[APPLICATION NOTE]

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Illicit Drug Analysis in Urine Using 2D LC-MS/MS for Forensic Toxicology

Claude Mallet[1] and Sabra Botch-Jones[2]
[1]Waters Corporation, Milford, MA, USA
[2]Boston University School of Medicine, Boston, MA, USA

APPLICATION BENEFITS

- Fast extraction protocol (20 min)
- 100 ppt detection level in urine
- Large volume injection

WATERS SOLUTIONS

ACQUITY UPLC® with 2D Technology

Xevo® TQD

Radar mode

Automated method development

KEY WORDS

Illicit drugs, urine, mixed-mode SPE

INTRODUCTION

The field of forensic toxicology encompasses several disciplines, such as analytical chemistry, and pharmacology with the main objective of assisting legal investigation of death, poisoning and drug use. The use of spot testing is perhaps the most known technique in forensic analysis for rapid results. For a complete legal identification, a more robust methodology is required and the current trend in forensic laboratories is the use of liquid chromatography coupled with mass spectrometry (LC-MS or LC-MS/MS). However, to achieve satisfactory results most often with a trace level detection requirements, extensive and time consuming sample preparation protocols are required to reach sub ppb levels. In recent years, advances in analytical capabilities with hyphenated instrumentation platforms have enabled analytical sensitivity and efficiency to detect trace level analytes. However, the bottleneck resides with the sample preparation techniques which have not yet been modernized. Although these techniques are "tried and true", there is much room for improvement. Utilizing Multi-Dimensional chromatography as a micro extraction technique can decrease sample preparation time while enhancing the separation integrity observed with current single dimension chromatography techniques.

The extraction process was performed using a reversed-phase solid-phase extraction in 1D, 2D optimized, 2D sequential and cumulative elution modes. The concept of sequential 2D extraction was designed to capture the retention behaviour of a target analyte in response to various extraction parameters (sorbent strength, elution polarity, solubility... etc.). Therefore, optimized conditions can be selected to excise a region of interest during extraction. Twelve illicit drugs were spiked in human urine and extracted using three extraction protocols for performance evaluation. The chosen 2D chromatography conditions used in this application were identified using a 6 x 6 automated methods development protocol (144 methods total). The manual extraction of urine samples was completed in less than 20 minutes. The analysis was performed using 200 µL of the final organic solvent (MeOH) extracts. The LOD for all drugs was measured at 100 ppt from a 1 mL sample volume. Several analytes showed excellent signal at 10 ppt.

EXPERIMENTAL

Two MRM transitions (quantification and confirmation) for all illicit drugs were selected and optimized. The MRM conditions are listed in Table 1. For this application, finding the optimum extraction and chromatographic condition for this multi-residue analysis poses a difficult challenge.

The chromatographic conditions were tested on several trapping chemistries (Oasis® HLB, XBridge® C_{18}, and XBridge C_8) and separation chemistries (ACQUITY UPLC BEH C_{18} and HSS T3). The loading (low pH, high pH and neutral pH) and eluting mobile phase (MeOH + 0.5% formic acid and ACN + 0.5% formic acid) were also optimized using an automated process.

The extraction process was performed using a reversed-phase/ion-exchange sorbent with a 3 cc Oasis MCX SPE barrel. The sorbent was conditioned by using 5 mL of methanol followed by 5 mL of water. The urine samples (1 mL) were extracted with 1 mL acetonitrile to precipitate residual protein material. The mix was then centrifuged at 4000 rpm for 5 min. The supernatant was diluted with 48 mL of Millipore water and the entire volume was loaded at a flow rate of 10 mL/min. The cartridge was washed with 5 mL water with 2% formic acid, followed by 5 mL of methanol. The illicit drugs were eluted with 1 mL of 100% methanol with 2% ammonium hydroxide.

Chromatography and MS/MS conditions

Loading conditions

Column:	Oasis HLB 20 µm
Loading:	MilliQ water (pH 7, no additives)
Flow rate:	2 mL/min
At-column dilution:	5% (0.1 mL/min pump A and 2.0 mL/min pump B)

UPLC conditions

UPLC system:	ACQUITY UPLC 2D configured for "Trap and Elute" with At-Column Dilution
Runtime:	10 min
Column:	ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm, 1.7 µm
Column temp.:	60 °C
Mobile phase A:	Water + 0.5% formic acid
Mobile phase B:	Acetonitrile + 0.5% formic acid
Elution:	5 minute linear gradient from 5% (B) to 95% (B)
Flow rate:	0.500 mL/min (pump C)
Injection volume:	200 µL

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI positive
Capillary voltage:	3.0 kV
Cone voltage:	30.0 V
Source temp.:	150 °C
Desolvation temp.:	550 °C
Desolvation gas:	1100 L/hr
Cone gas:	50 L/hr

Illicit drugs	Ion mode	Precursor ion	Cone	Product ion	CE
Cocaine	ESI +	304.2	30	105.0	40
				182.1	25
Lidocaine	ESI +	235.2	30	86.1	25
				NA	
THC	ESI +	315.2	30	135.1	25
				193.1	25
Heroin	ESI +	370.1	30	211.1	45
				328.2	45
Mescaline	ESI +	212.1	30	180.0	25
				195.0	15
THC Hydroxy	ESI +	331.2	30	193.1	30
				313.2	20
MDMA	ESI +	194.1	30	105.0	30
				163.0	10
Amphetamine	ESI +	136.1	30	91.0	20
				119.1	10
Mephedrone	ESI +	178.1	30	145.0	25
				160.0	15
5-APB	ESI +	176.1	30	131.0	25
				159.0	10
15I-NBOMe	ESI +	428.1	30	91.0	60
				121.0	30
LSD	ESI +	324.1	30	223.1	30
				281.2	25

Table 1. MRM transitions for illicits drugs.

RESULTS AND DISCUSSION

This application demonstrated the disruptive nature of the ACQUITY UPLC with 2D Technology with the Xevo TQD Mass Spectrometer for the analysis of illicit drugs in urine. The at-column dilution 2D configuration is a three pump variant using two quaternary and one binary pump. The chromatography performance utilizing at-column dilution is shown in Figure 1 for two illicit drugs (cocaine and amphetamine) between aqueous and organic extracts. The bottom chromatograms are aqueous extracts and clearly show lower signal when compared to the methanol and acetonitrile extracts. At trace level concentration (<ppb), a target analyte dissolved in an aqueous matrix will interact by ion-exchange with silanols in glass vials. When the same analyte is dissolved in an organic solvent (methanol, acetonitrile or acetone), ion-exchange is disrupted, resulting in a drastic signal increase.

