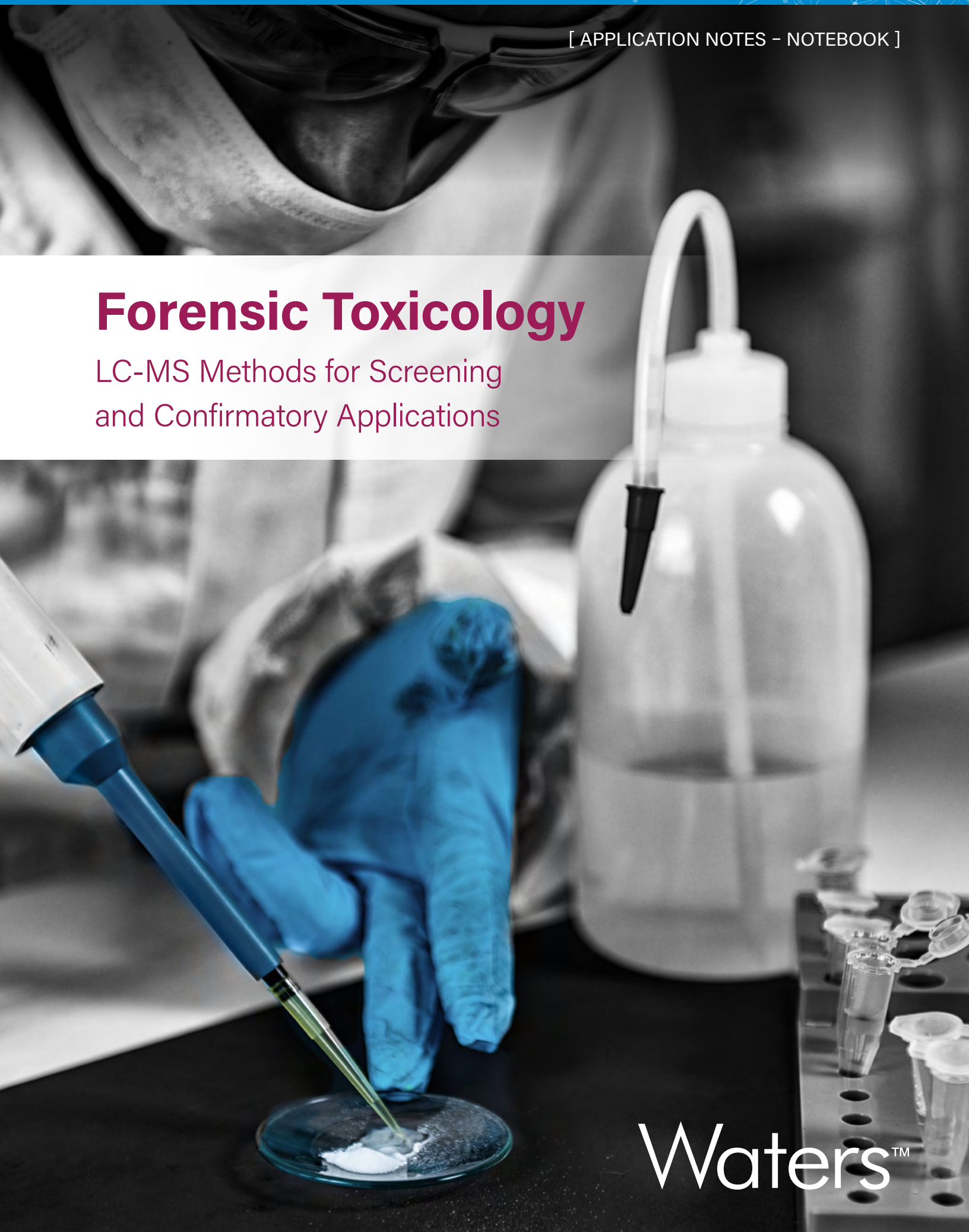

Forensic Toxicology

LC-MS Methods for Screening and Confirmatory Applications

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Application Note

Analysis of Phosphatidylethanol (PEth) in Whole Blood using SPE and UPLC-MS/MS for Forensic Toxicology

Jonathan P. Danaceau, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

Phosphatidylethanol (PEth) species are direct biomarkers of alcohol consumption that have attracted increasing interest recently due to their selectivity and relatively long half-life compared to other alcohol biomarkers. Waters has developed a rapid, clean, and robust method for the extraction of PEth 16:0/18:1 and 16:0/18:2 from whole blood that features Ostro Pass-Through Sample Preparation plates, an ACQUITY UPLC I-Class PLUS System and the Xevo TQ-S micro to achieve the performance and analytical sensitivity necessary for this method.

Benefits

- Rapid, simple, and clean extraction of PEth from whole blood
- Analytical sensitivity down to 10 ng/mL (0.014 µM)
- Rapid chromatography with no carryover
- Accurate and precise quantification across the analytical range

Introduction

Phosphatidylethanol (PEth) species are abnormal phospholipids formed in cell membranes in the presence of ethanol. More than 40 homologs have been reported. However the most abundant PEth species in whole blood are 16:0/18:1 and 16:0/18:2, accounting for approximately 37% and 25% of total PEths, respectively.[1] PEth is a direct marker of even moderate alcohol consumption and appears unaffected by factors such as age, gender, and liver disease. PEth can also be used to distinguish drinking patterns and behaviors, e.g., to identify moderate or excessive drinkers, as well as binge drinking. PEths have become popular markers for ethanol use recently due to their relatively long window of detection of up to 3–4 weeks vs 3–4 days for ethyl glucuronide (EtG) and ethyl sulfate (EtS) as monitored in urine. PEth also has a high degree of selectivity compared to other biomarkers and compounds such as EtG and EtS.[2-5] Baseline PEth concentrations are usually <10 ng/mL (0.014 µM) in abstinent individuals and cutoffs of 0.05 µM have been suggested to indicate moderate drinking behaviors.[3] Concentrations above 0.3 µM (210 ng/mL) have been suggested to indicate heavy or binge drinking.[2] However, the extraction and analysis of PEth from whole blood poses some unique analytical challenges due to the extreme hydrophobicity and unique chemical nature of these molecules.

This work details a solution for the quantitative analysis of PEth that uses solid phase extraction (SPE), followed by a rapid UPLC-MS/MS method for the analysis of PEth 16:0/18:1 and PEth 16:0/18:2 from whole blood. Ostro Pass-Through Sample Preparation plates were used in a simple 2-step process (load and elute). Method optimization revealed unique, multi-modal retention characteristics that appeared to have both reversed-phase and HILIC properties. Elution on a BEH C_8 Column combined with a strong mobile phase composed of 50:50 ACN:IPA resulted in a rapid method with no detectable carryover.

Experimental

Materials

PEth 16:0/18:1, PEth 16:0/18:2, and the deuterated analogue PEth 16:0/18:1-D_5 were obtained from Cerilliant (Round Rock, TX). PEth 16:0/18:1-D_5 was used as an internal standard for both molecules. Whole blood was obtained from Lampire Biological Products (Pipersville, PA).

Methods

One hundred microliters of whole blood was precipitated in two stages. First, 200 µL of isopropanol (IPA) containing 50 ng/mL deuterated internal standard was added and vortex-mixed for 5-10 seconds to fully mix the sample. Next, 800 µL of ACN containing 0.1% formic acid (FA) was added immediately, and the samples were once again vortexed. The samples were then centrifuged at 21K rcf, for 10 min, and the supernatant was loaded directly onto Waters Ostro Pass-Through Sample Preparation plates. Samples were eluted with 2 x 400 µL aliquots of 60:20:20 ACN:IPA:water. Twenty microliters were analysed using an ACQUITY UPLC I-Class PLUS System (FTN) in combination with a Xevo TQ-S micro Tandem Quadrupole MS.

Analytes were chromatographically separated on a 1.7 µm Waters BEH C_8 Column (2.1 x 50 mm) at 40 °C. Mobile phase A (MPA) was 5 mM ammonium formate with 0.1% formic acid; mobile phase B consisted of 50:50 ACN:IPA. The flow rate was 0.5 mL/min. The solvent ramp started at 50:50 MPA:MPB and increased to 100% MPB over three minutes. Two rapid 30 second ramps from 50:50 MPA:MPB to 100% MPB were added after the analytical run to minimize carryover. Samples were analyzed in negative ESI mode. Two transitions were monitored for each compound. MS parameters are listed in Table 1. Calibration curves ranged from 10–1000 ng/mL (0.014–1.4 µM). The method was validated for extraction recovery, matrix effects, linearity, accuracy, precision, analytical sensitivity, carryover, dilution integrity, and extracted sample stability.

Analyte	[M-H]⁻	MRM	Cone voltage (V)	Collision energy (eV)
PEth 16:0/18:1	701.6	255.2	10	30
		281.3	10	35
PEth 16:0/18:2	699.6	255.3	10	30
		279.3	10	35
PEth 16:0/18:1-d5	706.6	255.2	10	30
		281.3	10	35

Table 1. MS parameters.

Results and Discussion

Sample Clean-up

Method optimization revealed a possible multimodal retention mechanism for PEth on the Ostro sorbent. There appeared to be some HILIC character, as a substantial proportion of water (20%) and another protic solvent (MeOH or IPA) was necessary to elute PEth off the sorbent. At the same time, strong reversed-phase elution solvents were also required due to PEth's high lipophilicity. The use of IPA vs. methanol as a third co-solvent resulted in increased and more consistent recoveries. The final elution solvent after optimization was 60:20:20 ACN:Water:IPA.

This extraction method results in high recoveries with minimal ion suppression and readily meets the analytical sensitivity requirements of the method. The extraction method was simple, efficient, and clean. PEth 16:0/18:2 and PEth 16:0/18:1 had recoveries that averaged 88% and 79%, respectively. The extraction was reproducible with all %RSDs <10%. Matrix effects were minimal at <13% for both molecules. Results for recovery and matrix effects can be seen in Table 2.

Within batch results (N=6)						
	Recovery				Matrix effects	
Analyte	Mean	S.D.	%RSD		Mean	S.D.
PEth 16:0/18:1	75.1%	6.9%	9.3%		-12.4%	4.6%
PEth 16:0/18:2	89.6%	2.7%	3.1%		-9.1%	5.7%

Within batch results (N=6)						
	Recovery				Matrix effects	
Analyte	Mean	S.D.	%RSD		Mean	S.D.
PEth 16:0/18:1	79.0%	6.1%	7.7%		-7.6%	4.4%
PEth 16:0/18:2	87.8 %	3.0%	3.4%		-6.6%	2.2%

Table 2. Recovery and matrix effects.

Chromatography

The UPLC-MS/MS method was optimized to balance the required retention and selectivity of the method while minimizing carryover. This was achieved by using the C_8 Column with a strong mobile phase B consisting of 50:50 ACN:IPA. Initial method development indicated that a C_{18} Column was too retentive and mobile phase consisting of ACN only was not strong enough to adequately elute PEth and prevent carryover. In addition to the mobile phase optimization, a rapid "sawtooth" gradient was used after the analytical ramp to further minimize the potential for carryover. Using the conditions described here, PEth 16:0/18:1 and PEth 16:0/18:2 were baseline separated from one another, and from other interfering substances in whole blood. This can be seen in Figure 1 by the broad peaks that elute after PEth 16:0/18:1 and PEth 16:0/18:2. Retention times for PEth 16:0/18:2 and 16:0/18:1 were 2.54 and 2.64 min, respectively. No detectable carryover was seen even after injection of the high standard (1000 ng/mL).

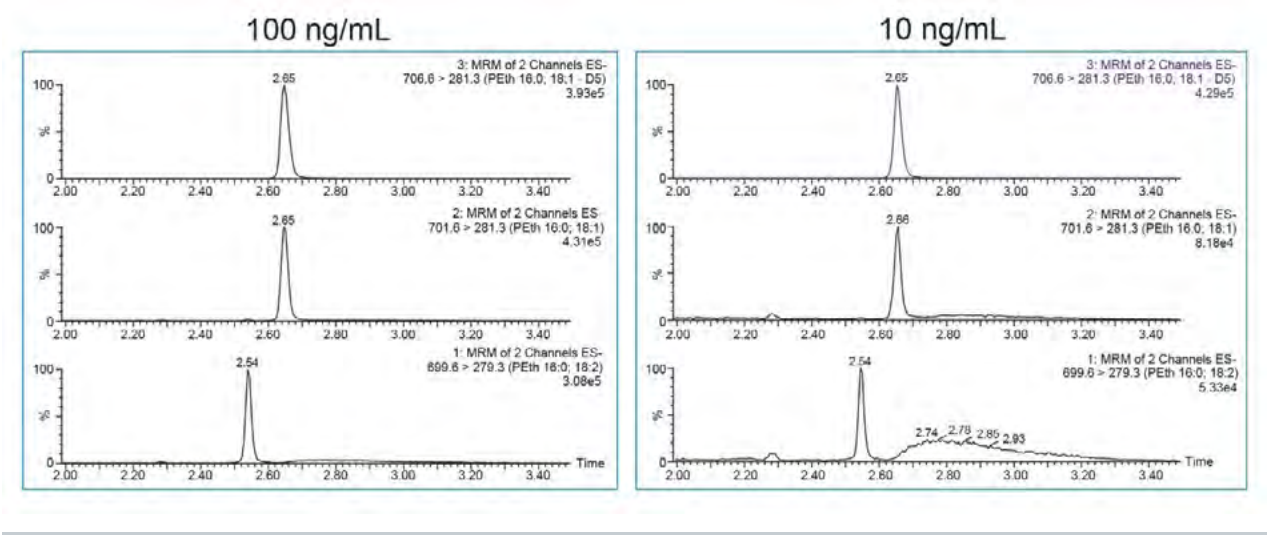

Figure 1. PEth chromatography.

Quantitative Validation

The analytical sensitivity of the method was more than adequate, with limits of quantification of 10 ng/mL (0.014 mM) for both molecules, easily meeting method requirements and limits of detection reported in the literature.[2,3] Although there was a small existing contribution of PEth in the blood matrix (~5 ng/mL), method validation revealed that the method could readily distinguish and accurately quantify the addition of 10 ng/mL at the lowest calibration level. The method was linear from 10 to 1000 ng/mL (0.014–1.4 µM). Calibration curves for PEth 16:0/18:1 and PEth 16:0/18:2 are shown in Figure 2.

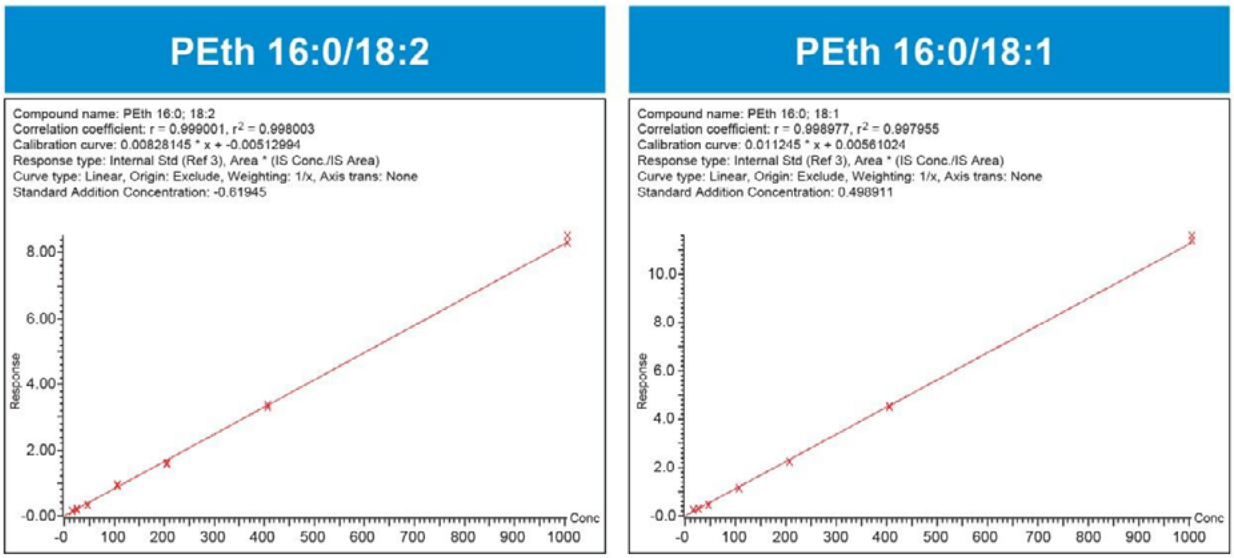

Figure 2. PEth calibration curves.

