction would be the optimal solid phase extraction choice. Agilent Bond Elut Certify mixed-mode solid phase extraction has a good balance of both hydrophobic and cation-exchange characteristics and is well suited for PCP analysis.

The conventional solid phase extraction workflow consists of conditioning, equilibration, sample loading, washing, elution, evaporation, and reconstitution, as shown in Figure 1. Some of the steps may be eliminated to reduce process time.

In this procedure, the solid phase extraction sorbent retains the compounds of interest until the elution step, while other interferences are removed in the wash step.



From Figure 2, the first three steps (condition, equilibrate, and sample load) typically do not require much variation to optimize the performance of solid phase extraction. Sometimes the conditioning and equilibration steps can be eliminated for some polymeric solid phase extraction sorbents. The most effective optimization can be done in the washing and elution steps. During sample loading, compounds of interest bind to the sorbent material along with interferences. The purpose of the washing step is to remove these interferences. The elution step then recovers the target compound by interrupting the interaction between it and the solid phase extraction sorbent. The maximum removal of interference while maintaining optimal recovery of the target compound is the key to successful method development in solid phase extraction. To verify method optimization, every step from sample loading to elution needs to be collected and analyzed by chromatography.

## Results and Discussion

After conditioning (2 mL MeOH) and equilibration (2 mL of 100 mM $KH_2PO_4$), spiked urine sample (1 mL) was loaded into a pipette, as shown in Figure 2. During the loading step, any eluate was collected in a vial. Washing was split into three aliquots of 1 mL each. The first wash was 1 mL 5 % acetic acid and the others were 1 mL MeOH. Each eluate was collected separately in a vial. Elution was done the same way, for example, 3 x 1 mL volumes, with ACN + 2 % $NH_4OH$. Each eluate was collected individually in a vial. The wash 2 vial in Figure 3 indicates that the majority of interference was removed by MeOH during the washing step. All collected eluates were injected into an LC/MS/MS and all chromatograms are shown in Figure 4.

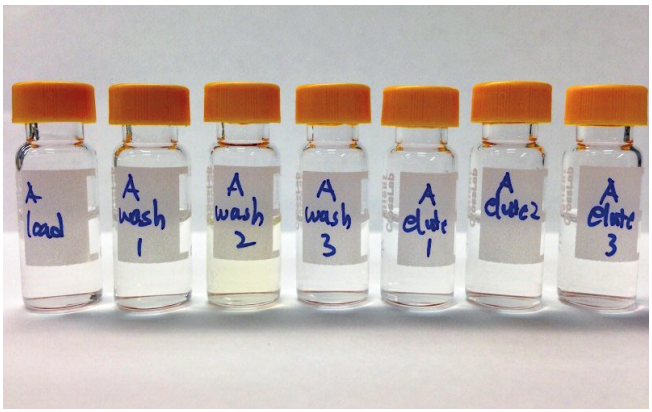



Figure 3. Collection eluates from every step in the solid phase extraction method (from left to right, eluate collections of sample loading, wash 1, wash 2, wash 3, eluate 1, eluate 2, and eluate 3).