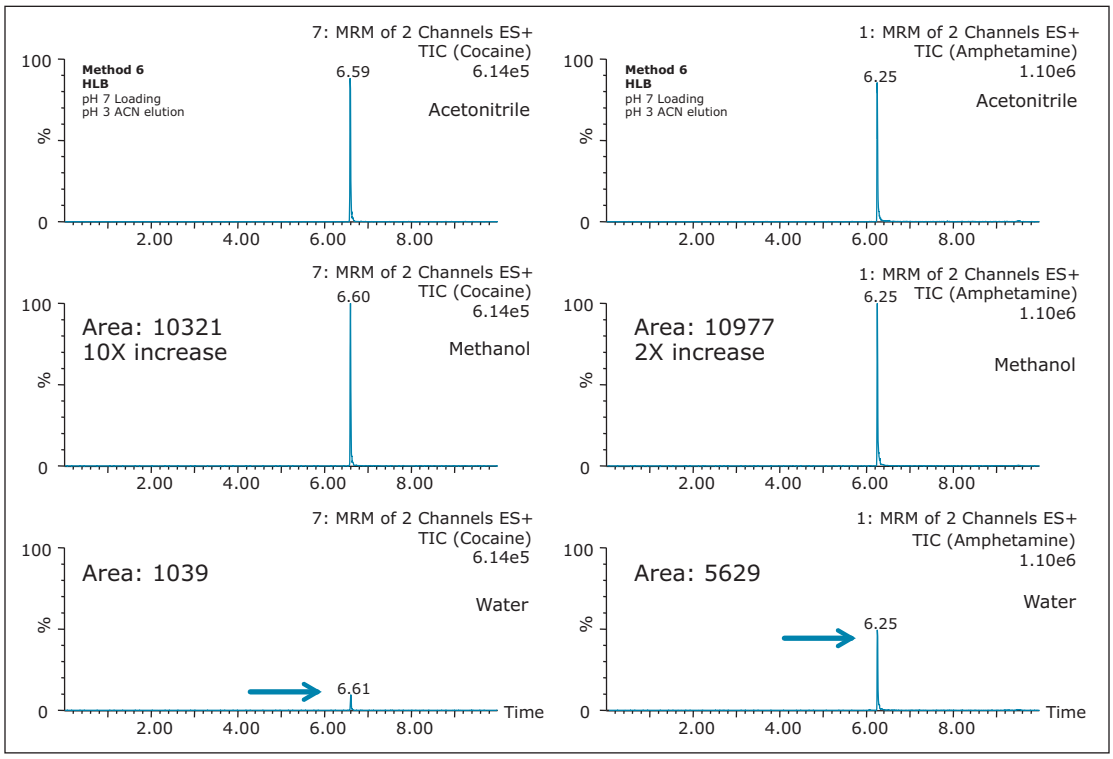

Figure 1.Aqueous vs organic extracts using at-column dilution.

The trap and elute 2D configuration is usually constructed with two circuits. The first one is to load the analyte of interest onto a trap material and the second circuit is used for backflush elution onto a high resolution analytical column. This configuration is limited to aqueous samples only. In recent development, adding a third stream (3 pump variant) has several advantages over the two stream design.[1,2] The optimization process for multi-dimensional chromatography instruments is a crucial process and can be time consuming to find optimum elution conditions. With 2D platforms, there are four key parameters to optimize that include (loading pH, trapping strength, elution strength and separation selectivity. Those parameters are split between the trap and the analytical column. For efficient trapping conditions, the loading pH, flow rate and retention chemistry are essential for ensuring a tight and narrow injection band on the trap sorbent. After the loading phase is completed, the target analytes are back flush eluted onto an analytical column for high resolution separation. For optimum separation, the usual chromatography parameters (flow rate, solvent, pH, and chemistry) are also optimized for peak performance. As such, finding the best chromatographic conditions can be quite difficult. By selecting the most common elution (Si-C_4, Si-C_8, Si-C_{18}, polymer, ion-exchange... etc.) , polarity (MeOH, ACN, acetone... etc.), dilution ratio (low vs. high) and pH (low, neutral and high) well over 400 permutations can be selected. A rational approach to select the most common parameters can be autonomously screened during an overnight run (18 hours). The At-column dilution ratio is calculated from the flow rate of the loader and dilutor pump. This ratio is crucial and if not set properly can lead to peak distortion. In most instances a dilution ratio set at 5% is an excellent starting point for most analytes in methanol, acetonitrile or acetone extract (decreasing polarity value). With a dilution ratio set at 5%, the analyte (cocaine) produce a Gaussian peak shape when dissolved in methanol and acetonitrile. However, with a less polar solvent like acetone, the analyte shows signs of peak distortion (see Figure 2). The distortion can be corrected by decreasing the dilution ratio. When set at 2.5%, all three extracts show excellent peak shape. With a parallel 2D configuration, the loading phase is done on the first trap, while the second trap loaded from the previous injection is eluted toward the analytical column.