Accuracy and Precision

Accuracy and precision results are shown in Table 3. All accuracies with within 15% of target values and most were under 10%. All %RSDs were under 13% with most under 10%. The limit of quantitation was defined as the lowest calibrator (10 ng/mL) and confirmed during validation. Accuracy was within 15% and %RSD was 5.1%.

	Low QC 75 ng/mL		Mid QC 250 ng/mL		High QC 750 ng/mL	
	Mean	%RSD	Mean	%RSD	Mean	%RSD
PEth 16:0/18:1	85.8%	4.1%	91.9%	3.5%	94.0%	10.4%
PEth 16:0/18:2	86.5%	4.6%	91.4%	3.1%	95.0%	12.3%

	Low QC 75 ng/mL		Mid QC 250 ng/mL		High QC 750 ng/mL	
	Mean	%RSD	Mean	%RSD	Mean	%RSD
PEth 16:0/18:1	85.9%	4.0%	95.1%	6.6%	99.5%	1.9%
PEth 16:0/18:2	85.8%	6.9%	93.3%	8.2%	99.8%	2.7%

Table 3. Accuracy and precision.

Conclusion

We have developed a bioanalytical method for the quantitative analysis of PEth 16:0/18:1 and PEth 16:0/18:2 in whole blood. The method uses protein precipitation followed by a simple extraction using Ostro Pass-Through Sample Preparation plates. Analysis using the Waters ACQUITY UPLC System and Xevo TQ-S micro Mass Spectrometer resulted in a method that was linear, accurate and precise, had no detectable carryover, and had the analytical sensitivity necessary to easily meet the suggested cutoff levels of 0.05 µM.

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MassLynx MS Software <https://www.waters.com/513662>

720007120, January 2021

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Application Note

Automated High Throughput Sample Preparation and UPLC-MS/MS Analysis for the Quantification of EtG and EtS in Human Urine Using the Andrew+ Pipetting Robot

Sarah Dunne, Danielle Cullen

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

For forensic toxicology use only.

Abstract

This work demonstrates an automated simple dilute and shoot sample preparation method using the Andrew+ Pipetting Robot from Andrew Alliance. The Andrew+ developed method prepares the sample preparation of biomarkers ethylglucuronide (EtG) and ethylsulfate (EtS). At each protocol step during experiment execution, Andrew+ executes the OneLab instructions, setting the required parameters on the labware and Bluetooth pipettes to be used. OneLab is very accessible and straight forward to use for creating and transferring methods.

Sample identification and quantification is performed on an ACQUITY UPLC I-Class (FTN) System and Xevo TQD Mass Spectrometer using an ACQUITY UPLC CSH Phenyl-Hexyl Column (p/n: 186005408 < https://www.waters.com/nextgen/us/en/shop/columns/186005408-acquity-uplc-csh-phenyl-hexyl-column-130a-17--m-21-mm-x-150-mm-1.html>).

Benefits

- Simple dilute and shoot sample preparation method
- Automated method transferability – comparable results to manual preparation
- Higher throughput with 96-well format
- Increase in efficiencies – automated pipetting enables better utilization of analyst's time
- Reduces the opportunity for human error
- OneLab Software is easily operated for creating/modifying methods

Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are important biomarkers for monitoring alcohol use. Identification and quantification of EtG and EtS as a biomarker of ethanol use is performed for a wide range of testing purposes. Detecting these metabolites has proven beneficial as alcohol abuse is highly prevalent in many cultures and contribute considerably to the global burden of health and social issues. As a result, there is a growing need for the detection and identification of ethanol use.

EtG and EtS are superior markers of alcohol intake due to a long detection window. EtG and EtS are minor water soluble II metabolites of ethanol and are detectable in urine up to 80 hours following ethanol consumption.[1]

The sample preparation method detailed here is based upon the "dilute and shoot" format. This method involves the dilution of samples with an internal standard before injection onto an LC-MS system.

The EtG/EtS "dilute and shoot" method on Andrew+ involves the development of accurate pipetting and mixing of samples on deck. This is a straightforward workflow to perform, however pipetting can still be a tedious and repetitive task especially when running larger sample numbers. The automation of this workflow frees up analyst expertise for other tasks and requires minimal training to run. The OneLab cloud native software guides the user when running a protocol with the required materials and deck setup. Andrew+ provides reproducible and consistent pipetting with every run, reducing the risk of user error that can be seen in manual sample preparation.

Experimental

Two scripts were developed on the Andrew+ for an automated "dilute and shoot" sample preparation of EtG and EtS in human urine.

Automated preparation – Andrew+ Pipette mix

- Following centrifugation (7200 rpm for three minutes) aliquots of 50 µL of sample are added into a 2 mL 96-well collection plate (p/n: 186002482)
- Samples were diluted with 500 µL of internal standard solution and pipette mixed
- Finally the user is prompted to seal and remove samples from Andrew+

Automated preparation – Andrew+ Microplate Shaker+

- Following centrifugation (7200 rpm for three minutes) aliquots of 50 µL of sample are added into a 2 mL 96-well collection plate (p/n: 186002482)
- Samples were diluted with 500 µL of internal standard solution and mixed on shaker at 1700 rpm for two minutes
- Finally the user is prompted to seal and remove samples from Andrew+

Results and Discussion

Two protocols were developed on the Andrew+ for a "dilute and shoot" automated method. Both protocol methods transferred a set of eight quality control low aliquots and eight quality control high aliquots to the first two rows of the 2 mL collection plate (p/n: 186002482 <https://www.waters.com/nextgen/us/en/shop/vials-containers--collection-plates/186002482-96-well-sample-collection-plate-2-ml-square-well-50-pk.html>) which were diluted with water (in place of internal standard). The difference in both automated methods was the mixing of the liquid in the wells. One automated method used pipette mixing and the other automated method used Microplate Shaker+ to perform mixing. The pipette liquid handling parameters were adjusted to ensure smooth transfer of samples. During development of protocol that performs pipette mixing of samples, adjustments were made to the pipette mixing parameters. An example of this was increasing the pipette mixing speed to ensure thorough mixing. Adjustments were straightforward to change in OneLab. This optimization helped increase reproducibility and precision.

The full Andrew+ deck layout for both scripts can be seen in Figures 1 and 2.

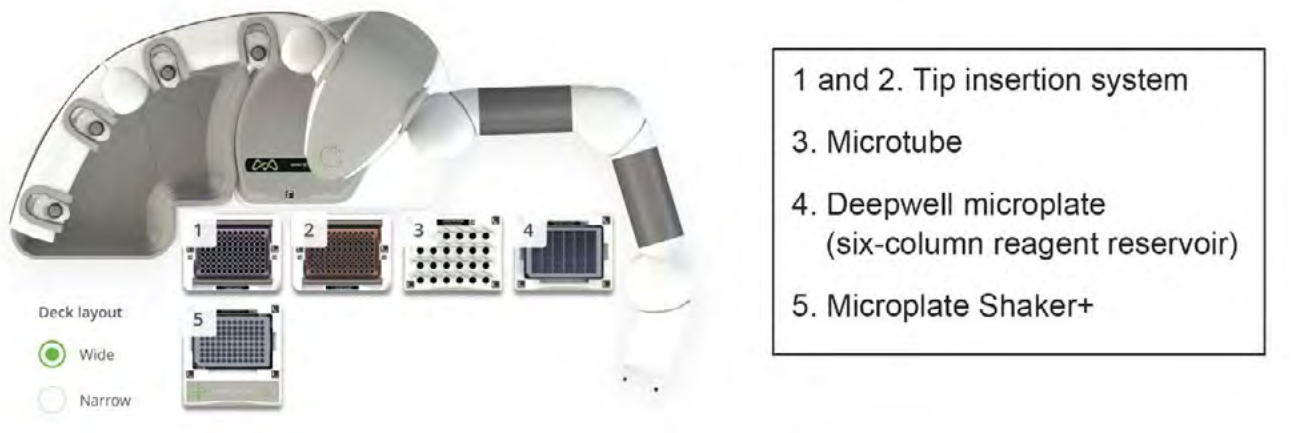

Figure 1. Andrew+ Deck layout for sample preparation of EtG and EtS in human urine using Andrew+ Microplate Shaker+.

Figure 2. Andrew+ Deck layout for sample preparation of EtG and EtS in human urine using Andrew+ pipettes for pipette mixing.

A manual preparation was also performed to compare the recovery and the precision of the automated workflow versus manual workflow.

Following sample preparation, multiple reaction monitoring (MRM) was performed using the previously specified transitions documented in Waters Application Note 720006273 < https://www.waters.com/content/dam/waters/en/app-notes/2018/720006273/720006273-en.pdf> . Two transitions were used for EtG and one transition for EtS. For EtG, a target quantifier/qualifier ratio was adopted using quality control concentration EtG/EtS:500/250 ng/mL.

Figure 3 is a representation of an automated injection. Results are equivalent to what was achieved in Waters Application Note 720006273 <https://www.waters.com/content/dam/waters/en/app-notes/2018/720006273/720006273-en.pdf> . Acceptability criteria included +/- 20% of target ion ratio.

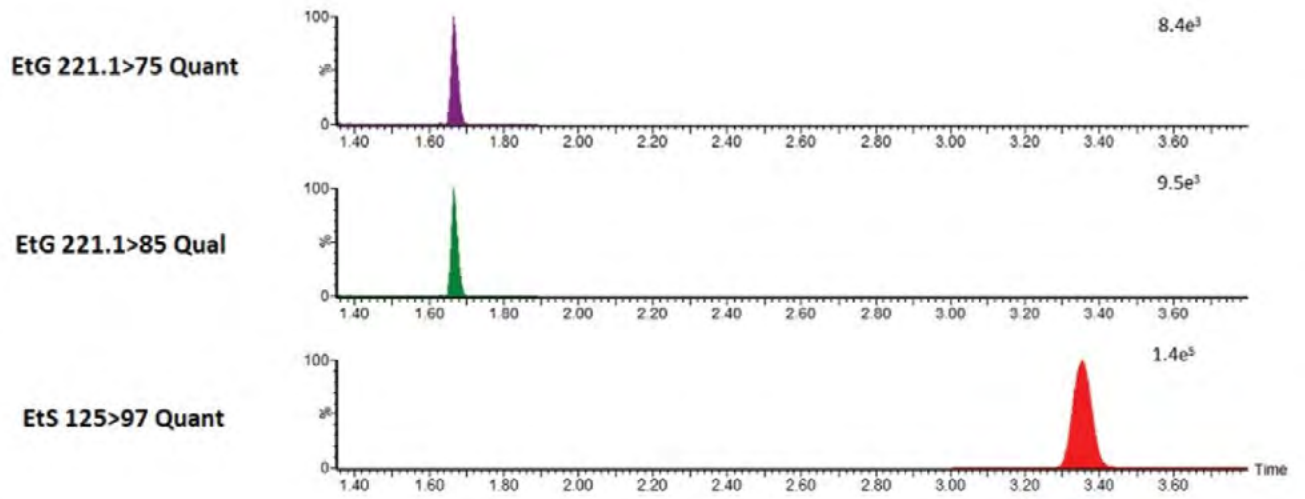

Figure 3. EtG: 500 ng/mL (0.5 mg/mL), EtS: 200 ng/mL (0.20 mg/mL).

The precision of all methods was assessed at two concentrations for EtG (200, 500 ng/mL) and EtS (800, 2000 ng/mL). The "dilute and shoot" automated assays for precision is comparable to manual preparation workflow. For EtS, the precision slightly improved. See Table 1 for precision results.

Table 1. Precision data

Precision (%CV) (n = 8)			
Compound	Manual preparation	Andrew+ Microplate Shaker+	Andrew+ pipette mix
EtG 500 ng/mL	3.4	3.4	3.3
EtG 2000 ng/mL	1.5	1.7	2.0
EtS 200 ng/mL	3.6	1.4	1.9
EtS 800 ng/mL	2.7	1.2	1.7

The comparison of recoveries for the automated workflows performed by the Andrew+ versus the manual workflow can be seen in Figure 4. The overall recovery of results (n = 8) achieved is +/- 2% compared to a manual workflow for both EtG and EtS, QC low and QC high samples.

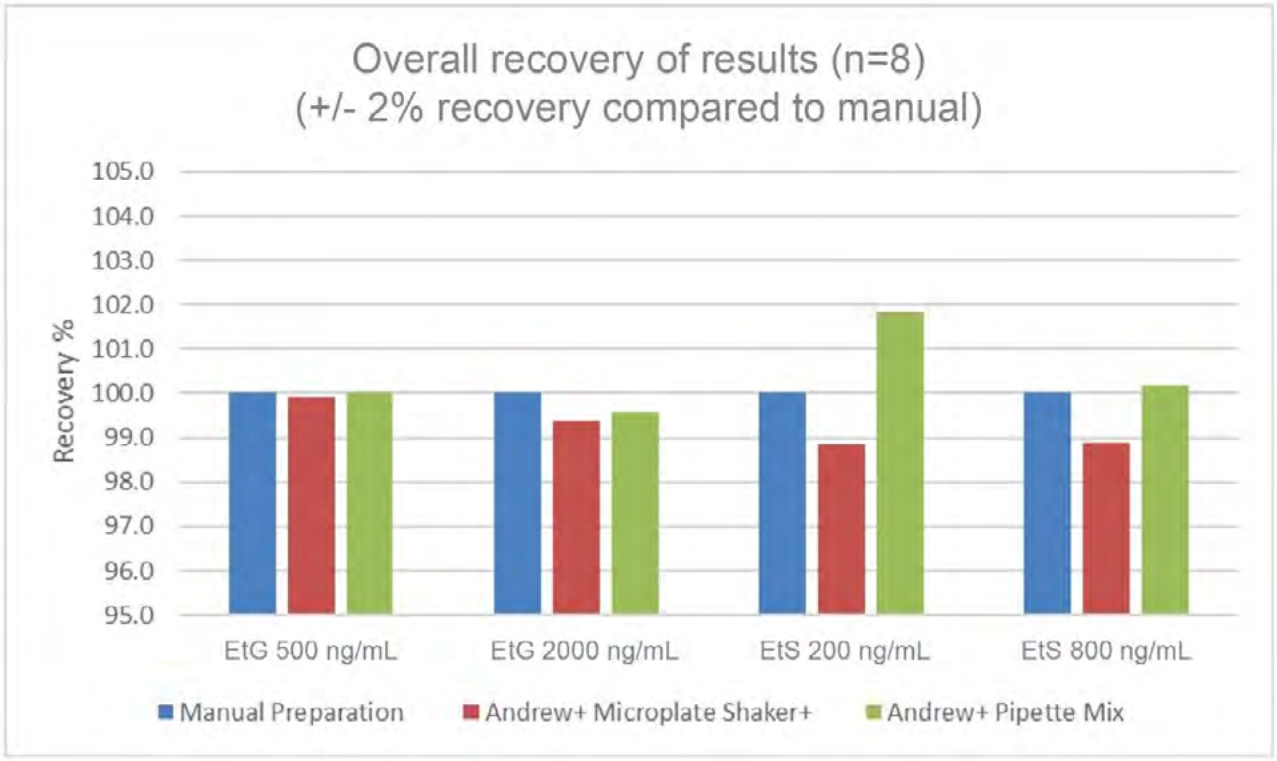

Figure 4. Recovery of Andrew+ sample preparation methods.