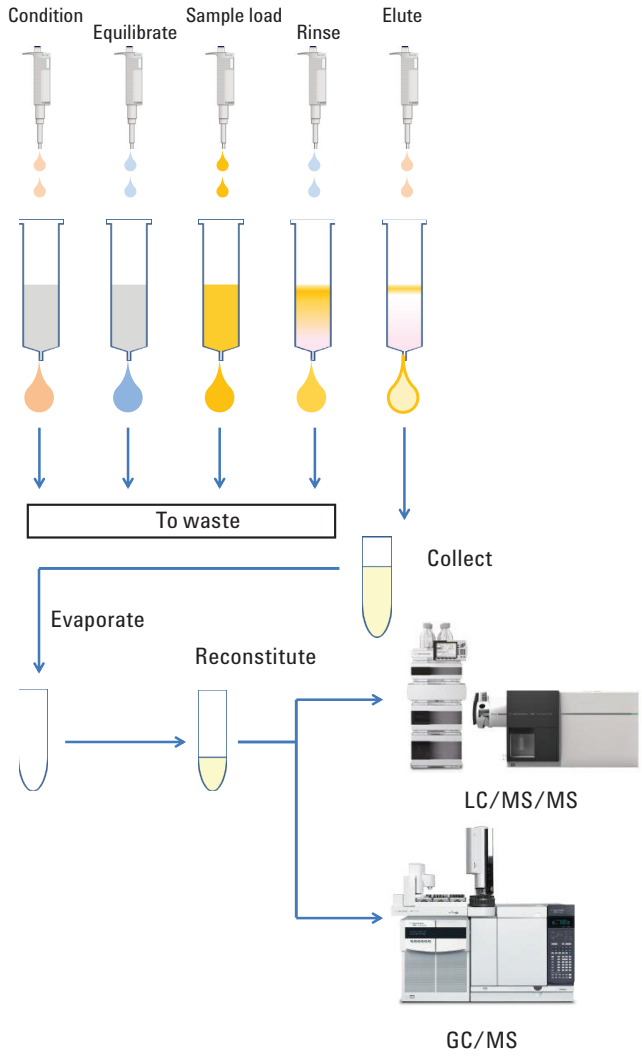



Figure 2. Schematic of forensic sample analysis using solid phase extraction.

From the information in Figure 4, it was clear that the target compound PCP (RT = 1.35 minutes) was not coming through the sorbent in the sample loading step. The chromatograms from washes 1 to 3 also confirmed that PCP was not washed off during the washing steps. The elution step should have PCP and, as the chromatograms show from eluates 1 to 3, target compounds were coming off of the sorbent.

The wash 3 vial was visually clear and the chromatogram from wash 3 did not have the target compound peak; hence, wash 3 can be skipped. Eluate 3 did not have a significant amount of PCP, which indicated that one aliquot of 2 mL of ACN + 2 % $NH_4OH$, by combining eluate 2 and 3, was sufficient for a single elution step.