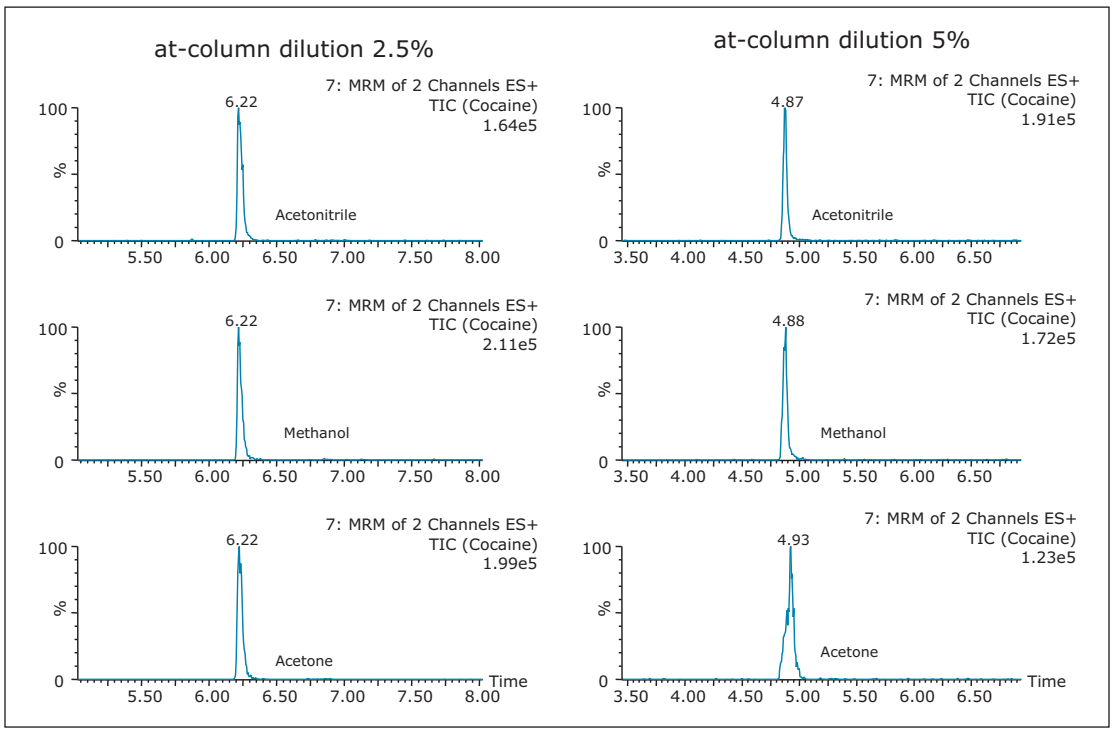

Figure 2. At-column dilution ratio: 5% vs 2.5%.

Method Development

Once the dilution ratio is selected, the next phase optimization of the chromatography (trapping and separation). As mentioned earlier, the task can be difficult to manage because of the huge amount of permutations. As a starting point, the 3 x 3 grid is designed to evaluate the effect of pH loading vs the trap retention strength while keeping the elution condition at a fixed value. By selecting three pH values (3, 7, and 10) and trap chemistries (C_8, C_{18} and HLB), a 3 x 3 grid will yield 9 permutations. With a 10 minutes run time, the results are tabulated in 4.5 hours (See Figure 3). The pH and retention strength for the trap are the primary parameters for efficient retention. By adding a low and high pH elution, a 3 x 6 grid gives 18 permutations (9 hours). The elution polarity is also a key elution parameter which increases the grid to a 6 x 6 with 36 permutations and can be completed in 18 hours. For representation purpose, a color code was selected to create a database between methods and end results. As seen in Figures 4 and 5, the end results for the 36 permutations are tabulated for each illicit drugs.

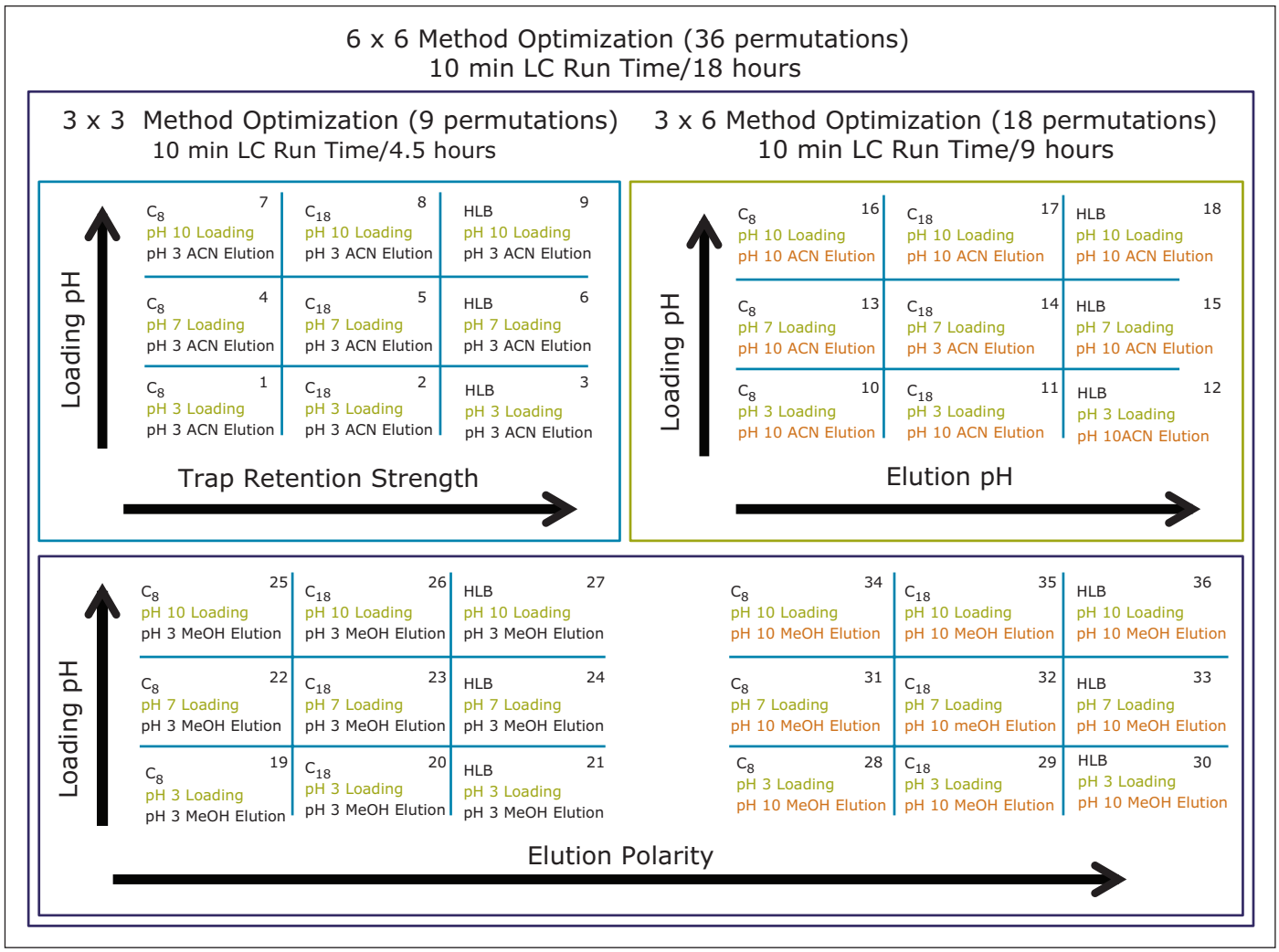

Figure 3. 6 x 6 Method optimization with 36 permutations.

Figure 4. Results for method 1 to 18. The "Hi" value reflects to the highest signal response.