Both automated methods developed on the Andrew+ have demonstrated very comparable and equivalent performance to a manual preparation workflow. This offers customers the option to use either automated methods for the quantification of EtG and EtS in human urine. In a high throughput setting workflow, efficiencies would be gained using the Shaker+ Microplate as the option for pipette mixing.

For further information on materials and LC-MS/MS methods used, refererence protocol 720006273 < https://www.waters.com/content/dam/waters/en/app-notes/2018/720006273/720006273-en.pdf> .

Conclusion

Automated methods for dilute and shoot sample preparation of EtG and EtS are demonstrated using the Andrew+ Pipetting Robot in a high throughput setting. The Andrew+ has demonstrated that mixing of samples can be performed by Pipette mixing on Andrew+ or the Microplate Shaker+.

The developed methods are accurate, precise, and comparable to manual preparation for the identification and quantification of biomarkers EtG and EtS.

References

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720007186, March 2021

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Application Note

UPLC-MS/MS Method for Quantitation of EtG and EtS in Human Urine

Patrice Y. Ohouo, Nicholas Dixon, Amy Lieu, Zahra Zavery, Thomas G. Rosano

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For forensic toxicology use only.

Abstract

This application note highlights the development of a rapid, simple dilute and shoot method for the definitive identification and quantitation of ethylglucuronide (EtG) and ethylsulfate (EtS) in human urine using UPLC-MS/MS, for forensic toxicology.

Benefits

Simple dilute and shoot sample preparation method.

Introduction

Ethanol consumption has been linked to significant socio-economic burdens worldwide.[1] As a result, there is a growing need for the detection and identification of ethanol use. Over the years, ethylglucuronide (EtG) and ethylsulfate (EtS) have emerged as reliable biomarkers of recent ethanol use.[2,3] EtG and EtS are minor water soluble phase II metabolites of ethanol and are detectable in urine up to 80 hours following ethanol consumption.[2,4] Definitive confirmation of EtG and EtS as a biomarker of ethanol use is performed for a wide range of testing purposes. The authors report the development of a rapid and simple dilute and shoot method for definitive identification and quantitation of EtG and EtS in human urine using UPLC-MS/MS.

Materials

Urine samples

Human urine samples for the preparation of calibrators and quality controls (QC) were obtained from volunteer donors with no recent (at least a week) use of ethanol. Prior to use, samples were confirmed negative for EtG by immunoassay analysis. Authentic samples were collected as part of routine casework. All samples were stored at -20˚C without addition of preservatives.

Reference standards

Drug reference material for EtG (Ethyl-β-D glucuronide, 1.0 mg/mL), and EtS (Ethylsulfate, 1.0 mg/mL) and deuterated analogues, EtG-D5 (Ethyl-β-D glucuronide D5, 1.0 mg/mL), and EtS-D5 (Ethyl-D5 sulfate, 1.0 mg/mL) were obtained from Cerilliant Corporation, TX, USA. Deuterated analogues were used for the purpose of internal standardization. Stock solutions containing a mixture of non-deuterated reference

material (EtG: 0.1 mg/mL and EtS: 0.05 mg/mL) or a mixture of internal standard (EtG-D5: 0.1 mg/mL and EtS-D5: 0.05 mg/mL) were prepared in methanol and stored at -20 °C. A daily working internal standard solution was prepared by a 400-fold dilution of the stock in distilled water.

Experimental

Sample preparation

Urine samples were initially clarified by centrifugation for three minutes at 7200 rpm (~4227 x g). Following centrifugation, 50 µL aliquots of urine were loaded into a 96-well plate (Waters 96-well Sample Collection Plate, 2 mL square well). Aliquots were diluted by adding 500 µL of the daily working internal standard solution. Following dilution, samples were mixed on a vortex for one minute.

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC CSH Phenyl-Hexyl 2.1 x 150 mm, 1.7 µm (P/N: 186005408)
Column temp.:	50 °C
Mobile phase A:	Water containing 0.1% formic acid
Mobile phase B:	Acetonitrile
Wash solvent:	Acetonitrile/isopropanol / dH_2O (1:1:1) (800 µL)
Purge solvent:	2% methanol in dH_2O (2400 µL)
Injection volume:	10 µL

Gradient

Time (min)	Flow rate (mL/min)	%A	%B	Slope
0	0.5	98	2	Initial
0.1	0.5	98	2	6
5	0.5	40	60	6
6.5	0.5	5	95	1
7	0.5	98	2	1

Table 1. Gradient conditions, total run time: 7.5 min.

MS conditions

MS system:	Xevo TQD Mass Spectrometer
Data acquisition and processing:	MassLynx v4.1 with TargetLynx
Ionization mode:	ESI
Capillary voltage:	2.5 kV
Acquisition mode:	Multiple reaction monitoring (MRM – Table 2)

MRM conditions

Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	Trace type
EtG	221.1	75.0	Quantifier
EtG	221.1	85.0	Qualifier
EtS	125.0	97.0	Quantifier
EtG-D5	226.1	75.0	Quantifier
EtG-D5	226.1	85.0	Qualifier
EtS-D5	130.0	98.0	Quantifier

Table 2. MRM conditions for EtG, EtS, and corresponding internal standards.

Results and Discussion

A series of calibrators and quality control (QC) samples were prepared by diluting the stock solution of non-deuterated EtG/EtS in negative human urine (Table 3). Following the simple sample preparation, multiple reaction monitoring (MRM) was performed using two transitions for EtG and EtG-D5, and one transition for EtS and EtS-D5 (Figure 1). For EtG a target quantifier/qualifier ion ratio was determined, using the threshold calibrator (EtG/EtS: 500/250 ng/mL), and subsequently used to monitor QC's and unknown samples. Acceptability criteria included +/- 20% of target ion ratio.

QC or Calibrator	% Threshold	EtG conc. (ng/mL)	EtS conc. (ng/mL)
S-200/LOD	40	200	100
S-500	100	500	250
S-1000	200	1000	500
S-2500	500	2500	1250
S-5000	1000	5000	2500
S-10000	2000	10000	5000
QCNEG	0	0	0
QC1	40	200	100
QC2	125	625	312.5
QC3	1600	8000	4000

Table 3. Method calibrators and QC's concentrations and corresponding percent of cut-off (EtG: 500 ng/mL, EtS: 250 ng/mL).

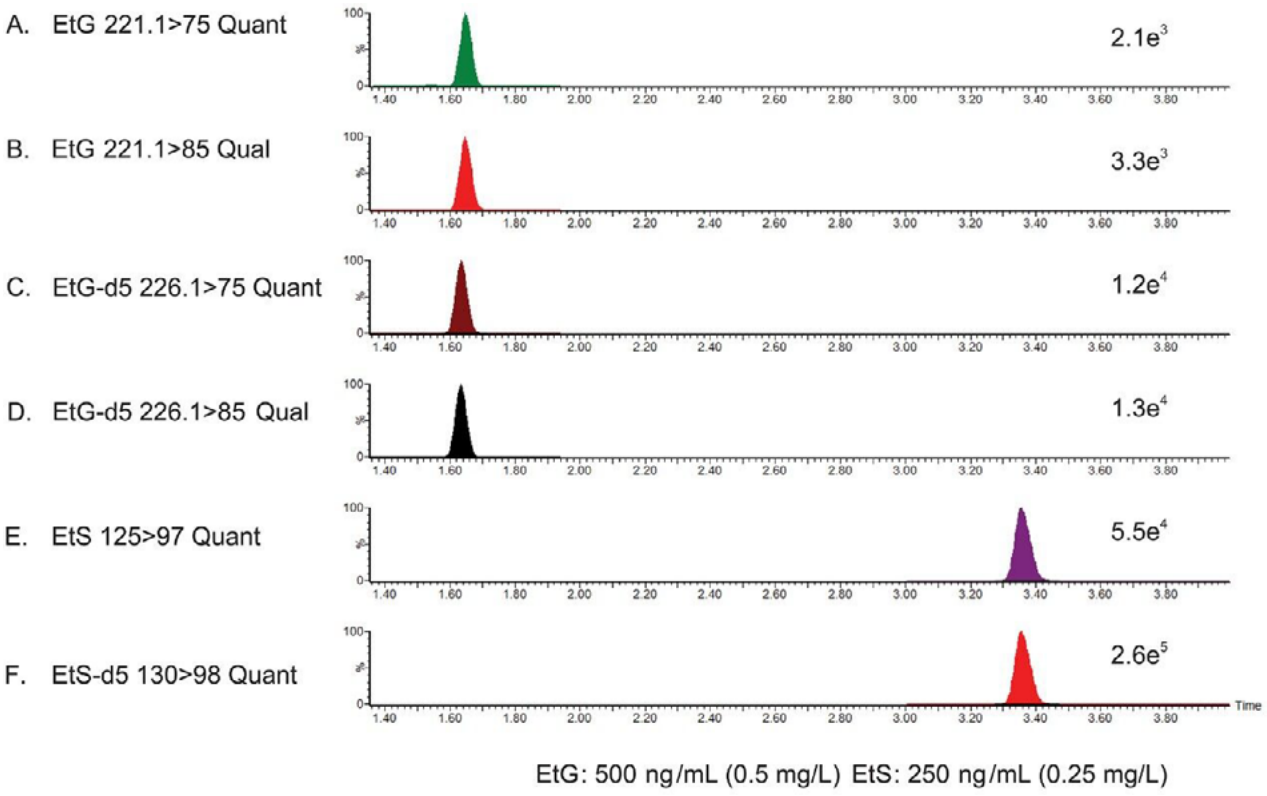


Figure 1. MRM chromatograms from a 10 µL injection of a 500/250 ng/mL EtG/EtS urine calibrator. (A) EtG quantifier ion, (B) EtG qualifier ion, (C) EtG-D5 quantifier ion, (D) EtG-D5 qualifier ion, (E) EtS quantifier ion, (F) EtS-D5 quantifier ion.

Calibration curves were generated based on the ratio of the response of the analyte's quantifier ion relative to the response of the quantifier ion for the respective deuterated internal standard. Regression lines were plotted using a 1/x weighting. Calibration curves for EtG (r^2 range: 0.991–0.999) and EtS (r^2 range: 0.997–0.999) were linear over the analytical ranges investigated, and extended from 200 to 10,000 ng/mL and 100 to 5,000 ng/mL for EtG and EtS, respectively (Figure 2). The cut-off for the assay was set at 500 ng/mL for EtG and 250 ng/mL for EtS. The limits of detection (LOD) were determined using the lowest non-zero calibrator approach. LOD's for EtG and EtS were set at 200 ng/mL and EtS 100 ng/mL, respectively.

The precision and accuracy of the method were assessed at three QC concentrations for EtG (200, 625, 8000 ng/mL) and EtS (100, 312.5, 4000 ng/mL). Based on 11 analytical runs, consisting of three or four replicates, the assay precision (%CV) and accuracy for EtG ranged from 8.4 to 19.6, and 98.4% to 103.6%, respectively. The assay precision and accuracy for EtS ranged from 4.7 to 18.2, and 96.4 to 110.8%, respectively. In all, the method showed good precision and accuracy as summarized in Table 4.

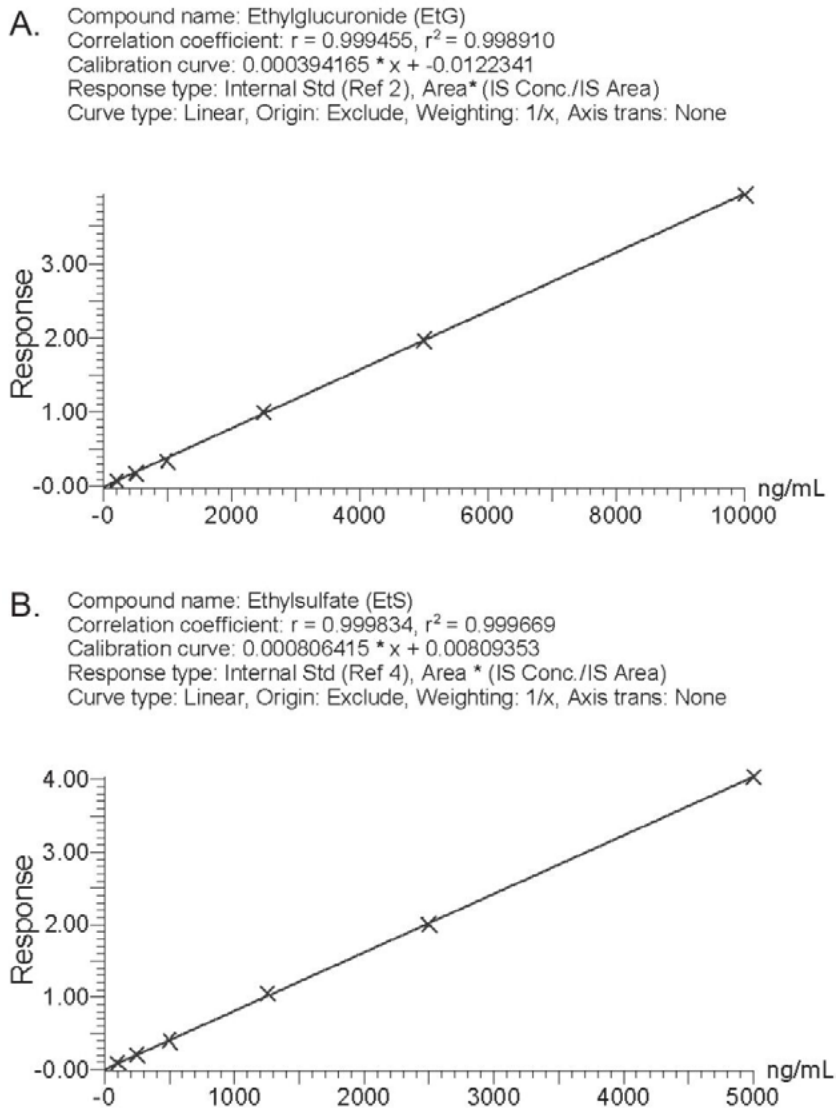


Figure 2. Representative calibration curves for (A) EtG (analytical range: 200 to 10,000 ng/mL) and (B) EtS (analytical range: 100 to 5000 ng/mL).

Compound	QC conc. (ng/mL)	Accuracy (%) (n=35)	Precision (% CV) (n=35)
EtG	8000	103.6	8.4
	625	98.4	12.8
	200	98.4	19.6
EtS	4000	102.9	4.7
	312.5	96.4	10.7
	100	110.8	18.2

Table 4. Summary of method precision and accuracy data.

Matrix effects were evaluated using aqueous versus urine based control samples through the analysis of 10 negative urine specimens and aqueous mobile phase spiked with EtG and EtS at 1000 and 500 ng/mL, respectively. Percent matrix effect was calculated using the following formula: [(A/B – 1) × 100%] where A represents the ion response in urine matrix and B represents the ion response without urine matrix present. Ion effects varied from 1% to -58% for EtG and -54% to 94.6% for EtS. Based on dilute and shoot sample injections, ion suppression of greater than 20% was anticipated, however for this reason analyte-matched deuterated internal standards were incorporated into the method to compensate for matrix effects. Normalization of the data using this approach resulted in a robust assay and satisfied the criteria for precision and accuracy. The stability of EtG and EtS were assessed in both primary specimens and prepared samples following a five day storage period at -10 °C and 4 °C, respectively. Results from reanalysis of primary specimens (n=6), calibrator, and QC samples were within 20% of the results obtained on initial analysis.