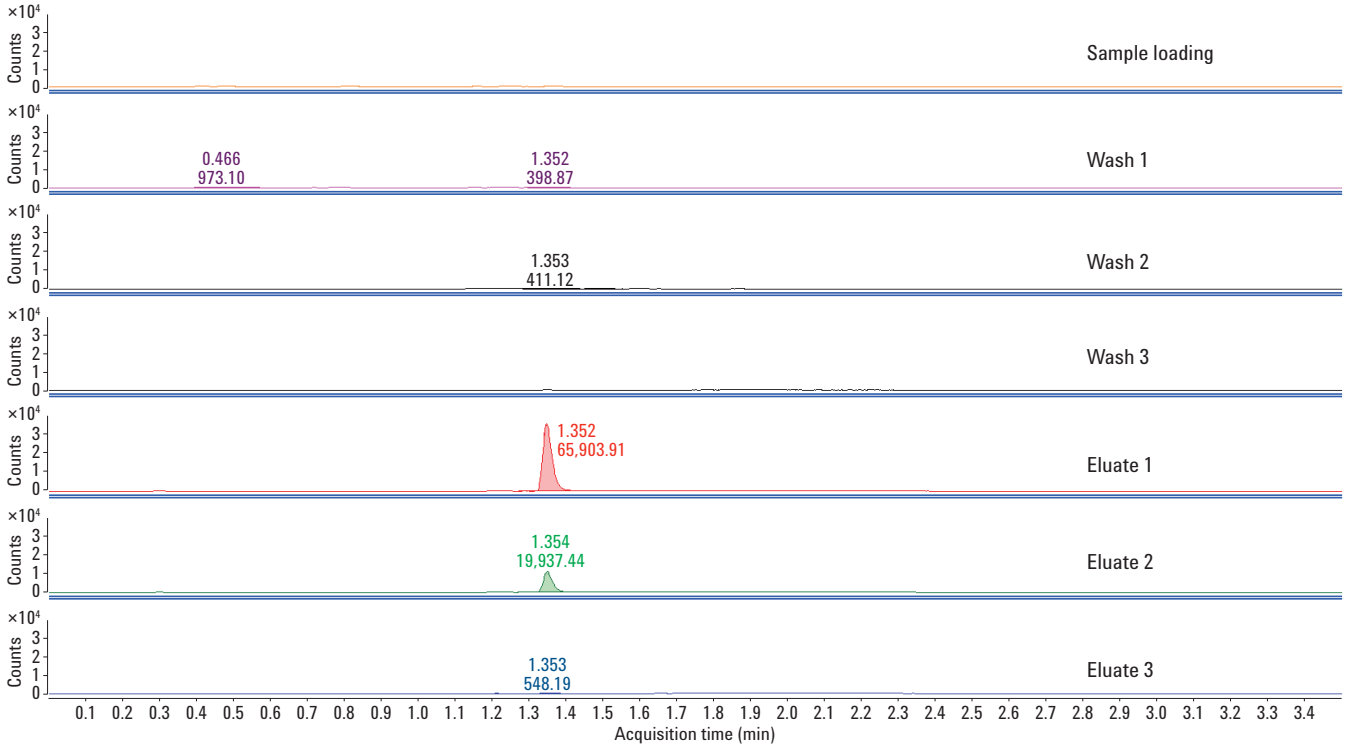
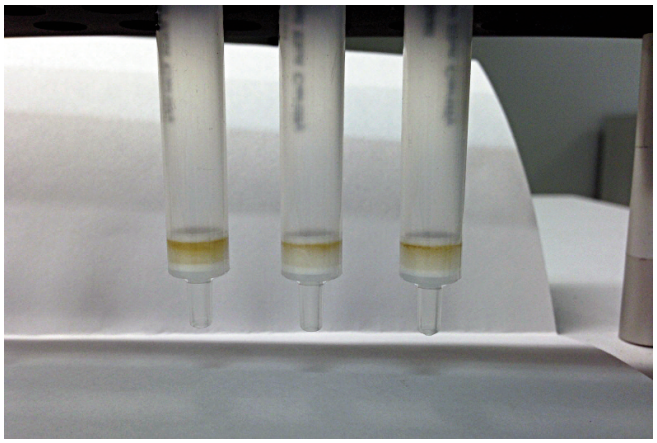
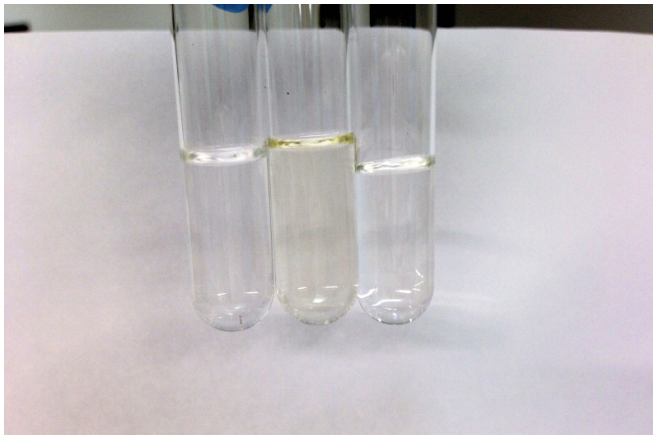



Figure 4. Chromatograms of PCP by LC/MS/MS for all eluates collected during solid phase extraction.

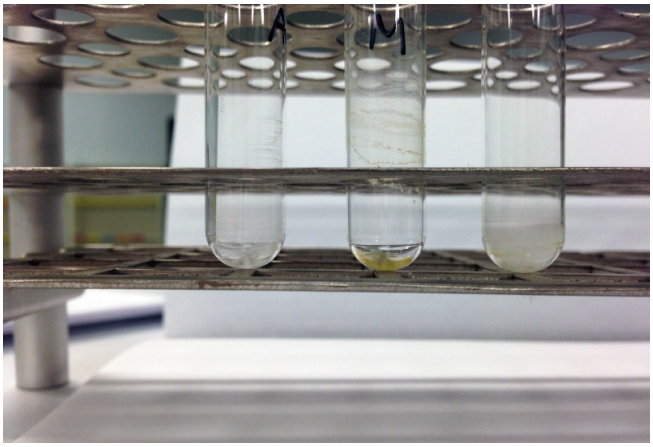
Different elution solvents can be tested for further elution step optimization. A widely accepted elution solvent mixture in forensic applications is DCM:IPA:$NH_4OH$ at 78:20:2. In addition, a typical elution solvent for LC/MS/MS applications is ACN or MeOH. When three different elution solvents were tested, different peak area counts and full-scan background information was obtained. Along with chromatograms, visual observation is also important and Figure 5 shows the effect of different elution solvents.