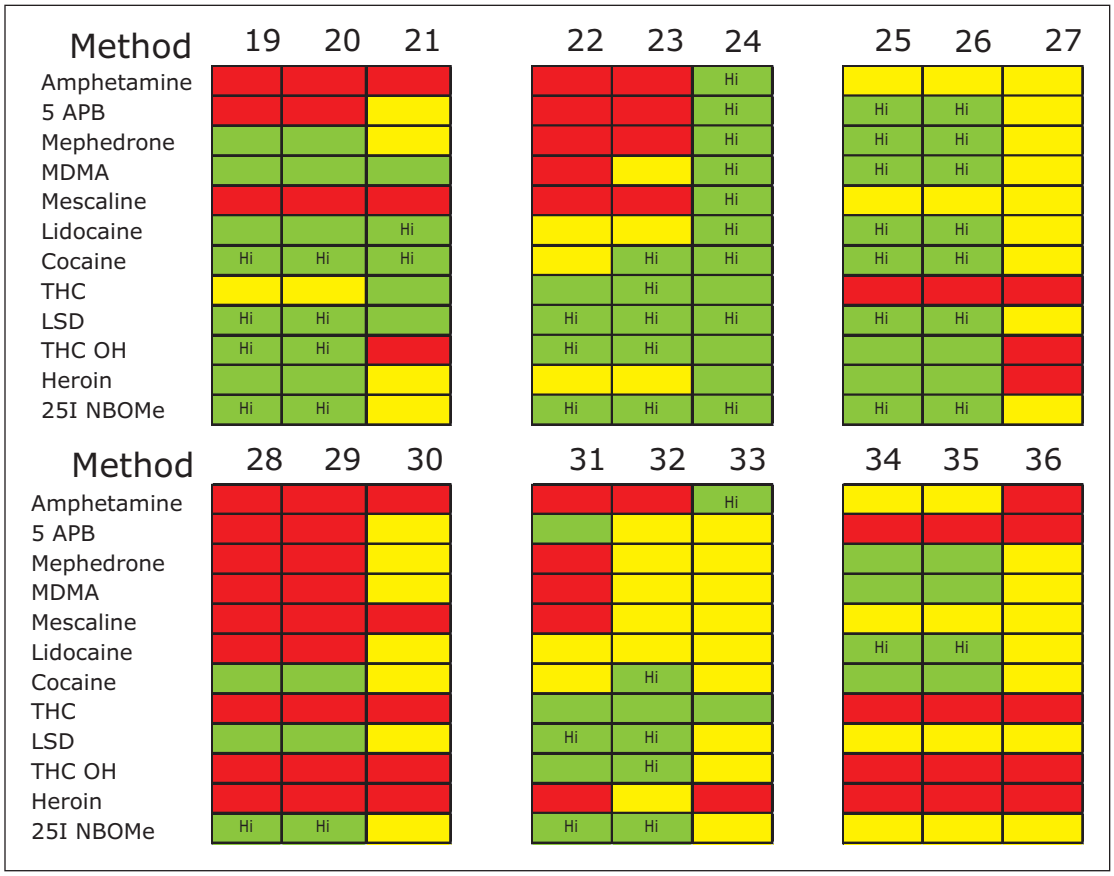

Figure 5. Results for method 19 to 36. The "Hi" value reflects to the highest signal response.

The yellow and red results are also crucial data points as they can give a clear insight as to the target analyte retention behavior during the extraction process. With the at-column dilution option, aqueous and organic extracts can be utilized to give additional information regarding an analyte's solubility. Overall, as more conditions are optimized, the elution behavior of an analyte becomes clearer. As a result, the illicit drugs were split into two methods (see Figure 6 and 7).

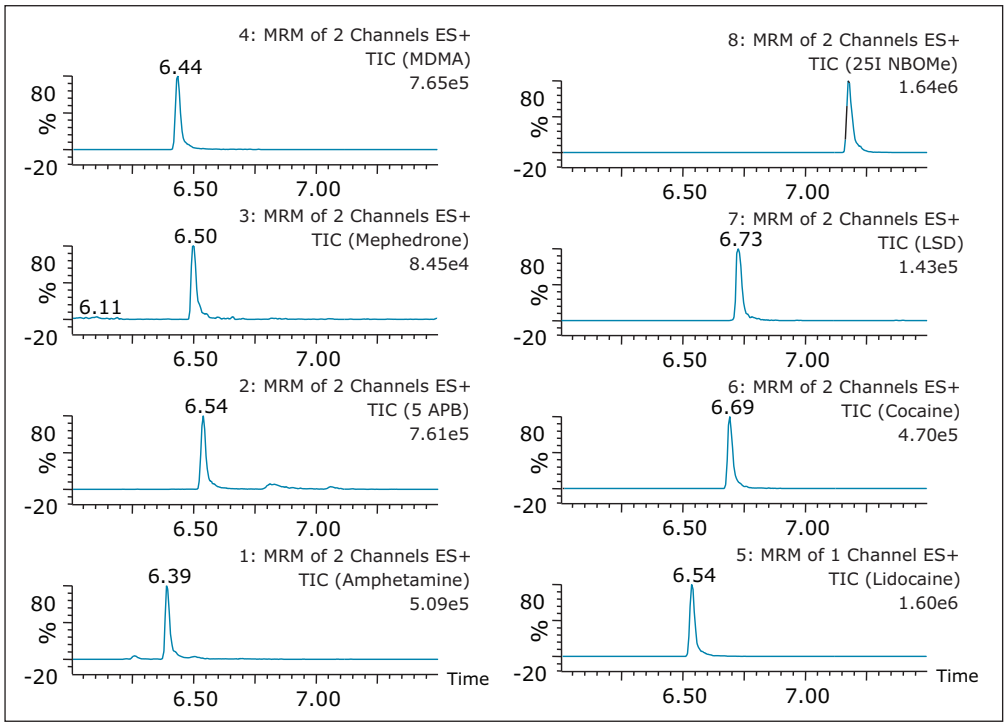

Figure 6. Chromatogram at 1 ppb with method 6: Oasis HLB in D1 and BEH C_{18} in D2, loading at neutral pH and elution with acetonitrile at 0.5% formic acid.

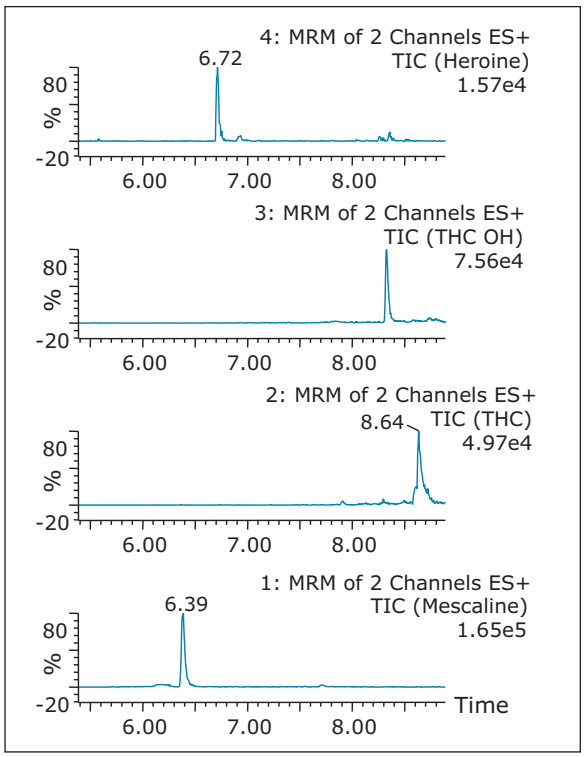

Figure 7. Chromatogram at 1 ppb with method 24: Oasis HLB in D1 and BEH C_{18} in D2, loading at neutral pH and elution with methanol at 0.5% formic acid.