	EtG (ng/mL)		EtS (ng/mL)	
Identifier	Reference method	Developed method	Reference method	Developed method
case 1	512	710	NA	
case 2	6871	8132	2118	2583
case 3	1431	1840	1411	1847
case 4	2194	1854	872	982
case 5	5892	8087	>5000	
case 6	1542	1506	510	586
case 7	942	1170	332	364
case 8	3174	4340	465	546
case 9	623	316	425	327
case 10	709	389	416	291
case 11	1772	1812	974	991
case 12	7632	7021	1497	1285
case 13	5360	5076	763	842
case 14	2770	2483	1332	1051
case 15	8431	8433	4024	3046
case 16	5220	3941	1056	863
case 17	1838	1503	429	285
case 18	5679	8146	1514	1023
case 19	6512	5063	2224	1695
case 20	2455	1796	332	274
case 21	1710	1581	355	362
case 22	1255	1273	964	842
case 23	2190	1532	744	614
case 24	704	783	250	165
case 25	8448	9354	2793	2761
case 26	1914	1526	1097	768
case 27	1414	1642	333	373
case 28	1759	1391	346	272
case 29	3605	1953	452	284
case 30	1763	1206	1013	1086
case 31	5314	4667	1312	1168
case 32	2166	4603	2341	2018
case 33	1124	927	267	227
case 34	2483	2468	NA	

Table 5. Quantitative EtG and EtS results obtained from reference method (MedTox Laboratories, Inc.) and developed method. Data from the developed method was not tabulated (shaded cells) and indicated when quantitation from the reference method was not available (NA).

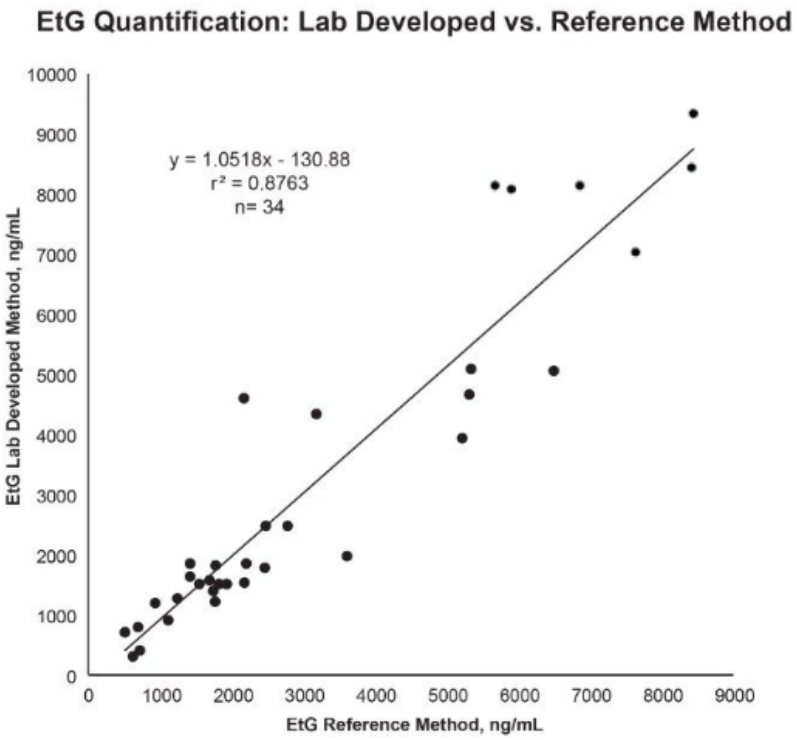
Method correlation studies were performed using de-identified casework specimens (n=34) with positive presumptive and confirmatory results for EtG and/or EtS. Initial presumptive results were obtained using a qualitative Microgenics DRI® EtG Enzyme immunoassay analysis with a 500 ng/mL cutoff. Quantitative EtG and EtS results were obtained from MedTox Laboratories, Inc. (Minnesota, USA) using a currently validated

confirmatory LC-MS/MS method (reference method). Following initial analyses, the specimens were stored at -10 °C for a period of six months. Table 5 shows the results from the reference and developed methods. Analysis of the concentrations obtained by both methods shows dispersion in the data as expected between laboratories (Figure 3). However, statistical analysis did not show method bias based upon linear regression analysis using 95% confidence limit for the slope and y-intercept (Table 6).

EtG		
	Regression statistics	
Multiple R	0.9361	
R Square	0.8763	
Adjusted R square	0.8725	
Standard error	964.1767	
Observations	34	
	Coefficients	
Intercept	-130.8756	
X Variable 1	1.0518	
Standard error	*t Stat*	
275.7429	-0.4746	
0.0699	15.0581	
P-value	Lower 95%	Upper 95%
0.6383	-692.5455	430.7942
4.45922E-16	0.9095	1.1941

EtS		
	Regression statistics	
Multiple R	0.9545	
R Square	0.9111	
Adjusted R square	0.9081	
Standard error	236.7427	
Observations	31	
	Coefficients	
Intercept	55.4750	
X Variable 1	0.8526	
Standard error	*t Stat*	
67.6134	0.8205	
0.0494	17.2436	
P-value	*Lower 95%*	*Upper 95%*
0.4186	-82.8099	193.7599
8.75782E-17	0.7514	0.9537

Table 6. Summary of statistical analysis for correlation study between developed and reference methods.



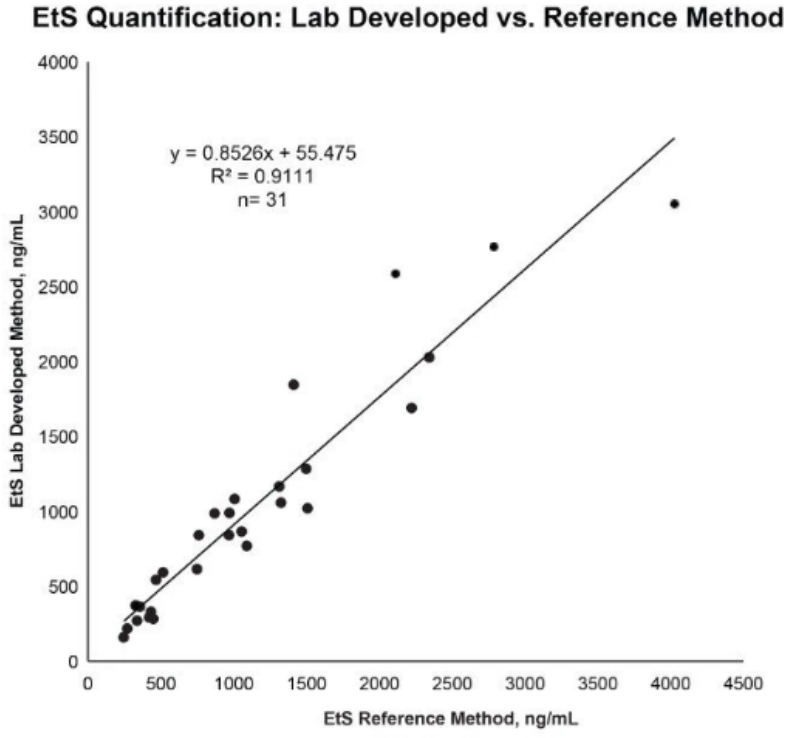


Figure 3. Analysis of the concentrations obtained from developed method compared to reference method (MedTox laboratories, Inc).

2. Jaakonmaki PI, Knox KL, Horning EC, Horning MG. Eur. *J. Pharmacol.* (1967) 1:60–70.

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Application Notes

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Application Note

Using UPLC-MS/MS for the Determination of cTHC in Preserved Oral Fluid

Robert Lee, Michelle Wood

Waters Corporation

For forensic toxicology use only.

Abstract

This application brief describes a simple UPLC-MS/MS method for the analysis of cTHC in preserved oral fluid at low pg/mL concentrations that can be used for confirmation analysis to support various forensic or roadside drug testing schemes.

Benefits

A simple, sensitive UPLC-MS/MS method for the determination of cTHC in oral fluid collected using the Quantisal Oral Fluid Collection Device.

Introduction

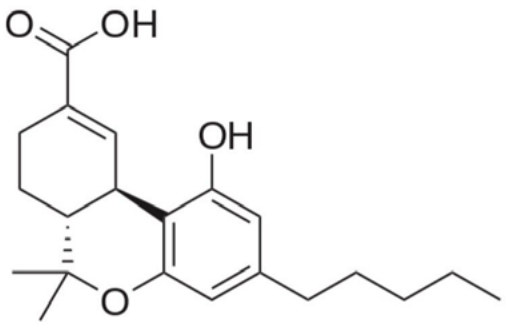
Background

The requirement to analyze drugs at low levels in oral fluid has become an important requirement for many forensic laboratories around the world. The use of oral fluid as a biological matrix for forensic and roadside testing has significantly increased in popularity over the last decade. The presence of parent drugs in oral fluid can provide a better indication of current impairment or intoxication than when measured in urine, and for some drug substances the levels in oral fluid represent a convenient marker for circulating levels in blood. Oral fluid collection is a non-invasive technique and can be achieved without the privacy and adulteration issues associated with urine collection and, in contrast to blood samples, oral fluid does not require medically trained staff to collect the sample. The Quantisal Oral Fluid Collection Device (Immunalysis, USA) allows 1 mL of sample to be collected into a stabilizing buffer which promotes stability of the sample during transportation to the testing laboratory.

Cannabis is the most widely used illicit substance in the world and long-term use can lead to dependency. Cannabinoids are one of the most commonly detected classes of illegal drugs; consequently their analysis is of key importance in forensic testing.

Δ-9-tetrahydrocannabinol (THC) is the major psychoactive element present in the plant Cannabis sativa and produces a number of metabolites including 11-nor-9-carboxy-Δ-9- tetrahydrocannabinol (cTHC). The advantage of measuring cTHC in oral fluid is that cTHC is not detected in cannabis smoke and hence documents active cannabis consumption. Monitoring this metabolite is therefore important as it helps

differentiate active cannabis intake from passive environmental cannabis smoke exposure. However analysis of this metabolite is challenging as it is typically only found at low pg/mL concentrations; thus high sensitivity analytical techniques are required.



Experimental

Sample collection:

Control oral fluid was collected and preserved using the Quantisal Oral Fluid Collection Device according to the manufacturer's directions. It is generally understood that the collected oral fluid is diluted by a factor of four once it has been added to the buffer in the device, and the concentrations stated in this technical brief relate to those in neat oral fluid. Once collected the samples were stored at 4 °C for at least 24 hours prior to analysis.

Sample preparation and UPLC-MS/MS analysis:

Control preserved oral fluid was spiked at a concentration equivalent to 20 pg/mL in neat oral fluid. A liquid-liquid extraction method was used to extract cTHC from the samples. Following evaporation of the organic layer which contains the cTHC, the sample was reconstituted in 50% acetonitrile.

The ACQUITY UPLC I-Class FTN was fitted with a 50 µL extension loop, which allowed 25 µL of sample to be analysed; cTHC was separated using a water/acetonitrile gradient on a CORTECS C_{18} Column.

Two MRM transitions were monitored for cTHC i.e., 343.2 > 245.2 (quantifier) and 191.1 (qualifier). The internal standard (cTHC-d3) was monitored using the transition 346.2 > 248.2.

Results and Discussion

In this technology brief the Xevo TQ-S micro was used in conjunction with the ACQUITY UPLC I-Class System to detect cTHC at very low concentrations.

A comparison of a control (blank) preserved oral fluid extract with a spiked preserved oral fluid extract is shown in Figure 1. The figure shows the smoothed and integrated quantifier MRM trace for both samples.

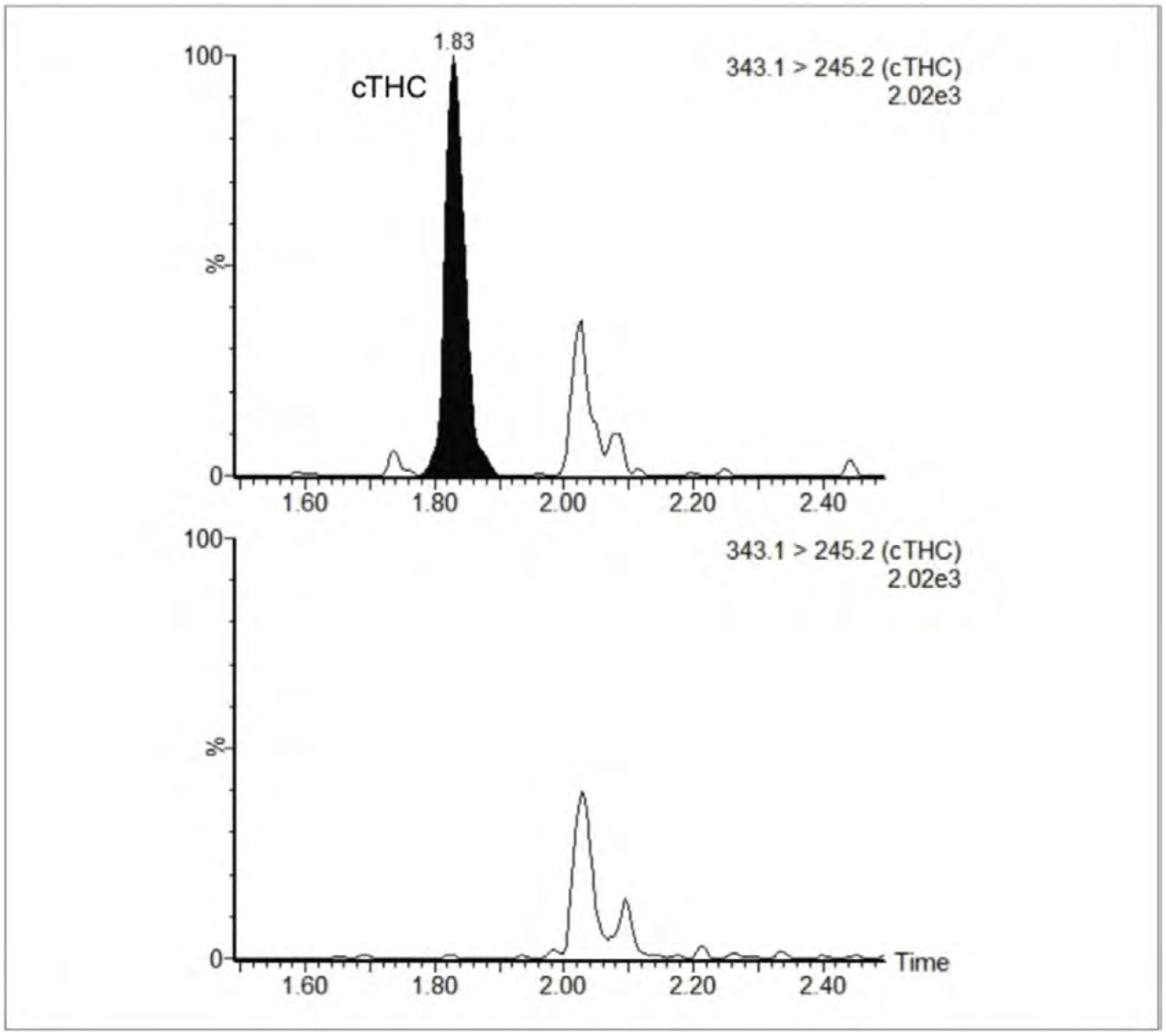


Figure 1. Chromatograms showing quantifier MRM transition for a control (0 pg/mL cTHC) preserved oral fluid sample (lower trace) and a spiked preserved oral fluid sample (top trace). The spiked sample was prepared to give a concentration equivalent to 20 pg/mL in neat oral fluid.

The signal-to-noise (peak-to-peak on unsmoothed raw data) for both MRM transitions in the spiked sample is shown in Figure 2 along with those for an equivalent concentration matrix-free standard.