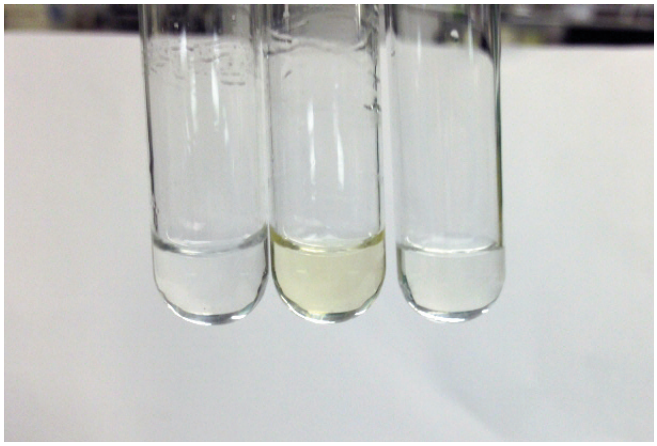
**A)** Agilent Bond Elut Certify cartridges after elution



**B)** Elutions by three different solvents



**C)** After evaporation



**D)** Reconstituted in initial mobile phase

Figure 5. Elution solvent variation study (from left to right, elutions made by ACN + 2 % $NH_4OH$, MeOH + 2 % $NH_4OH$, and DCM:IPA:$NH_4OH$ at 78:20:2).

Elution by MeOH + 2 % $NH_4OH$ had the dirty eluate, whereas ACN +2 % $NH_4OH$ and DCM:IPA:$NH_4OH$ at 78:20:2 had visually clean extracts. Chromatographically supporting data confirmed that MeOH-based elution was not appropriate, with lower recovery (Figure 6) and more LC/MS background noise (Figure 7).

Based on the data shown in Figures 5 to 8, ACN-based elution performed the best, minimizing interference and maximizing the peak area count of the target compound.