Extraction and quantification

After the optimization process, the work will concentrate on the extraction process. This application targets illicit drugs in urine. With At-column dilution enabled, the extraction optimization can be streamlined by using a micro extraction protocol. As a starting point, the workflow will start with the reversed-phase protocol listed in Figure 8. The initial sample volume is limited at 1 mL using a 1 cc cartridge with a 1 mL elution volume. The elution is transferred directly (no evaporation/reconstitution) into a 2 mL vial. In this application, since the micro extraction can be completed in less than 15 minutes, the workflow can evaluate several elution profiles in a short time. In this case, the micro extraction protocol evaluated the effect of solvent polarity and pH on the recovery for the illicit drugs. The results for amphetamine with various elution conditions are depicted in Figure 9. The results were compared to an un-extracted standard and a recovery percentage was calculated for all illicit drugs (see Figure 10).

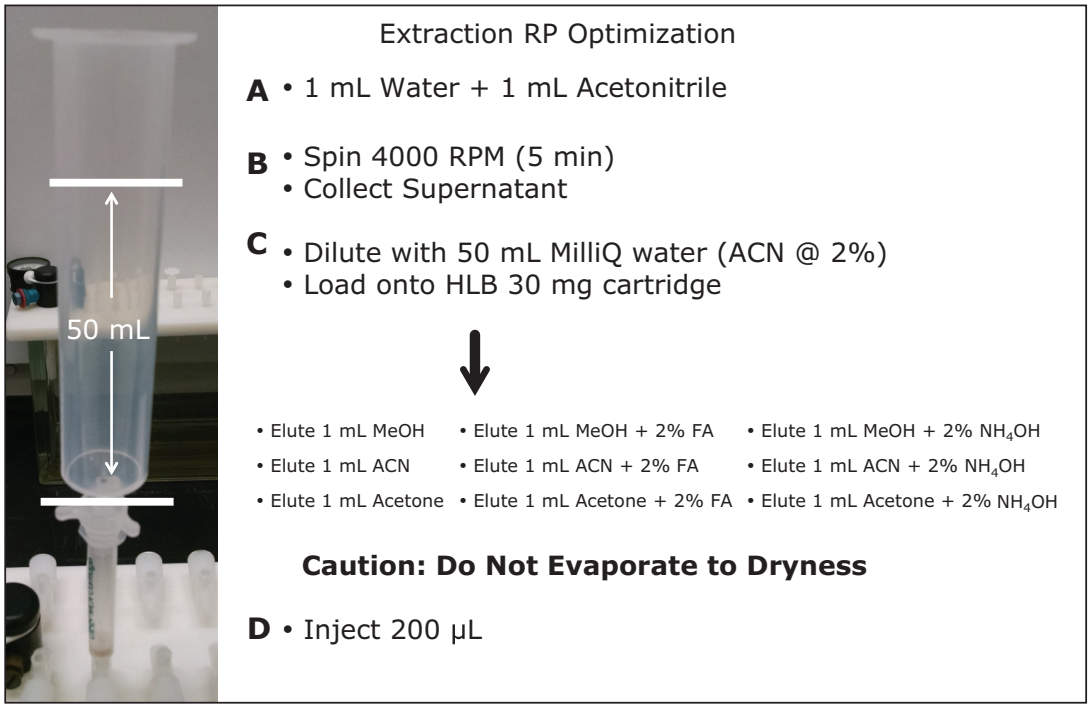

Figure 8. Optimization of the reversed-phase extraction protocol.

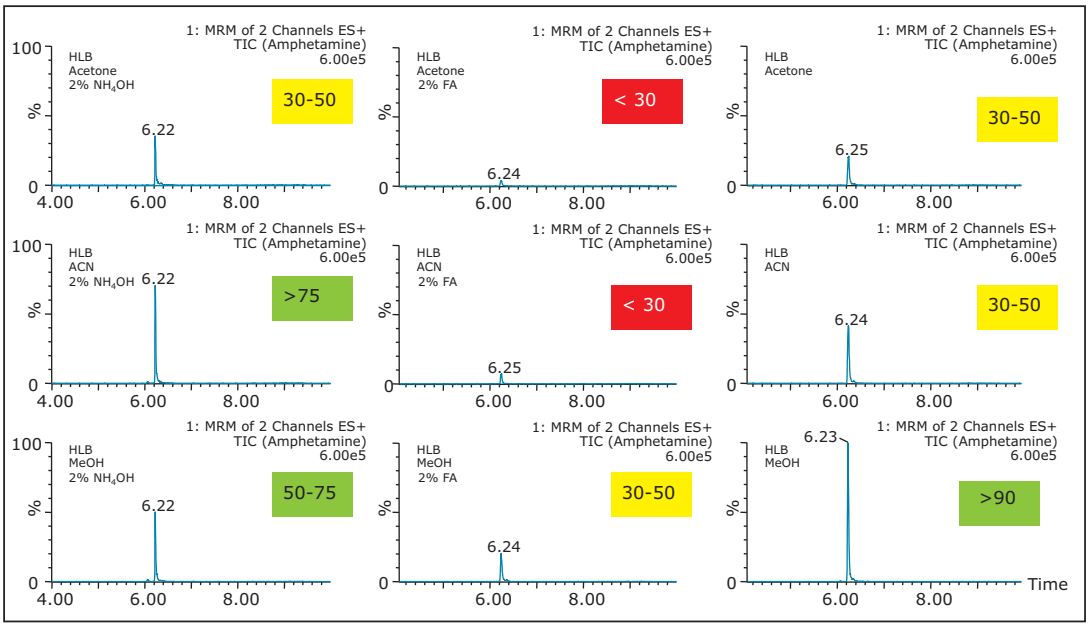

Figure 9. Results from the reversed-phase extraction optimization process.