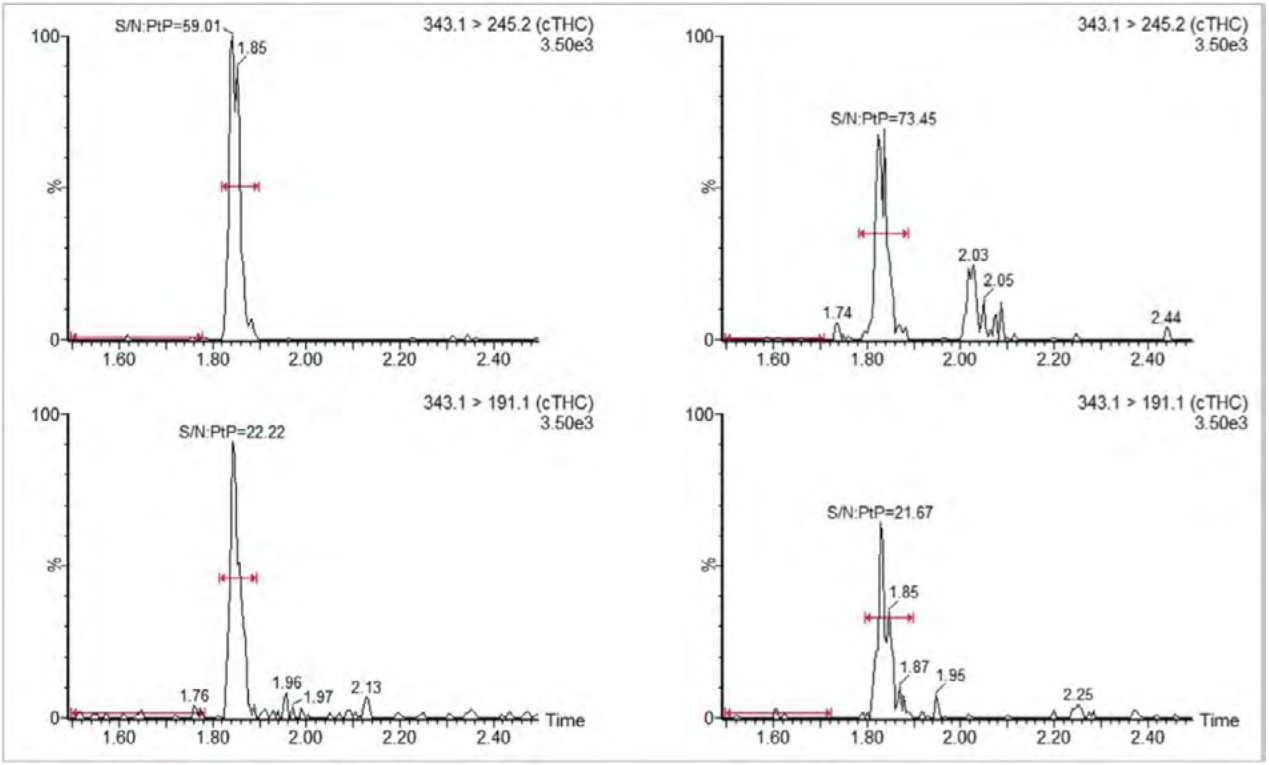


Figure 2. Chromatograms showing signal-to-noise calculations, for both MRM transitions, for a matrix free standard at the equivalent concentration (left hand trace) and the spiked preserved oral fluid sample (right hand trace). The spiked sample was prepared to give a concentration equivalent to 20 pg/mL in neat oral fluid.

Conclusion

The increasing use of oral fluid in forensic and roadside drug testing has highlighted the need for a quick, sensitive, accurate, reliable, and robust method to quantify illicit drugs in this frequently used biological matrix. The use of preserved oral fluid allows for simple, supervised and non-invasive collection of a matrix which contains analytes commonly measured in such testing schemes.

The Xevo TQ-S micro has demonstrated the required analytical sensitivity to detect cTHC in preserved oral fluid at low pg/mL concentrations.

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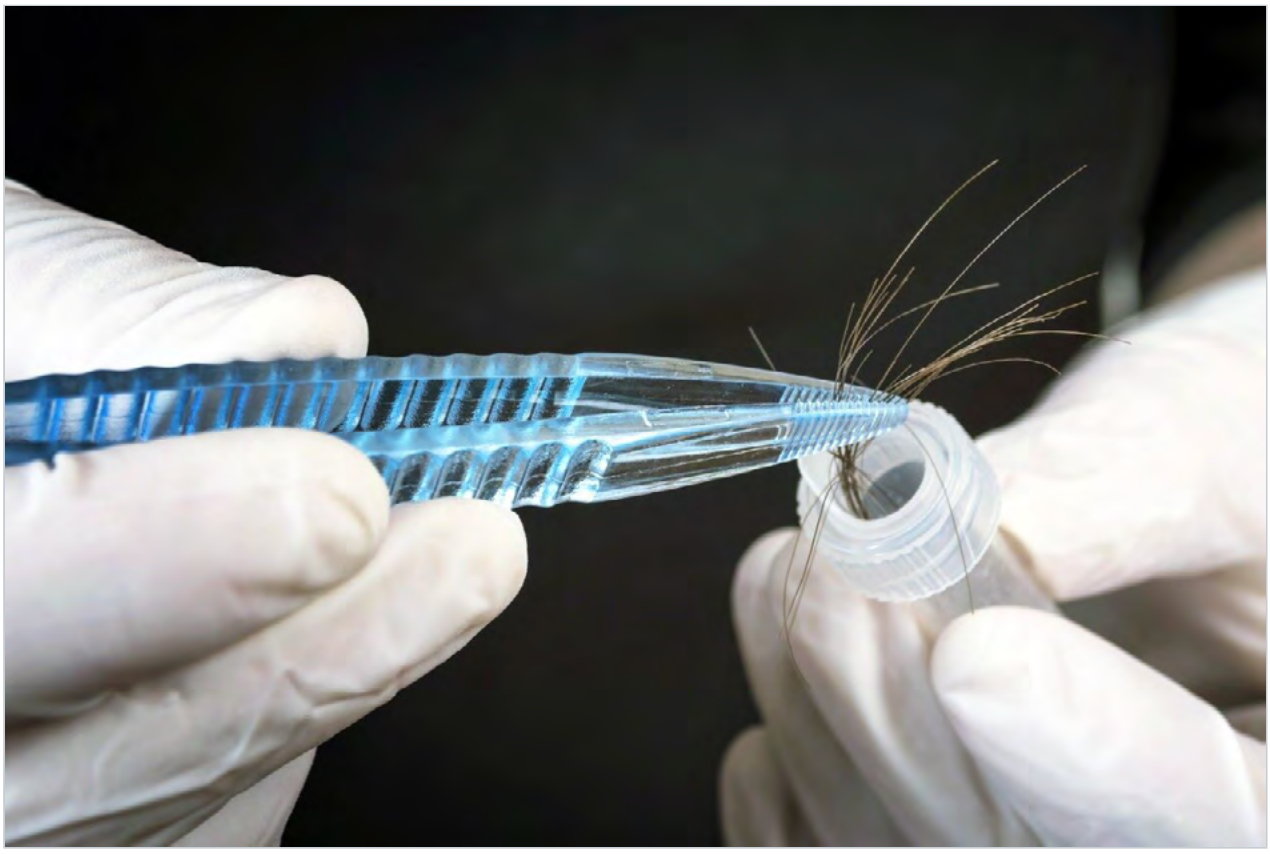
Application Note

A Complete Workflow for the Determination of Drugs that Conforms to the Society of Hair Testing (SoHT) Guidelines

Robert Lee, Massimo Gottardi, Andrea Gardumi, Simone Donzelli, Michelle Wood

Waters Corporation, Comedical



For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

The use of hair as a biological matrix for forensic toxicology testing has increased in popularity over the last decade, with laboratories testing for a panel of drugs as recommended by societies such as the Society of Hair Testing (SoHT) or the European Workplace Drug Testing Society (EWDTS).[1,2] A robust sample preparation workflow and sensitive UPLC-MS/MS method has been developed that allows the user to determine such panels of drugs of abuse in hair to meet these guidelines. The workflow allows the user to determine multiple classes of drugs, such as opiates, amphetamines, cocaine, ketamine, buprenorphine, benzodiazepines, and their metabolites, as well as tetrahydrocannabinol (THC) and its metabolite carboxy-THC (cTHC), from a single hair specimen. Following incubation in a commercially available extraction buffer, the analytes were separated from the remaining hair matrix using a single solid phase extraction (SPE) method using OASIS PRiME MCX 96-Well Plates. Two ACQUITY UPLC I-Class System/Xevo TQ-S micro System LC-MS/MS methods were developed; one to measure 29 "basic drugs" and the second to measure THC, cannabidiol, and cannabinol. A different sample preparation method, using the remaining incubation mixture, along with a new LC-MS/MS method are required to measure carboxy-THC to confirm the presence of THC.[3] This workflow allows all the analytes to be quantified at concentrations below the recommended confirmation cut-offs of the two societies, in under 10 minutes per sample, allowing for a large number of samples to be analyzed in a short period of time.

Benefits

- Single sample preparation protocol using OASIS PRiME MCX 30 mg 96-Well Plate
- Multiple drug classes separated on an ACQUITY UPLC BEH C_{18} Column using an ACQUITY UPLC I-Class System
- Excellent sensitivity of the Xevo TQ-S micro Mass Spectrometer allows for the analytes to be quantified at low concentrations

Introduction

Previously, we have discussed the benefits of hair as a biological matrix for forensic toxicology testing and have described a highly sensitive method for carboxy-THC, a metabolite of THC that can be used to definitively prove active use of cannabis.[3] The procedure utilized 20 mg of hair and extraction of drugs from

the hair matrix was achieved using M3 Reagent followed by sample clean-up using OASIS PRiME HLB and analysis using an ACQUITY UPLC I-Class System/Xevo TQ-S micro Mass Spectrometer LC-MS/MS method.

In routine practice, however, cTHC is only one compound from a large panel of drugs that need to be analyzed routinely and are included in recommended guidelines by societies such as the SoHT or EWDTS (see Table 1). The larger panel also includes opiates, amphetamines, cocaine, ketamine, buprenorphine, benzodiazepines, and their metabolites as well as tetrahydrocannabinol (THC). The recommended cut-offs for each drug class are different, and for some analytes are very low. Consequently, some laboratories apply several different procedures to ensure coverage of all the analytes in this panel. In practice, this may involve the use of differing extraction procedures, separate sample clean-up protocols, and even differing subsequent analytical methods to be applied on the same specimen to ensure appropriate sensitivity for the different drug classes. In addition to the inconvenience of applying multiple protocols, this potentially increases the amount of sample required, which can be problematic as the amount of hair available is usually limited. Thus, a more streamlined approach is required; a simplified sample preparation workflow that can improve laboratory efficiency and allow these analytes to be quantified in hair by a fast, robust, and sensitive analytical method is preferable. The workflow must allow for a large volume of tests to be carried out, while conforming to the guidelines recommended by societies such as the SoHT and the EWDTS.

Group	Analytes	SoHT cut-off (ng/mg)	EWDTS cut-off (ng/mg)
Amphetamines	Amphetamine	0.2	0.2
	Methamphetamine	0.2	0.2
	MDA	0.2	0.2
	MDMA	0.2	0.2
	MDEA		0.2
Cannabinoids	THC	0.05	0.05
	Carboxy-THC	0.0002	0.0002
Cocaine	Cocaine	0.5	0.5
	Ecgonine methyl ester	0.05	
	Benzoylecgonine	0.05	0.05
	Norcocaine	0.05	
	Cocaethylene	0.05	0.05
Opiates	Morphine	0.2	0.2
	Codeine	0.2	0.2
	6-MAM	0.2	0.2
Methadone	Methadone	0.2	0.2
	EDDP	0.05	0.05
Buprenorphine	Buprenorphine	0.01	0.01
	Norbuprenorphine	0.01	0.01
Ketamine	Ketamine		0.5
	Norketamine		0.1
Benzodiazepines/Z-drugs			0.05
Alcohol	Ethyl-glucuronide		0.03

Table 1. SoHT and EWDTS recommended confirmatory cut-off concentrations for drugs in hair.

Results and Discussion

Control hair was collected from volunteers and, following successive decontamination with dichloromethane, methanol, and diethyl ether, it was scissor-minced into 1–2 mm segments. M3 Reagent was supplied by Comedical, Trento, Italy, http://www.comedical.biz/ <http://www.comedical.biz/> .

Control hair (10–20 mg) was weighed into a glass centrifuge tube with a sealed cap and spiked with a panel of drugs of abuse at concentrations ranging from 0.5x cut-off to 200x cut-off. An internal standard (ISTD) mixture was added at 1 ng/mg along with M3 Reagent. The samples were heated for 60 min at 100 °C in an incubator and, once cooled, 100 µL of the sample was diluted with phosphoric acid before loading onto an

OASIS PRiME MCX 30 mg 96-Well Plate (p/n: 186008916 < https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186008916-oasis-prime-mcx-96-well-plate-30-mg-sorbent-per-well-30--m-1-pk.html>). The sample was washed with 2% formic acid followed by 50% methanol. Analytes were eluted with acetonitrile/methanol (9:1 v/v) containing ammonia. A 25-µL aliquot of the SPE eluant was taken for analysis of the "basic drugs" and a second 25-µL aliquot was taken for the analysis of THC.

The ACQUITY UPLC I-Class System with FTN (flow-through needle) was fitted with a 30-µL needle, which allowed 15 µL of sample to be analyzed. The two reconstituted samples were separated using an ammonium formate/acetonitrile gradient on an ACQUITY UPLC BEH C_{18} Column (p/n: 186002350 < https://www.waters.com/nextgen/global/shop/columns/186002350-acquity-uplc-beh-c18-column-130a-17--m-21-mm-x-50-mm-1-pk.html>) by two different inlet methods but the same mobile phases. Two MRM transitions were monitored for each analyte along with an MRM transition for each ISTD. The combined runtime of the two LC-MS/MS methods was 10 mins.

A chromatogram showing the separation of the "basic drugs" spiked into 10 mg of hair at the confirmation cut-off recommended by the SoHT and EWDTS is provided in Figure 1 and shows the quantifier MRM trace for 29 analytes. In this panel, the recommended cut-off levels for buprenorphine and norbuprenorphine are particularly challenging. Figure 2 shows the calibration curve and residuals plot for buprenorphine spiked into 20 mg of hair at concentrations ranging from 0.005 ng/mg to 2 ng/mg.