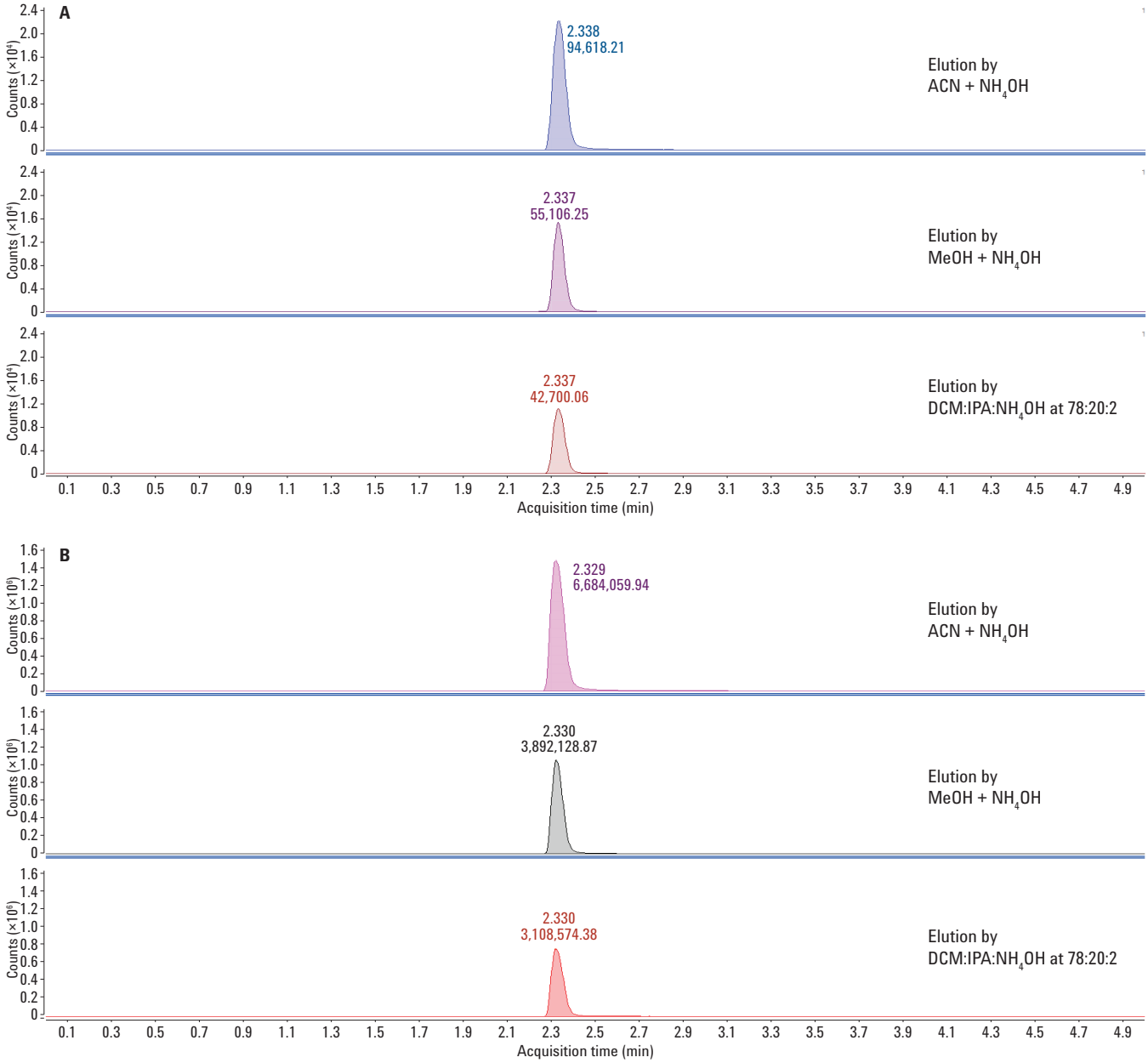



Figure 6. LC/MS/MS chromatograms of PCP (A) and PCP-d5 (B) using three different elution solvents (top to bottom: ACN + 2 % $NH_4OH$, DCM:IPA:$NH_4OH$ at 78:20:2, and MeOH + 2 % $NH_4OH$).