	Water Spike @ 1 ppb								
	RP MeOH	RP MeOH Low pH	RP MeOH High pH	RP ACN	RP ACN Low pH	RP ACN High pH	RP Ace	RP Ace Low pH	RP Ace High pH
Amphetamine	>90	30-50	50-75	30-50	<30	>75	30-50	<30	30-50
5 APB	>75	>75	>90	>75	30-50	>75	>75	<30	50-75
Mephedrone	>90	>90	>75	>75	30-50	>75	50-75	<30	50-75
MDMA	>75	50-75	>90	50-75	<30	>75	30-50	<30	30-50
Mescaline	30-50	50-75	50-75	>90	30-50	30-50	50-75	<30	30-50
Lidocaine	>75	>75	>90	>75	30-50	>75	50-75	<30	50-75
Cocaine	>90	>90	>75	>90	50-75	50-75	>75	30-50	30-50
THC	50-75	50-75	50-75	>90	>90	50-75	>90	>90	>90
LSD	>75	>90	>75	>75	>75	>75	>75	>75	50-75
THC	50-75	50-75	50-75	>90	>90	>75	>90	>90	>90
THC OH	>90	>75	50-75	50-75	50-75	50-75	50-75	50-75	50-75
Heroin	50-75	30-50	<30	>90	30-50	<30	50-75	<30	30-50
25I NBOMe	>90	>90	30-50	>75	>75	30-50	>75	>75	50-75

Figure 10. Recovery calculations for reversed-phase extraction protocols.

From the recovery results, two elution conditions were selected for first round of extraction with urine sample. In this instance, a reversed-phase sorbent was used for the extraction and eluted with 1 mL of methanol (first set) and 1 mL acetonitrile (second set). From the aqueous screening results, a urine sample was tested with two elution conditions (see Figure 11). The acetonitrile added to the urine sample was use to precipitate all remaining protein material. As seen in Figure 11, a 1:1 ratio urine/acetonitrile created a clear supernatant. After centrifugation, the supernatant is simply diluted to 50 mL, thus reducing the percentage of acetonitrile to 1%. At that organic percentage, the illicit drugs are not at risk of breakthrough. In radar mode, a full scan data and multiple reaction monitoring channels (MRM's) can be acquired during the same run. This feature gives a unique insight to the quality of the extraction procedure and showcases the amount of potential interferences (scan mode) versus a target analyte signal to noise ratio in MRM mode.

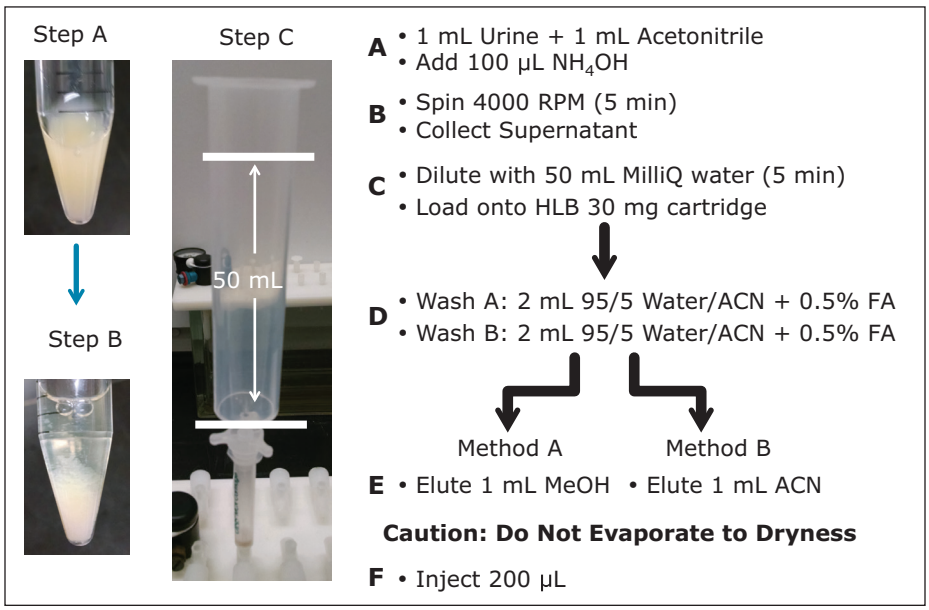

Figure 11. Optimized reversed-phase extraction protocol for urine matrix.

As to be expected with a generic SPE method, a urine sample shows a higher level of interferences that an LC-MS/MS grade water (used as a reference for this application) (Figure 12). The recovery results (Figure 13) demonstrate an interference closely eluting to the target analyte. Overall, the 3 target analytes were un-detected using a reversed-phase extraction protocol when using methanol as well as acetonitrile (see Table 2). The recovery values with acetonitrile for 5 target analytes are below the acceptable range of 70% – 120%.

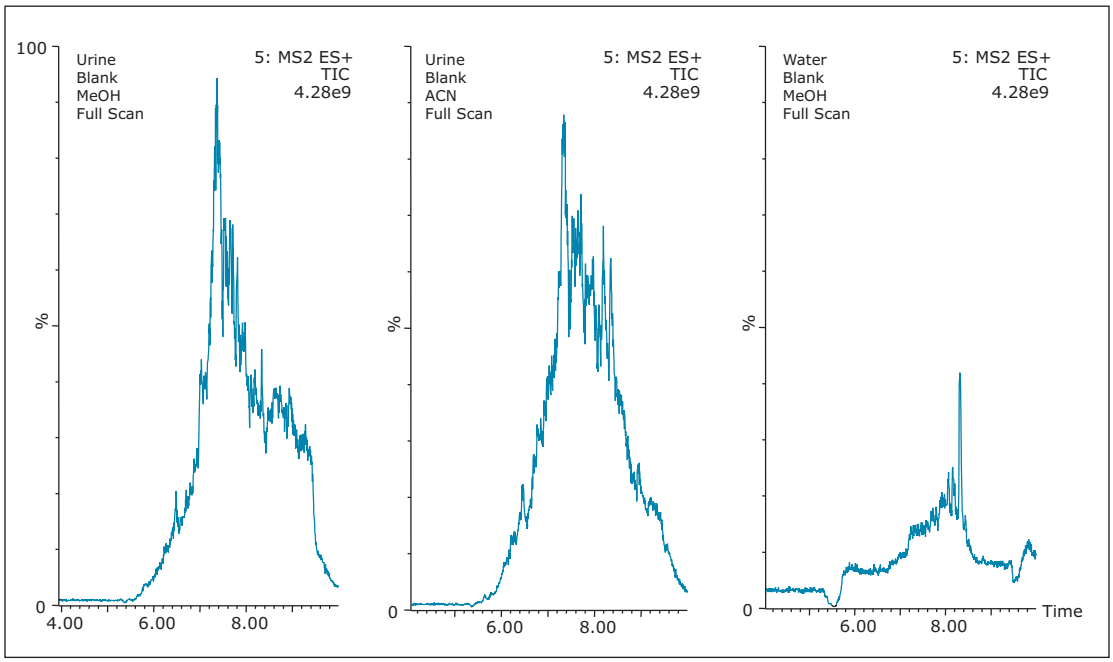

Figure 12. Scan MS data for MeOH and ACN urine reversed-phase extracts.