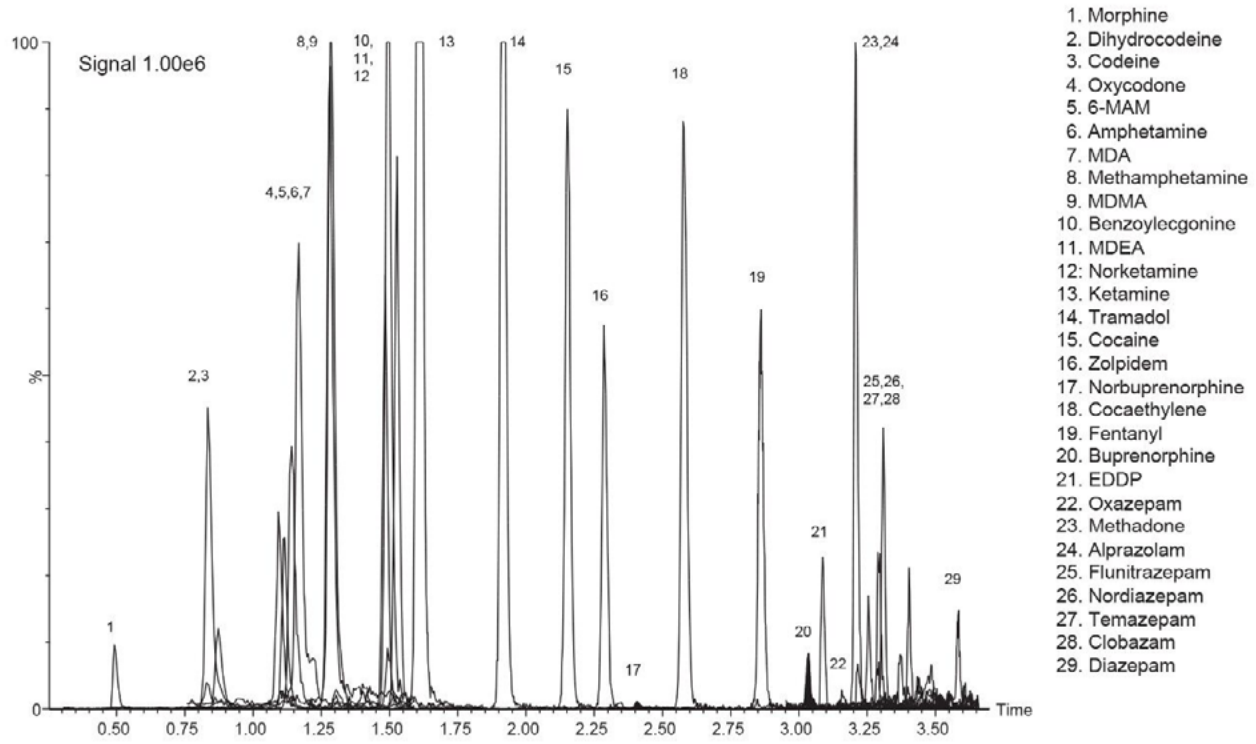


Figure 1. Chromatogram showing the quantifier MRM transitions for all analytes (except cannabinoids) spiked into 10 mg hair at SoHT/EWDTS confirmation cut-off concentrations. Norbuprenorphine (No. 17, 2.41 min) and buprenorphine (No. 20, 3.04 min) are highlighted.

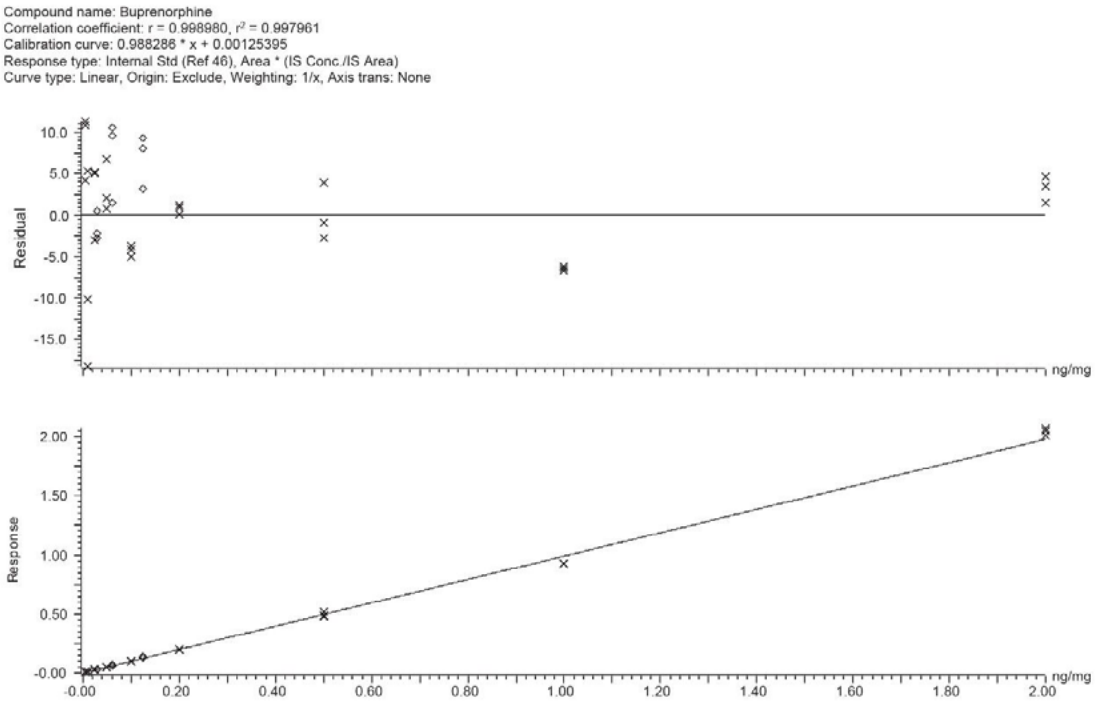


Figure 2. Calibration curve for buprenorphine spiked into 20 mg hair. Calibration range is from 0.5x cut-off to 200x. Cut-off values are those recommended for confirmation by SoHT and EWDTS.

Figure 3 shows the quantifier MRM traces for THC, cannabidiol, and cannabinol spiked into hair at 0.05 ng/mg. To confirm the presence of THC in a sample, the remaining incubation mixture was used to detect cTHC by a separate analytical method as reported previously. The alcohol biomarker ethyl glucuronide can be measured from the M3 incubation mixture following the method described by Joya, ***et al.***[4] A potential complete workflow schematic is shown in Figure 4.

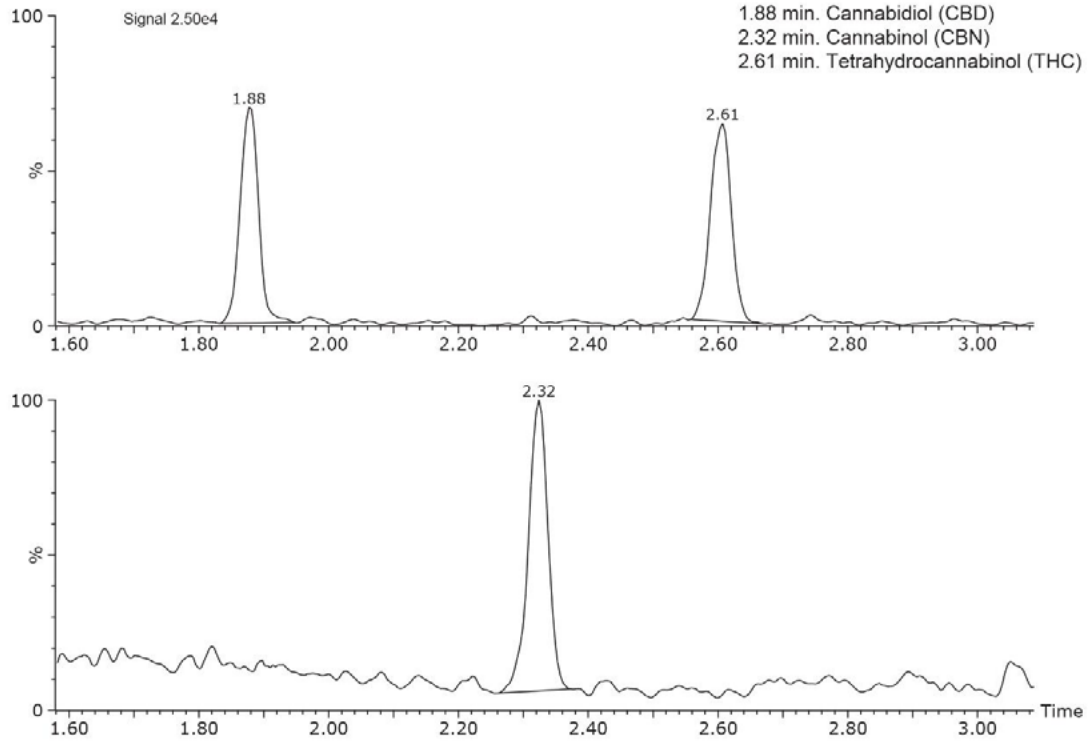


Figure 3. Chromatogram showing the quantifier MRM transition for THC spiked into 10 mg hair at SoHT/EWDTS confirmation cut-off concentration. Also shown are the quantifier MRM transitions for cannabidiol and cannabinol spiked at the same concentration (0.05 ng/mg).

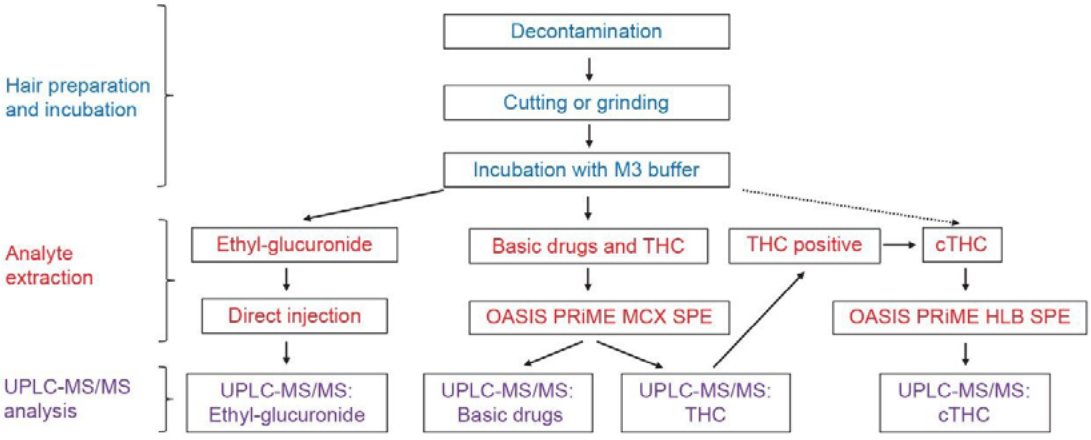


Figure 4. A potential complete workflow schematic for the analysis of drugs in hair.

Conclusion

The increased use of hair for drug testing has highlighted the need for quick, accurate, reliable, and robust methods to quantify drugs of abuse at very low concentrations. This note details a workflow that can be used to quantify a panel of drugs at cut-off concentrations recommended by both the SoHT and EWDTS from a single hair specimen using a commercially available extraction buffer and OASIS PRiME MCX 96-Well Plate. In combination with the ACQUITY UPLC I-Class System and Xevo TQ-S micro Mass Spectrometer, it is possible to quantify a large number of compounds at low concentrations, ensuring high sample throughput.

References

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720006989, Revised October 2020

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Application Note

Determination of Drugs of Abuse in Hair by UPLC-MS/MS: View from Brazil

Danilo Pereira

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

Here we describe a robust UPLC-MS/MS method for the analysis of various drugs of abuse in hair that can be used in routine testing to allow the granting of commercial driving licenses in Brazil.

Benefits

A robust and sensitive UPLC-MS/MS method for the determination of a panel of drugs of abuse in hair.

Introduction

The use of hair as a biological matrix for forensic toxicology testing has increased in popularity over the last decade. In contrast to traditional matrices, such as blood and urine, hair offers another means of detection and can be used to provide a chronological history of drug exposure over several months to years, if segmental analysis is performed. Human hair is known to grow at approximately one centimeter per month. Drugs can be incorporated into the hair by several mechanisms including passive diffusion from the blood supply at the follicle into the growing hair matrix, diffusion into the hair shaft from sweat or sebum, and through external contamination such as smoke or contaminated hands. Hair collection is a non-invasive technique and can be achieved without the privacy and adulteration issues associated with urine collection and, in contrast to blood samples, hair does not require medically trained staff to collect the sample. Furthermore, hair samples can be easily stored.

In certain geographies, such as Brazil, the granting of commercial driving licenses is linked to the applicant being able to prove that they have not taken any of the drugs of abuse that are on the government sanctioned list. This list includes opiates, amphetamines, cocaine, and tetrahydrocannabinol (THC) and their metabolites. In Brazil, it is estimated that this leads to more than one million tests per year. To ensure that this extremely large volume of tests can be carried out, a very fast, robust analytical method is required which can also conform to the guidelines recommended by the Society of Hair Testing (SoHT).[1]

Results and Discussion

Control hair was collected from volunteers, and following decontamination with methanol, it was finely cut into 1–2 mm segments using scissors. The minced hair was stored at 4 °C until required.

Ten milligrams of chopped hair (spiked or real samples) was placed into polypropylene tubes containing methanol. The sample was pulverized and incubated at 50 °C for 15 hours. Following incubation, the sample was centrifuged, and the supernatant simply transferred to a Waters Total Recovery Vial.

To achieve the very fast separation required, an ACQUITY UPLC I-Class (FTN) System was used and the analytes of interest were separated using a gradient of formic acid/acetonitrile on an ACQUITY UPLC BEH C_{18} Column (p/n: 186002349). At least two MRM transitions were monitored for each analyte using the Xevo TQ-S micro Mass Spectrometer. Where available, equivalent deuterated internal standards were added to the samples at a concentration of 0.4 ng/mg to ensure robust quantitation. The run time for the chromatographic method was 1.2 minutes and provided separation of all analytes including the isobaric norcocaine and benzoylecgonine. A chromatogram of the separated analytes is shown in Figure 1. The figure shows the smoothed and integrated quantifier MRM trace for the analytes of interest spiked at concentrations equivalent to the confirmation cut-off concentration recommended by SoHT.

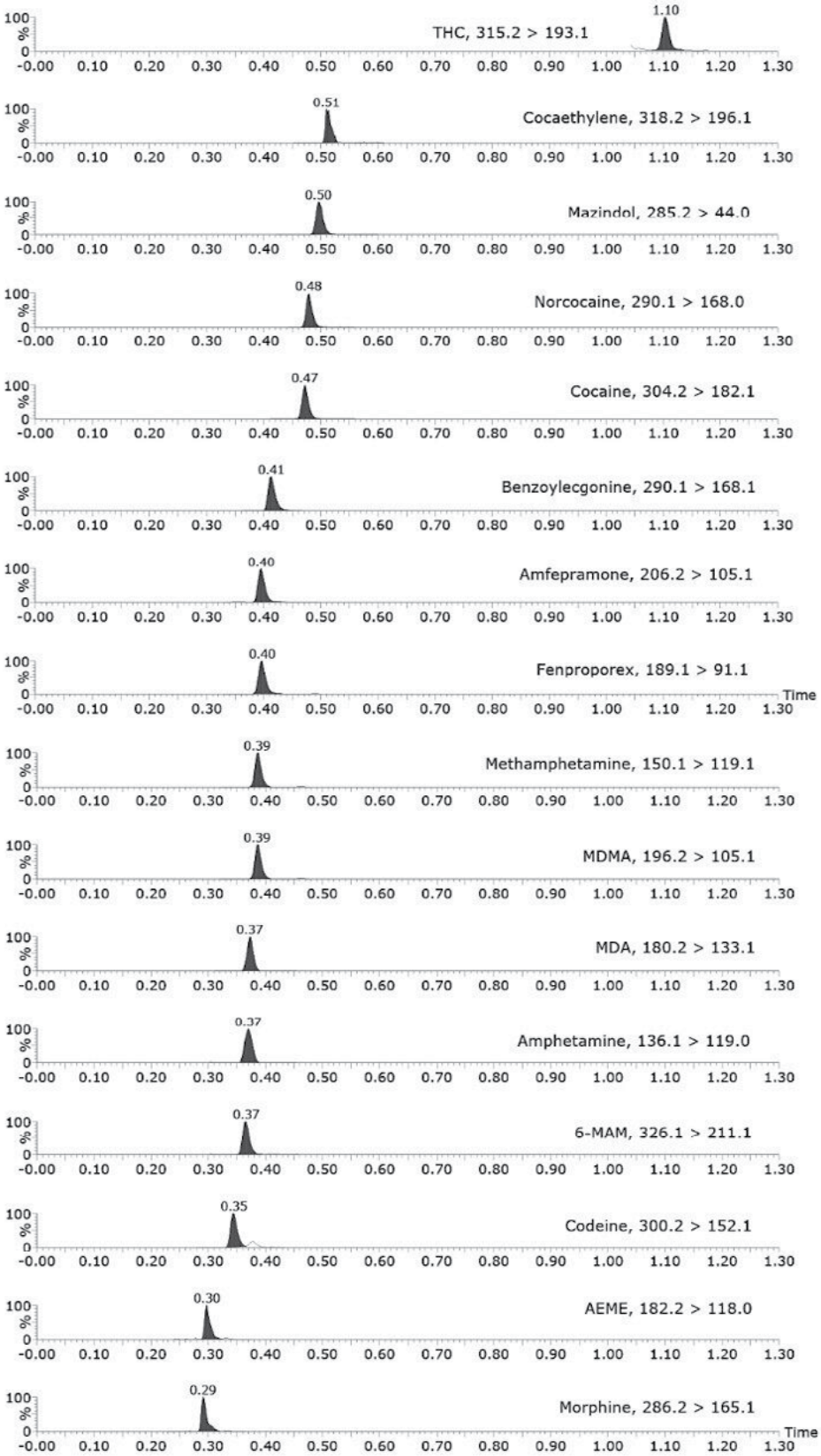

Figure 1. Smoothed and integrated chromatogram showing the analytes spiked into control hair

Forensic Science International 2012, 218, 20-24.