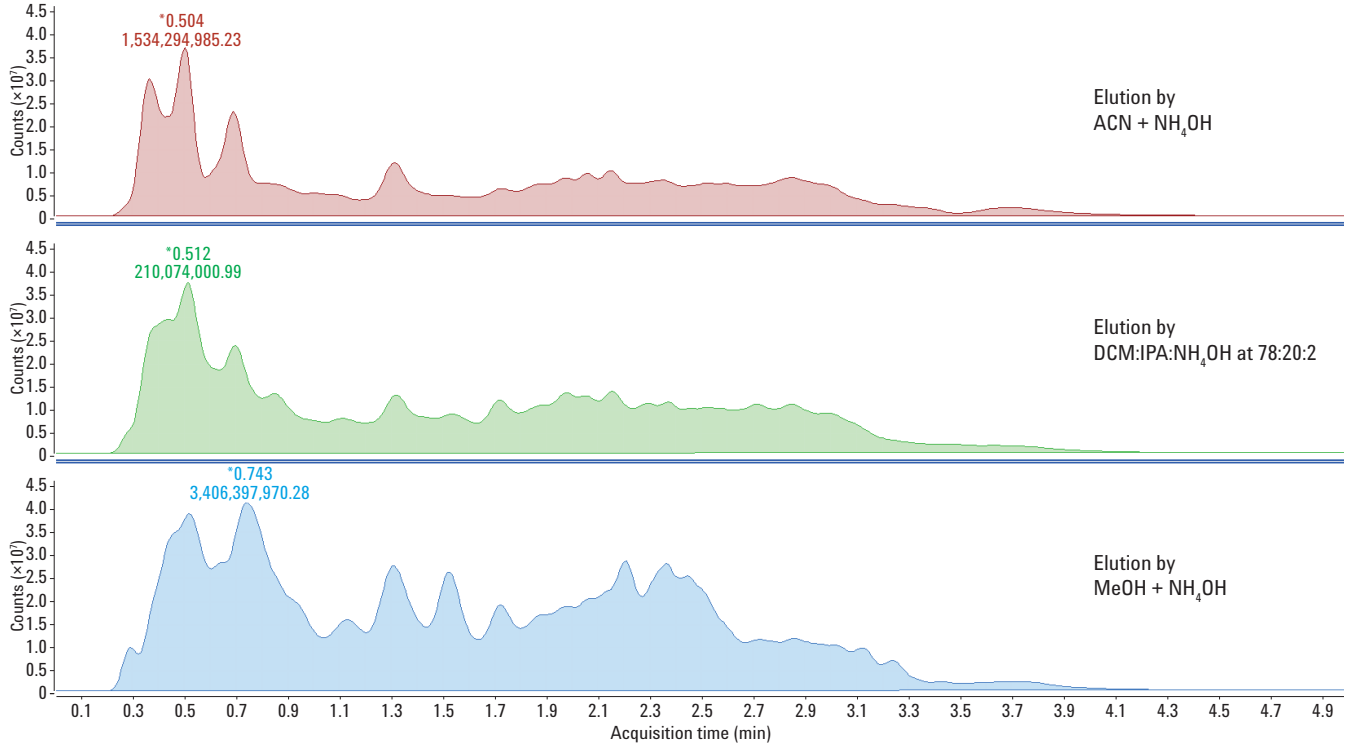



Figure 7. Full-scan LC/MS/MS chromatograms of blank urine prepared by Agilent Bond Elut Certify with different elution solvents.