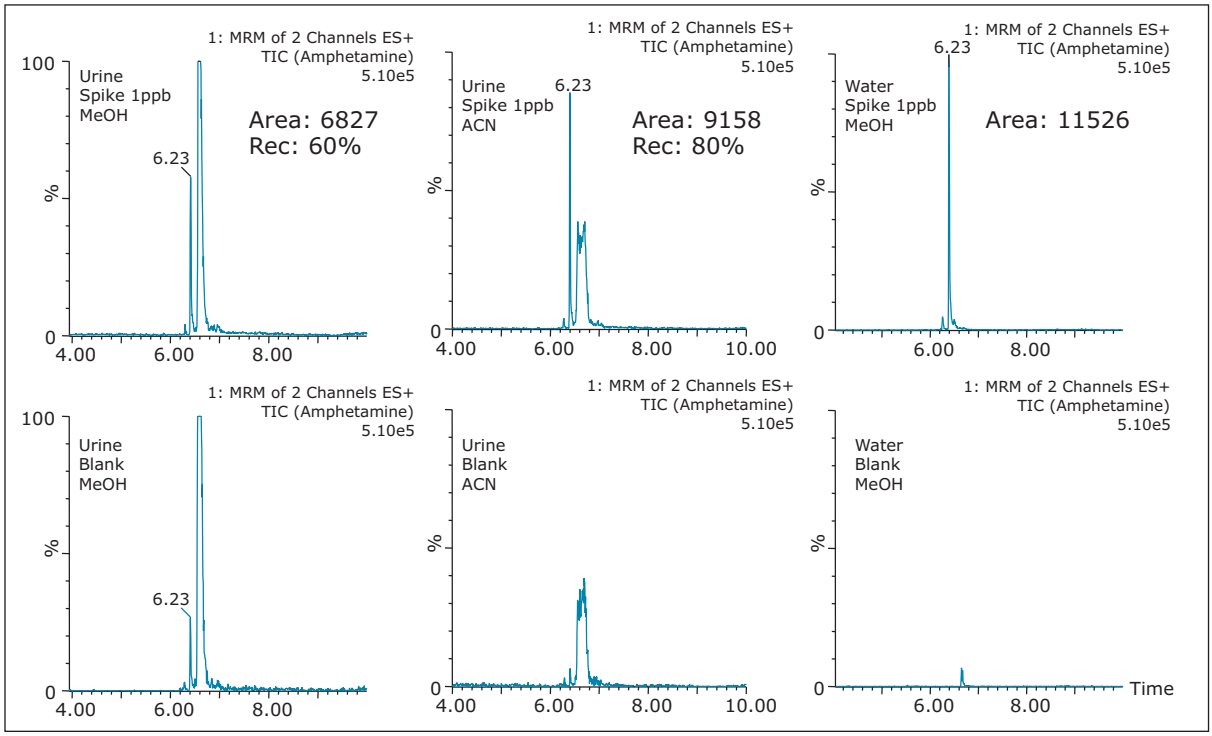

Figure 13. MRM data for MeOH and ACN urine reversed-phase extracts.

	Urine Spike @ 1 ppb with RP 1D				
	Water MeOH	Urine MeOH	REC	Urine ACN	REC
Amphetamine	11526	6827	60%	9158	80%
5 APB	15631	2732	17%	NA	
Mephedrone	1608	NA		827	51%
MDMA	19258	NA		16586	86%
Mescaline	3226	3228	101%	2957	92%
Lidocaine	20461	5396	27%	13240	65%
Cocaine	7354	2950	40%	4328	59%
THC	2002	NA		NA	
LSD	4139	2230	45%	5190	104%
THC OH	4974	2230	45%	5190	104%
Heroin	255	315	123%	NA	
25I NBOMe	45799	3961	9%	23013	50%

Table 2. Recovery percentage with MeOH and ACN for the optimized reversed-phase protocol.

In order to reach better performance, the extraction protocol efficiency will need to be upgraded with a dual reversed-phase/ion-exchange protocol (see Figure 14). In this instance, since the elution with acetonitrile gave better results than methanol, the RP/IE protocol was evaluated with acetonitrile only. The stronger washes of the RP/IE protocol removed more interferences than the RP 1D and RP 2D protocols (Figure 15). The chromatograms in Figure 16 shows the trace signal for amphetamine using the RP 1D, RP 2D and RP/IE, respectively. The results in Table 3 (Getting this from the author) shows the RP/IE protocol producing excellent results for the entire mix of illicit drugs and also capturing un-detected analytes with the RP 1D protocol.

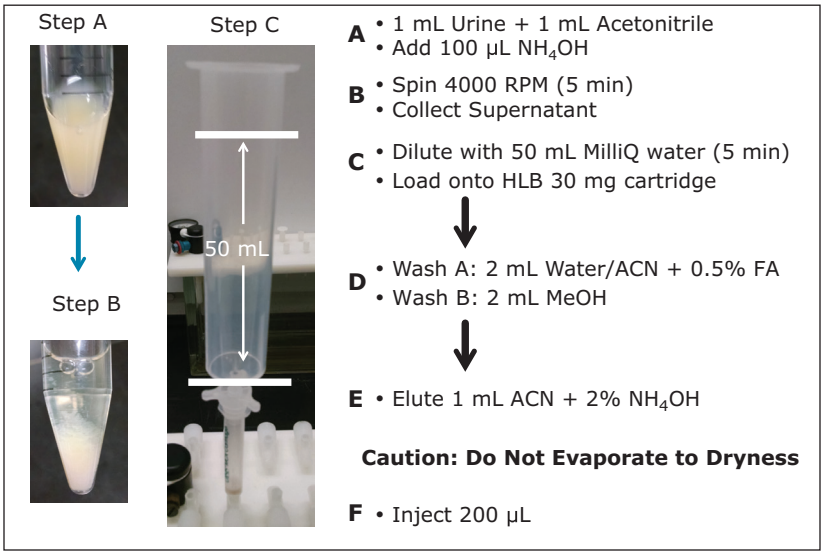

Figure 14. Optimized reversed-phase/Ion-exchange extraction protocol for urine matrix.