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720006680, October 2019

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Application Note

Using UPLC-MS/MS for the Determination of Carboxy-THC (cTHC) in Hair for Forensic Toxicology

Massimo Gottardi, Francisco Ferron, Robert Lee, Andrea Gardumi, Simone Donzelli, Michelle Wood

Comedical, Waters Corporation

For forensic toxicology use only.

Abstract

This application brief describes a robust UPLC-MS/MS method for the analysis of cTHC in hair that can routinely meet the guidelines for the confirmation cut-off concentration recommended by the Society of Hair Testing (SoHT).[1]

Benefits

Robust and sensitive UPLC-MS/MS method for the determination of cTHC in hair.

Introduction

Background

The use of hair as a biological matrix for forensic toxicology testing has increased in popularity over the last decade. In contrast to traditional matrices, such as blood and urine, hair offers an extended detection window and can be used to provide a chronological history of drug exposure over several months to years if segmental analysis is performed. Human hair is known to grow at approximately 1 centimeter per month. Drugs can be incorporated into the hair by several mechanisms including, passive diffusion from the blood supply at the follicle into the growing hair matrix; diffusion into the hair shaft from sweat or sebum, and through external contamination such as smoke or contaminated hands.

Hair collection is a non-invasive technique and can be achieved without the privacy and adulteration issues associated with urine collection and, in contrast to blood samples, hair does not require medically trained staff to collect the sample. Furthermore, hair samples can be easily stored.

Cannabis is the most widely used controlled substance in the world and long-term use can lead to dependency. Cannabinoids are one of the most commonly detected classes of drugs; consequently their analysis is of key importance in forensic testing. Delta-9-tetrahydrocannabinol (THC) is the major psychoactive element present in the plant Cannabis sativa and produces a number of metabolites including 11-nor-9-carboxy-Δ9- tetrahydrocannabinol (cTHC).

To differentiate between actual cannabis intake from passive environmental cannabis smoke exposure, the SoHT requires that the positive identification of THC in hair samples must be confirmed by measuring the

endogenous metabolite cTHC. However, analysis of this metabolite is very challenging, as it is typically found at low pg/mg concentrations and the amount of sample available is often very limited, thus high sensitivity analytical techniques are required.

Results and Discussion

Control hair was collected from volunteers and following successive decontamination with dichloromethane, methanol, and diethyl ether it was scissor minced into 1 to 2 mm segments. The minced hair was stored at 4 °C until required. M3 Reagent was supplied by Comedical, Trento, Italy. http://www.comedical.biz/ < http://www.comedical.biz/>

Control hair (20 mg) was weighed into a centrifuge tube with a sealed cap and spiked with cTHC at concentrations ranging from 0 to 10 pg/mg. Internal standard (80 pg of cTHC-d3) was added along with M3 Reagent. The samples were incubated for 60 min at 100 °C in an incubator and once cooled the entire sample was loaded onto an OASIS PRiME HLB 30 mg Cartridge (p/n 186008055 < http://www.waters.com/waters/partDetail.htm?partNumber=186008055&locale=en_US>). The sample was washed with an acetonitrile solution followed by hexane. The cTHC was eluted with acetonitrile/ methanol (9:1 v/v) and following evaporation of the solvent, the samples were reconstituted in a methanol solution and transferred to Waters Total Recovery Vials.

The ACQUITY UPLC I-Class (FTN) System was fitted with a 30 µL needle, which allowed 15 µL of sample to be analyzed; cTHC was separated using a gradient of ammonium fluoride pH 9.5 and methanol on a BEH C_{18} Column. Two MRM transitions for cTHC were monitored using the Xevo TQ-S micro Mass Spectrometer i.e., *m/z* 343 > 191 (quantifier), and *m/z* 343 > 245 (qualifier). The internal standard (cTHC-d3) was monitored using the transition *m/z* 346 > 194.

A comparison of a control (blank) hair extract with a spiked hair extract is shown in Figure 1. The figure shows the smoothed and integrated quantifier MRM trace for both samples. The concentration of the spiked sample is 0.2 pg/mg, which is the confirmation cut-off concentration recommended by the SoHT. The signal to noise calculations (peak to peak) for the quantifier and qualifier MRM transitions from a 0.2 pg/mg cTHC spiked hair sample are shown in Figure 2. The linearity of the assay was investigated over the range 0 to 10 pg/mg and the calibration curve along with the residuals plot are shown in Figure 3.

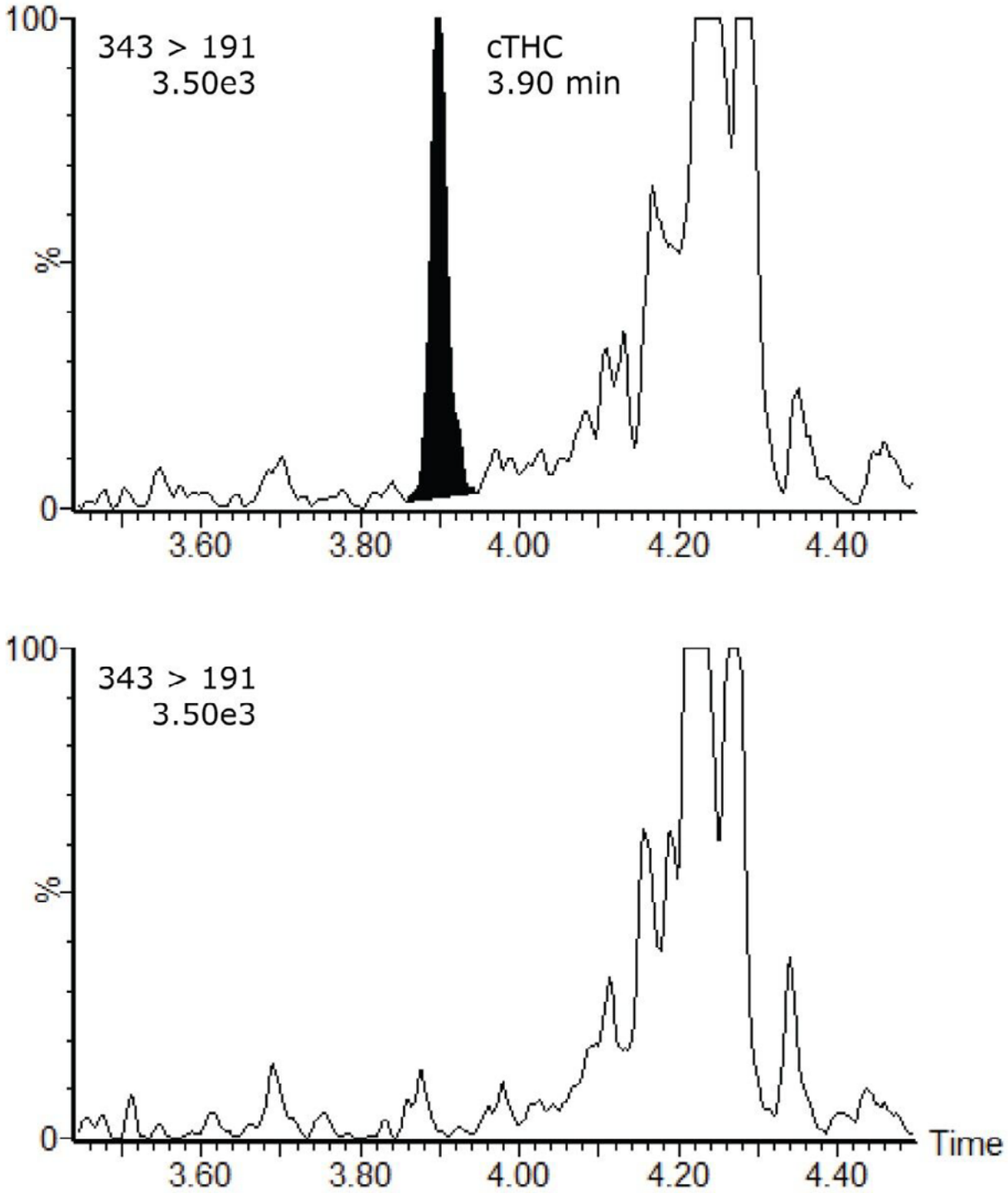

Figure 1. Chromatograms showing quantifier MRM transition for a control (0 pg/mg) hair sample (lower trace) and a cTHC spiked (0.2 pg/mg) hair sample (upper trace).

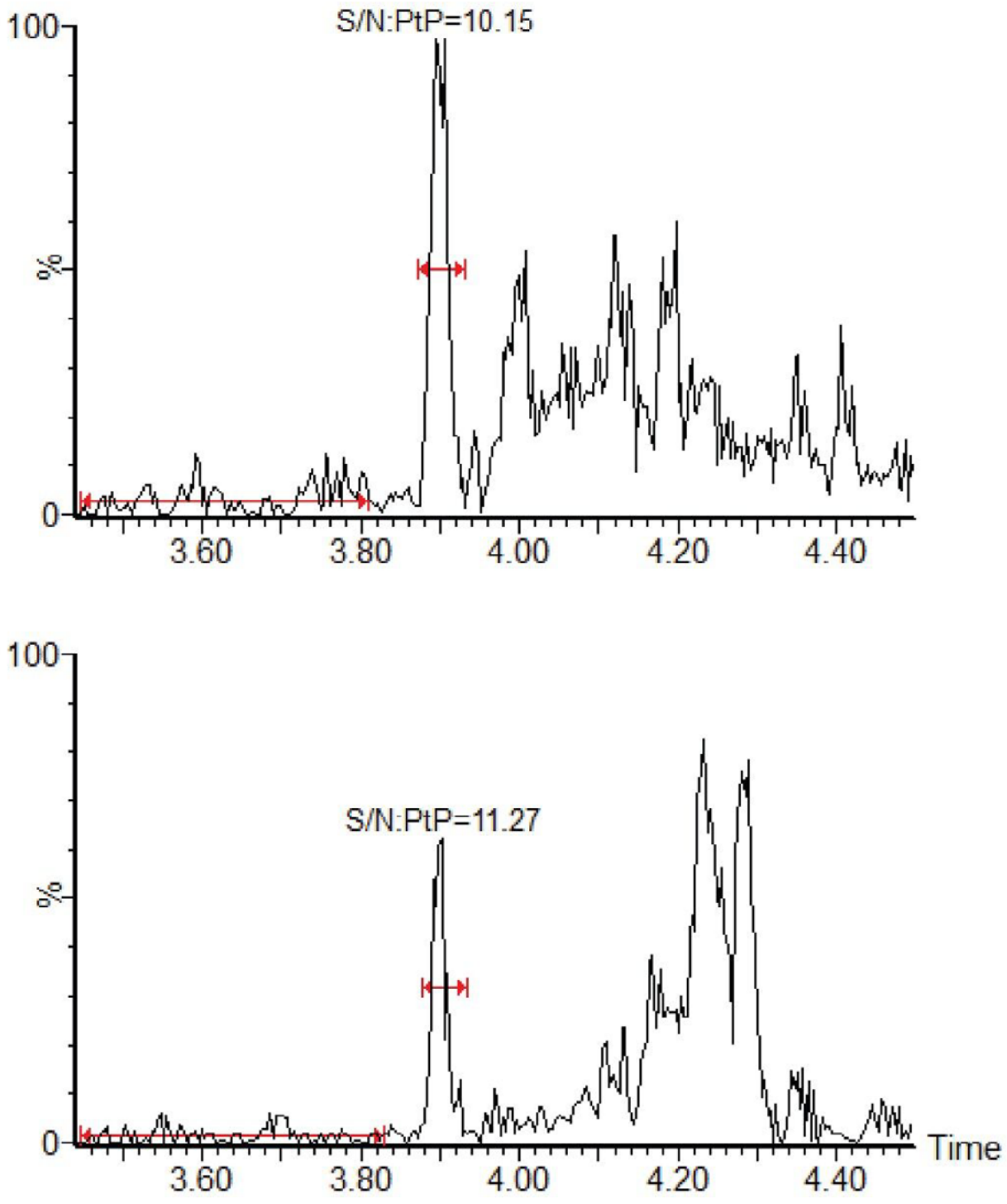

Figure 2. Chromatograms showing signal to noise calculations for both quantifier (lower trace) and qualifier (upper trace) MRM transitions for a 0.2 pg/mg spiked hair sample.

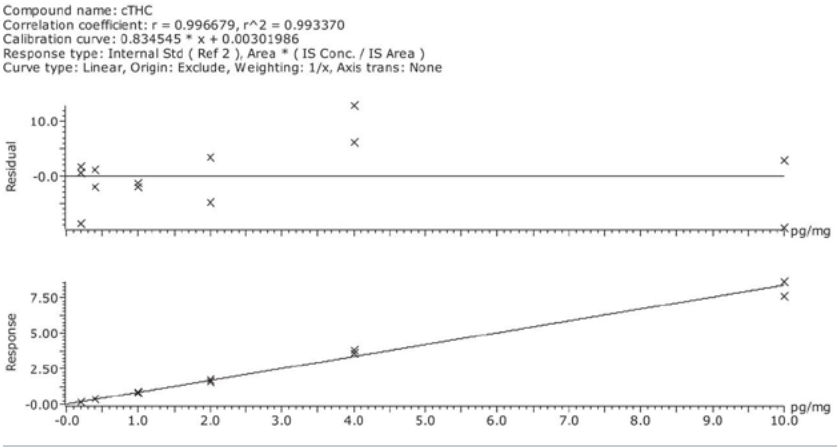

Figure 3. Linearity of cTHC spiked into control hair over the range 0 to 10 pg/mg.

Conclusion

The rise of forensic toxicology testing has highlighted the need for a quick, accurate, reliable, and robust method to quantify compounds in various biological matrices. The use of hair allows for simple, supervised, and non-invasive collection of a matrix which contains analytes commonly measured in such testing schemes.

The ACQUITY UPLC I-Class/Xevo TQ-S micro System has demonstrated the required analytical sensitivity to detect cTHC in hair at sub pg/mg concentrations.

References

1. G.A.A. Cooper, R. Kronstrand, P. Kintz. Society of Hair Testing guidelines for drug testing in hair. Forensic Science International 218 (2012) 20–24.

This technology brief was produced in partnership with COMEDICAL s.r.l. Trento, Italy.

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720006363, December 2018

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Application Note

Incorporation of Tetrahydrocannabinol into a Mixed Drug Substances Panel – Application to Three Commercially Available Oral Fluid Collection Devices

Robert Lee, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief demonstrates the development of a single sample preparation method, using Waters Oasis MCX 96-well µElution Plate, for the analysis of a mixed drug panel containing Tetrahydrocannabinol (THC) that can be applied to different oral fluid collection devices.