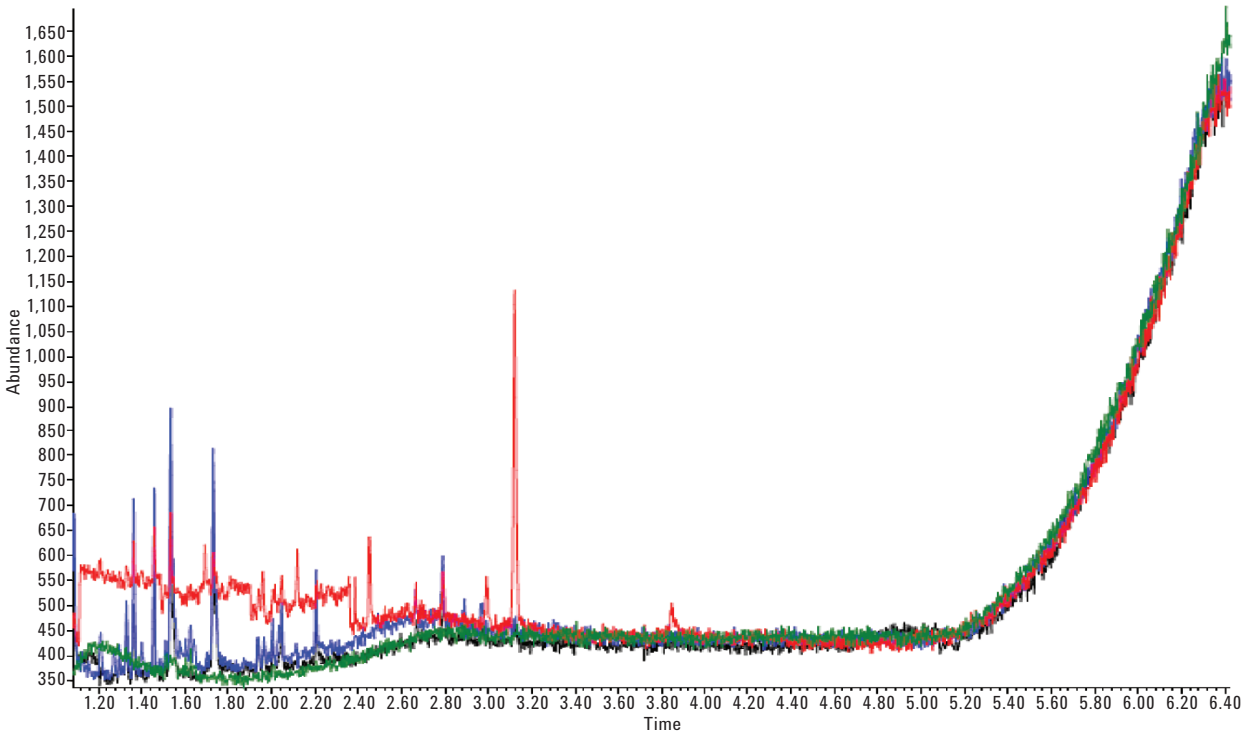


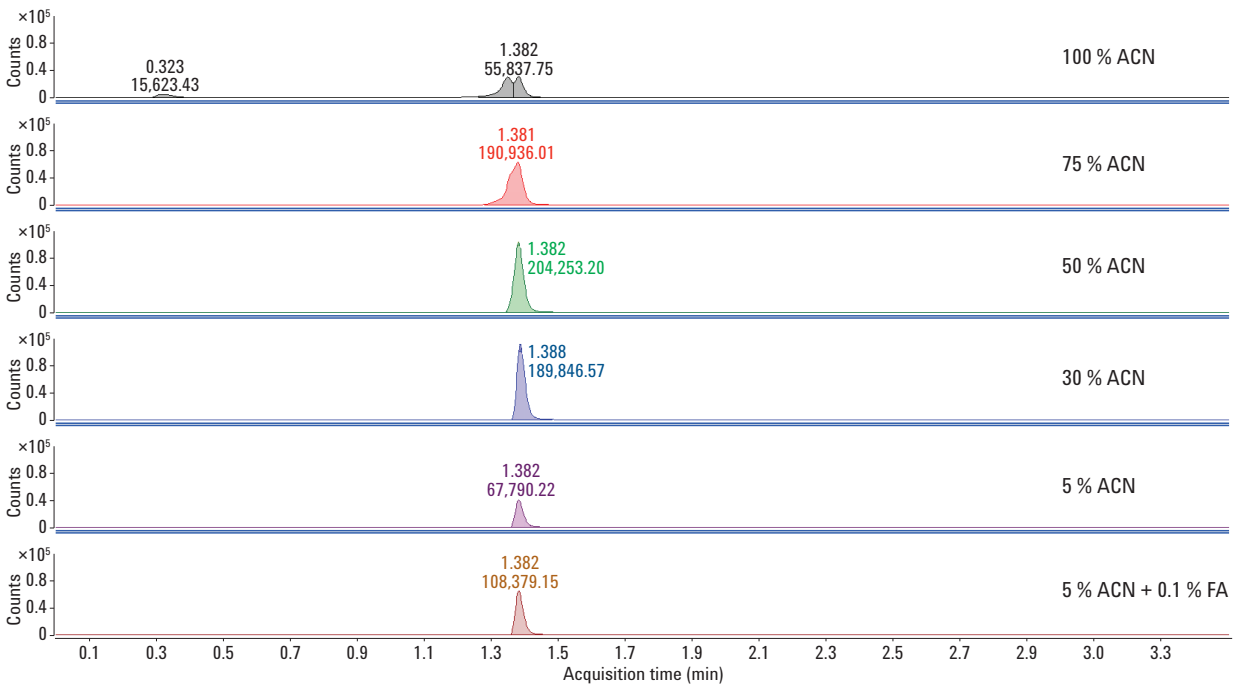

Figure 8. Full-scan GC/MS chromatograms of blank urine prepared by Agilent Bond Elut Certify with different elution solvents.

Injection volumes for LC/MS/MS can vary from sub-µL to 20 µL. With UHPLC systems, injection volumes tend to be smaller. However, if large-volume injection is required for better detection limits, a sample solvent and injection volume study can be done. The general rule is to have initial mobile phase as the sample solvent. Typically, the higher the organic solvent composition in the sample solvent, the higher the LC/MS/MS response, due to improved desolvation in the ionization source. Different injection volumes and sample solvent compositions were tested, as shown in Figure 9.

From Figure 9, it was evident that for smaller injection volumes such as 2 µL, the percentage of organic solvent had minimal impact on the peak shape and peak area. For larger injection volumes, for example, 5 to 10 µL, having sample solvent composition close to the initial mobile phase improved the peak shape and sometimes the peak area count as well. These experimental data can differ, depending on the LC/MS/MS conditions and the compounds of interest.

Final experimental conditions and results with human urine samples, including sample preparation, and instrument parameters, have been published [1].