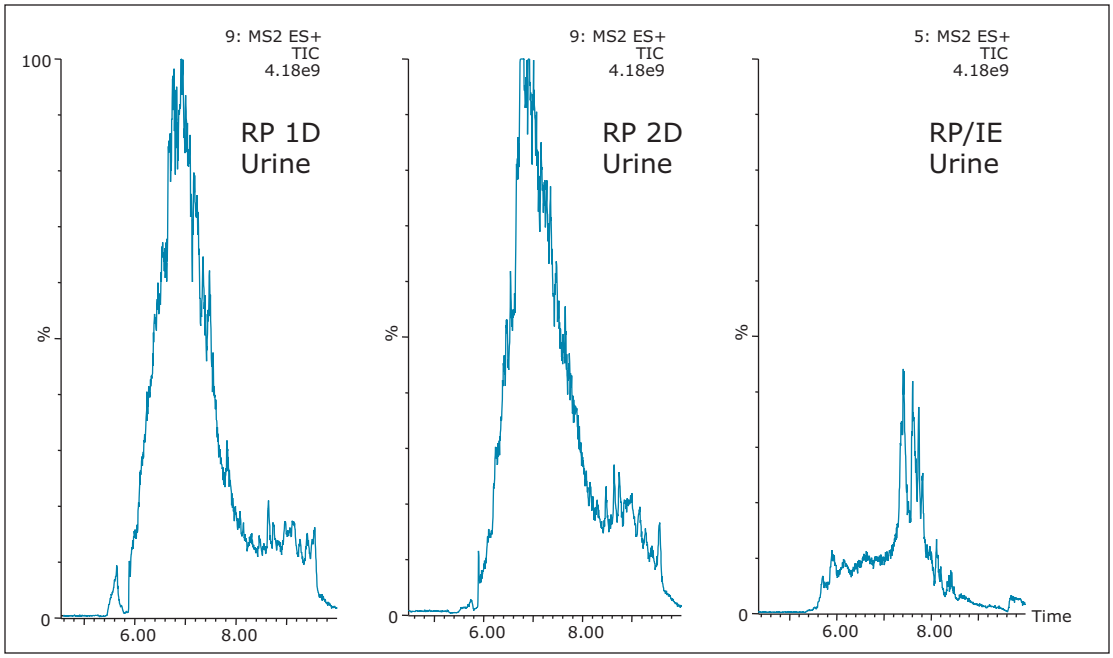

Figure 15. Scan MS data for MeOH and ACN urine reversed-phase/ion-exchange extracts.

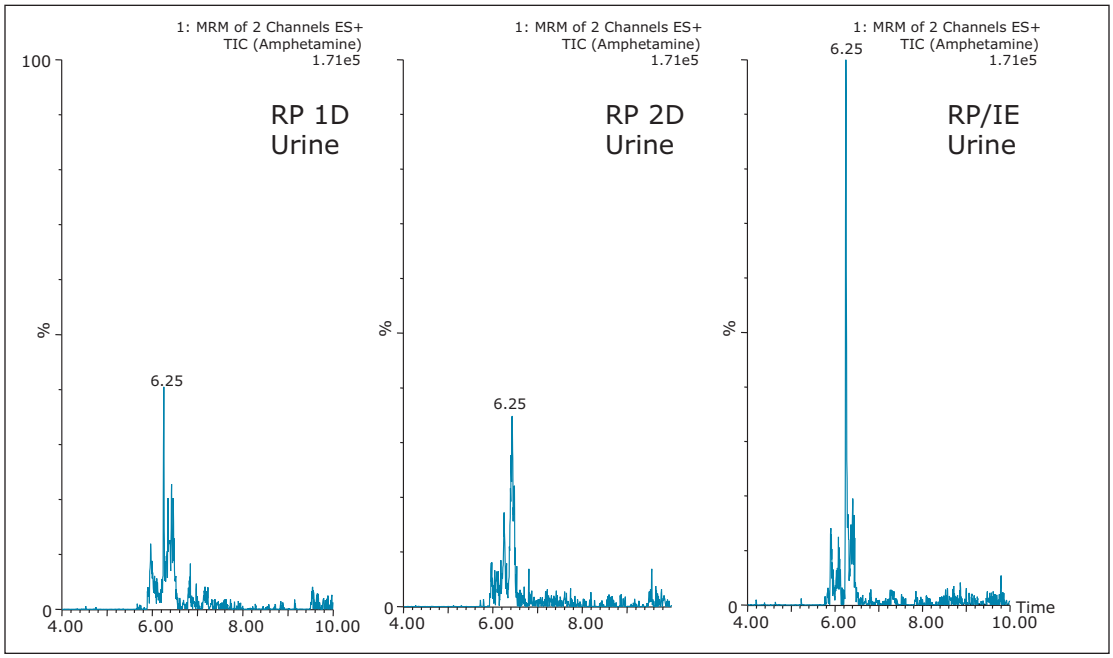

Figure 16. MRM data for MeOH and ACN urine reversed-phase/ion-exchange extracts.

	Urine Spike @ 1 ppb with RP and RP/IE				
	REC MeOH RP 1D	REC ACN RP 1D	Water MeOH RP/IE	Urine MeOH RP/IE	REC MeOH RP/IE
Amphetamine	60%	80%	4577	4400	96%
5 APB	17%	ND	3358	3079	91%
Mephedrone	ND	51%	2335	1784	76%
MDMA	ND	86%	5142	4729	92%
Mescaline	101%	92%	8657	8350	96%
Lidocaine	27%	65%	31753	26787	84%
Cocaine	40%	59%	8040	7095	88%
THC	ND	ND	4989	3874	79%
LSD	30%	83%	3103	2867	92%
THC OH	45%	104%	3387	3166	93%
Heroin	123%	ND	487	409	84%
25I NBOMe	9%	50%	5228	4925	94%

Table 3. Recovery Percentage with MeOH and ACN for the Optimized Reversed-phase/Ion-exchange Protocol.

CONCLUSIONS

This application demonstrated the automated and fast method development capability of the ACQUITY UPLC System with 2D Technology for the analysis of illicit drugs in urine. The quantification limit for all illicit drugs was set at 1 ppb using a 1 mL urine sample. The micro extraction protocol offered the option to evaluate several elution parameters in a short time period. The elution optimization was completed within a 4 hrs hands-on work and the 2D LC results were analyzed using an over-night run multi-methods sample list (18 hrs). With the extraction protocol optimized, the final protocol produced a clean extract in 15 minutes without any evaporation to dryness and reconstitution into initial mobile phase conditions. The reversed-phase/ion-exchange extraction protocol gave a 90% recovery average for the 12 illicit drugs in urine.

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2. Mallet, C.R, Multi-Dimensional Chromatography Compendium: Trap and Elute vs AT-column dilution, 720005339EN 2015.

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Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com