**10 µL injection**



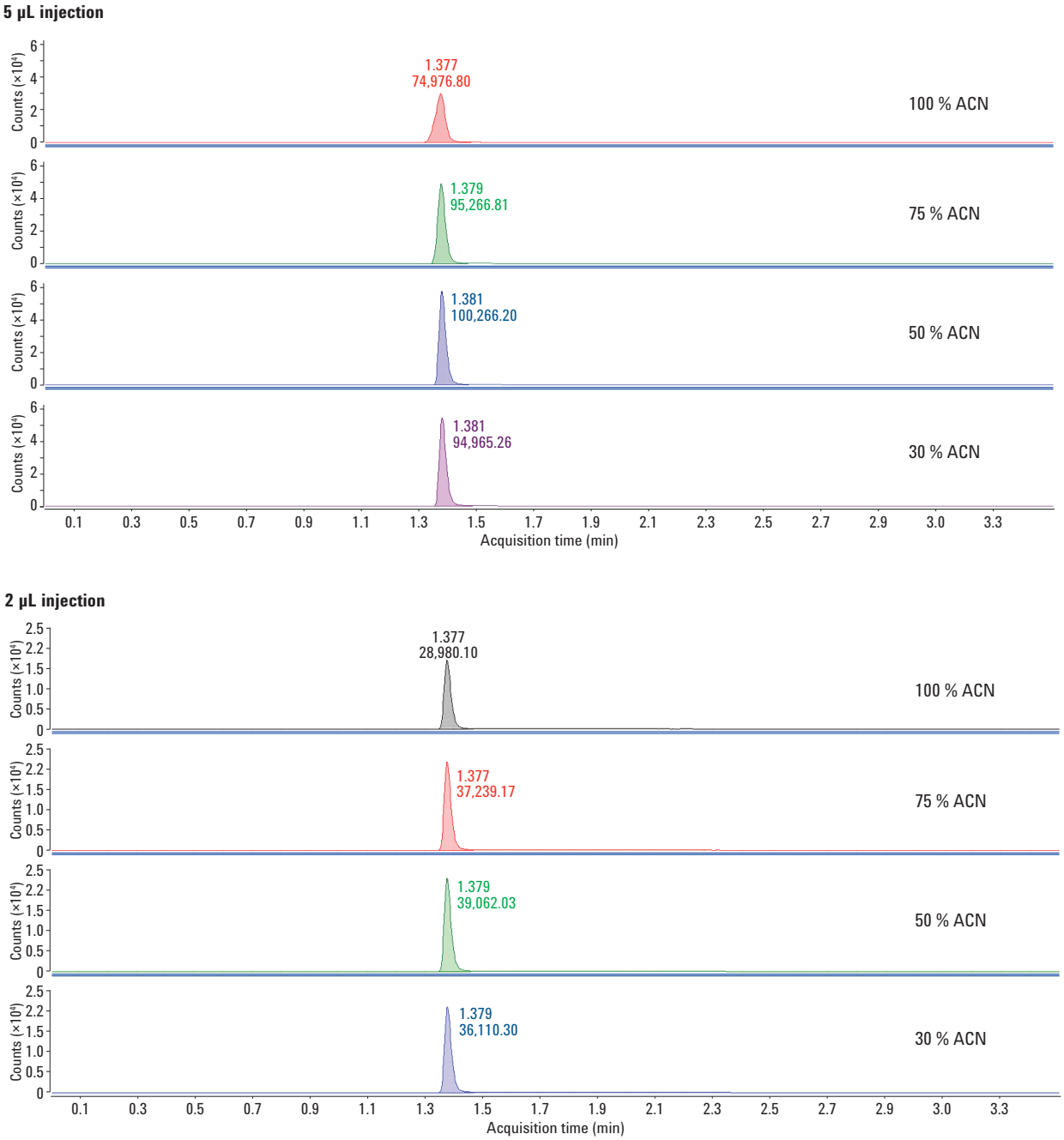



Figure 9. Injection volume and sample solvent effect in LC/MS/MS (injection volume is shown at the top and sample solvent composition is shown in every chromatogram).

## Conclusions

The sample preparation process using mixed-mode solid phase extraction was illustrated with real samples and chromatographic data, producing optimized conditions for improved quality of data for forensic applications. A good balance between the removal of interference and retention of the target compounds, followed by elution optimization for cleaner extracts, is essential for successful method development in solid phase extraction.

## Reference


1. Mike Chang. *Analysis of Phencyclidine in Urine to U.S. SAMHSA Guidelines with LC/MS/MS and GC/MS.* Application note, Agilent Technologies, Inc. Publication number 5991-4575EN (2014).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