Benefits

Inclusion of THC into an oral fluid mixed analyte panel using a single sample preparation method

Introduction

The requirement to analyze drugs at low levels in oral fluid has become an important requirement for many forensic laboratories around the world. Many different collection devices are commercially available which offer a simple way of collecting oral fluid samples in a non-invasive, yet supervised, manner. These devices provide the laboratory with either neat oral fluid or oral fluid that has been diluted in a variety of different preservative buffers. The lack of standardization for collection devices highlights the need for a sample preparation strategy suitable for all analytes that can be used with all the commonly available devices. The most commonly detected drug in these schemes is THC, however many other drug classes such as opiates, opioids, amphetamines, and benzodiazepines must also be measured, and as sample volume can be limited, this has to be performed using a single sample preparation method. Oasis MCX µElution offers the ability to extract all of these analytes from limited volumes of this complex matrix with sufficient efficiency to meet current guidelines such as those applied by the European Workplace Drug Testing Society (EWDTS).[1]

Results and Discussion

The oral fluid collection devices tested in this study were the Salivette saliva collection device from Sarstedt, the Quantisal Oral Fluid Collection Device from Immunalysis, and the Saliva Collection System from Greiner

Bio-One. Control oral fluid samples were collected as per the manufacturer's instructions and spiked with a mixture of 27 illicit or prescription substances commonly measured in oral fluid.

The analytes were extracted from the matrix using a simple Oasis MCX 96-well µElution Plate (P/N 186001830BA) protocol, based on previously reported methods.[2,3] The volume of sample loaded onto the plate for each collection device equated to the same volume (<75 µL) of neat oral fluid. The data was collected using a dual transition MRM method (quantifier and qualifier ions for each analyte) and processed using the TargetLynx Application Manager.

The chemical properties of THC are very different to the other analytes in the panel, and as such, THC requires a different chromatographic gradient to be able to meet the 2 ng/mL cut-off for confirmation tests recommended by the EWDTS guidelines for oral fluid analysis, as seen in Figure 1.

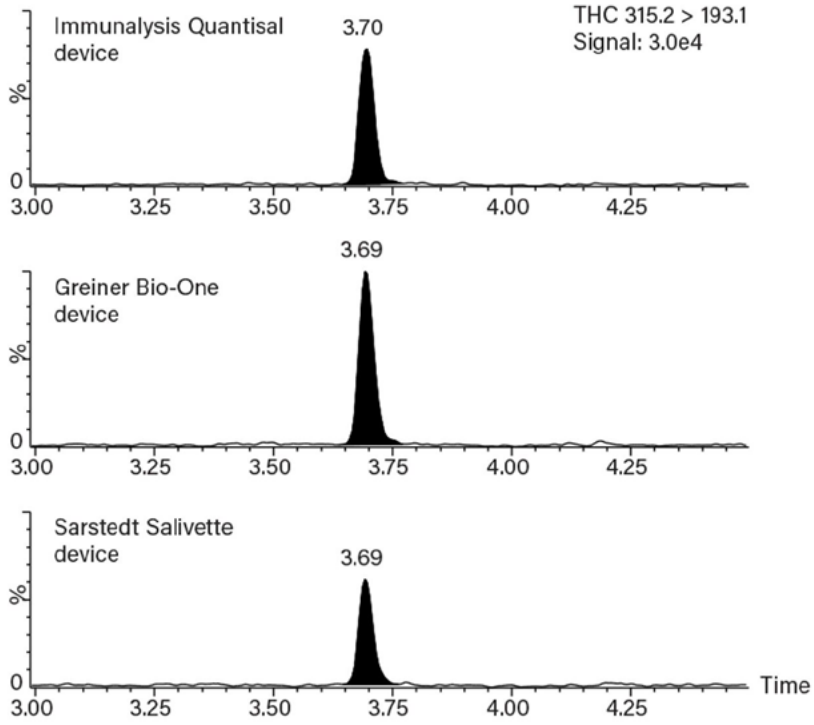

Figure 1. Chromatograms showing the quantifier MRM transition for THC spiked into oral fluid collected by the three different collection devices. The samples were prepared to give a concentration equivalent to 1 ng/mL in neat oral fluid.

The eluant from the Oasis MCX µElution Plate was split into two equal aliquots in a 96-well Sample Collection Plate; 700 µL Round well (P/N 186005837). Following evaporation under nitrogen the separate

aliquots were reconstituted in one of two alternative solvents. One aliquot was reconstituted in 50% acetonitrile and analyzed specifically for THC; the other aliquot was reconstituted in 5% acetonitrile containing 0.1% blank human plasma and used to determine all the other compounds. Figure 2 shows the chromatographic separation of all the analytes (except THC) scaled to the most intense.

To avoid the need for column switching, the two analytical methods were run on the same ACQUITY UPLC BEH C_{18} Column (P/N 186002352) using the same mobile phases, allowing for the methods to be run consecutively.

The high sensitivity Xevo TQ-S micro mass spectrometer in conjunction with the ACQUITY UPLC I-Class System (FTN) is ideally suited to this application as the analyte concentrations in oral fluid can be relatively low in comparison to other biological matrices.

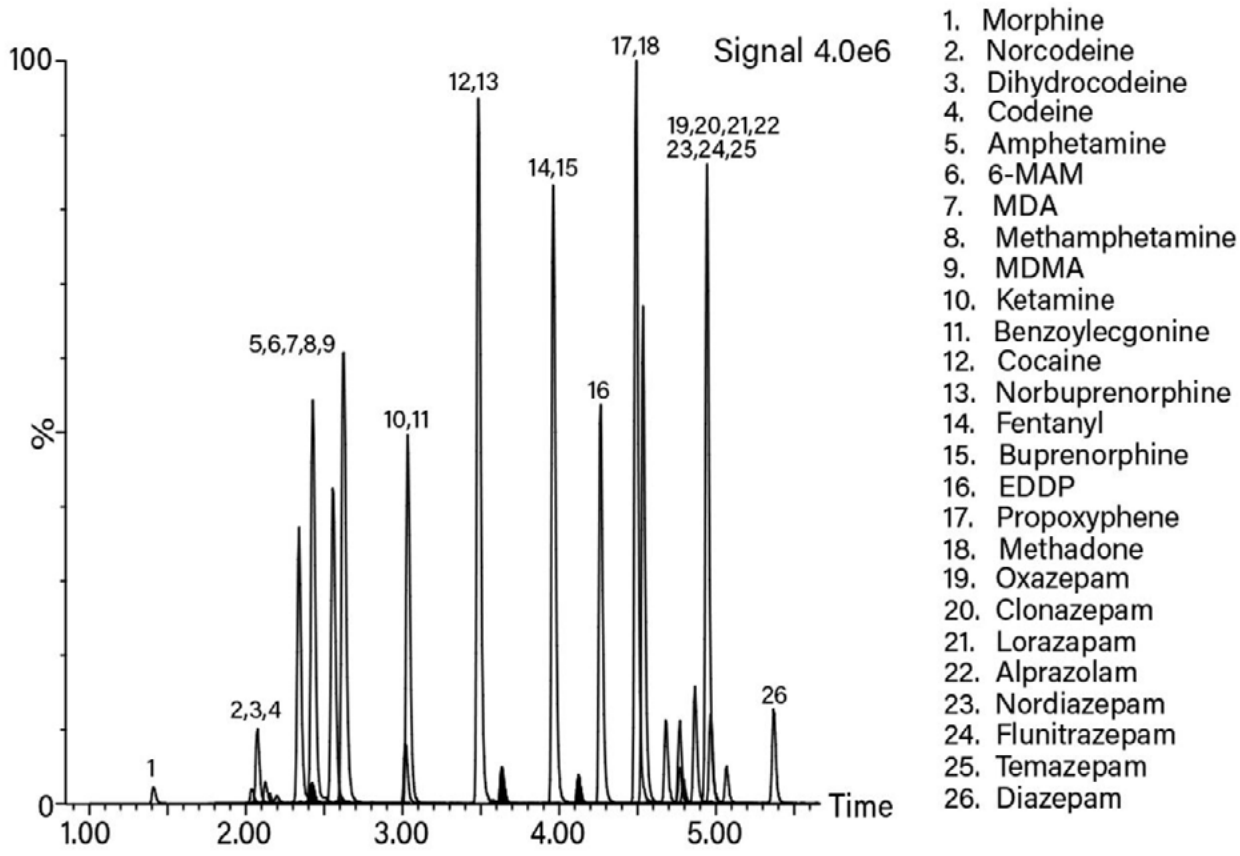

Figure 2. Chromatogram showing the quantifier MRM transitions for all analytes (except THC) spiked into oral fluid collected using the Immunalysis Quantisal Collection Device. The sample was prepared to give a concentration equivalent to 2 ng/mL in neat oral fluid. 6-MAM (peak #6), norbuprenorphine (peak #13), and buprenorphine (peak #15) are highlighted as compounds with EWDTS cut-off concentrations (≤2 ng/mL).

Conclusion

The presence of multiple chemical classes with very different chemical properties in a drug panel, in combination with the limited sample volume that is often provided by oral fluid collection devices, creates a very challenging application. The use of a single Oasis MCX µElution sample preparation method and two UPLC-MS/MS methods allow for the analysis of a mixed drug panel, which includes THC, in less than 15 minutes and at concentrations which meet current guidelines such as those applied by the EWDTS. This sample preparation method can be easily automated to increase sample throughput.

References

1. *European Workplace Drug Testing Society Guidelines*. http://www.ewdts.org (accessed 29 June 2017).
2. Danaceau et al. Direct Analysis of Opioids and Metabolites in Oral Fluid by Mixed-Mode µElution SPE Combined with UPLC-MS/MS for Forensic Toxicology. 2013. Waters Application Note. 720004838EN.
3. Lee et al. Using UPLC-MS/MS for the Quantitation of Illicit or Prescription Drugs in Preserved Oral Fluid. 2016. Waters Application Note. 720005584EN.

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720006081, August 2017

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Application Note

Improved Extraction of THC and its Metabolites from Oral Fluid Using Oasis PRiME HLB Solid Phase Extraction (SPE) and a UPLC CORTECS C18 Column

Xin Zhang, Jonathan P. Danaceau, Erin E. Chambers

Waters Corporation

For forensic toxicology use only.

Abstract

This application note details the extraction of THC-OH, THC-COOH, and THC from oral fluid samples using a novel SPE sorbent, Oasis PRiME HLB, in a µElution format for forensic toxicology applications.

This sorbent enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. In addition, the µElution format enabled the direct injection of extracts without evaporation or reconstitution, minimizing the risk of nonspecific binding and sample losses. The unique nature of oral fluid resulted in some ion suppression that was not seen in other matrices. In order to overcome this signal suppression, a CORTECS C_{18} Column was utilized for high efficiency and the SPE wash step was optimized with the addition of 5% strong ammonia. These changes resulted in a method with negligible matrix effects (<10%) and calibration curves R^2 value all greater than 0.999.

In conclusion, Oasis PRiME HLB has been successfully used to achieve consistent recoveries with minimal matrix effects as well as accurate quantification 4 orders of magnitude from oral fluid samples.

Benefits

- Semi-validated method for a non-invasive, easy to collect matrix–oral fluid
- Faster, simplified sample preparation workflow compared to traditional SPE sorbents
- Excellent and consistent recoveries and minimal matrix effects
- No evaporation or reconstitution necessary with µElution plate format
- Linear, accurate, and precise results for all analytes

Introduction

Cannabis continues to be a highly abused recreational drug. The increasing number of states legalizing it for medical use combined with the trend towards legalization for recreational purposes, means that there is a growing need for analytical methods for the quantification of Δ-9-tetrahydrocannabinol (THC), its metabolites including the active metabolite 11-hydroxy Δ-9-THC (THC-OH) and non-active metabolite 11-nor-9-Carboxy-Δ-9-THC (THC-COOH).[1] While urine has traditionally been used to assess cannabis use, oral fluid

has become increasingly popular as a matrix. Collection of oral fluid is relatively easy to perform, non-invasive and can be achieved under close supervision. Moreover, drug and metabolite concentrations in oral fluid provide better indications of current impairment than urine concentrations, so there is a higher probability that the subject is experiencing pharmacological effects at the time of sampling.[2,3] The cut off level for THC use was reported as 2 ng/mL in oral fluid,[5] which means any analytical method should be able to accurately quantify at this concentration.

This method details the extraction and analysis of THC and its major metabolites, 11-THC-OH and 11-THC-COOH from oral fluid using the Oasis PRiME HLB µElution Plate, followed by UPLC-MS/MS analysis. The SPE procedure is simple and very efficient, with elution in LC compatible solvents, allowing for direct injection, without evaporation and reconstitution of samples. Analysis is rapid with all analytes eluting in 3 minutes. Recoveries were excellent (all greater than 75% with %RSDs <6) and matrix effects were minimal (<10% ME) for all compounds. Quantitative results were highly reproducible. All calibration curves were linear and R^2 values were greater than 0.999. Quality control results were within 10% of expected concentrations with average %RSDs less than 3%.

Experimental

LC conditions

UPLC system:	ACQUITY I-Class UPLC System
Column:	CORTECS UPLC C_{18} Column 1.6 µm, 2.1 x 100 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Mobile phase A (MPA):	Water with 0.1% formic acid
Mobile phase B (MPB):	ACN with 0.1% formic acid

Strong wash solvent:	70:30 ACN:Water with 2% formic acid
Weak wash solvent:	10% ACN
Injection volume:	5 µL

The gradient ramp is shown in Table 1.

Gradient

Time (min.)	Flow (mL/min.)	%A	%B
0.0	0.6	50	50
1.0	0.6	50	50
3.0	0.6	5	95
5.0	0.6	5	95
5.6	0.6	50	50
6.0	0.6	50	50

Mass spectrometry

MS system:	Xevo TQ-S Mass
Spectrometer Ionization mode:	ESI Positive
Capillary voltage:	2.0 kV
Cone voltage:	Optimized for each analyte

Cone gas:	150 L/hr
Desolvation temp.:	500 °C
Source temp:	150 °C

Materials

All standards and stable isotope labelled internal standards were purchased from Cerilliant (Round Rock, TX, USA). Stock standards at 100 µg/mL were prepared in 40% methanol (THC, THC-OH and THC-COOH). A working internal standard solution, consisting of 100 ng/mL THC-D3, THC-OH-D3 and THC-COOH-D3 was also prepared in 40% methanol. Individual calibrators and quality control standards were prepared daily in 40% methanol. 200 µL of each working calibrator or QC standard was added to 1800 µL of oral fluid to make calibration curves and QC samples. Calibrator concentrations ranged from 0.05–100 ng/mL for all analytes. Quality control samples were prepared at 0.375, 1.75, 7.5 and 37.5 ng/mL, in oral fluid.

Sample preparation

Sample pre-treatment

Oral fluid samples were collected with Quantisal collection device from Immunalysis according to the manufacturer's directions. The collection applicator was saturated with oral fluid (spiked), and then placed in a collection vial, which contained 3.0 mL of sample stabilization buffer. Per Quantisal instruction, this was claimed to be the equivalent of collecting 1.0±0.1 mL of sample. 1 mL acetonitrile was then added to the collection vial to help improve extraction. The collection kit was stored in a refrigerator overnight to simulate the transit time of the sample and to allow for complete equilibration between the sample in the pad and the stabilization buffer mix in the collection vial.

SPE with an Oasis PRiME HLB µElution Plate

500 µL aliquots of buffer stabilized oral fluid samples (equivalent to 100 µL oral fluid) were pre-treated by adding 200 µL 4% H_3PO_4 and 10 µL of working IS mixture (100 ng/mL in 40% MeOH).

The entire pre-treated sample (total of 710 µL) was directly loaded on to the Oasis PRiME HLB µElution Plate without conditioning or equilibration, followed by washing with 2 x 250 µL 5% NH_4OH in 25:75 methanol:water. All the wells were then eluted with 2 x 25 µL 90:10 ACN:MeOH and diluted with 50 µL of water. 5 µL was injected onto the UPLC-MS/MS system. The SPE extraction procedure is summarized in Figure 1.

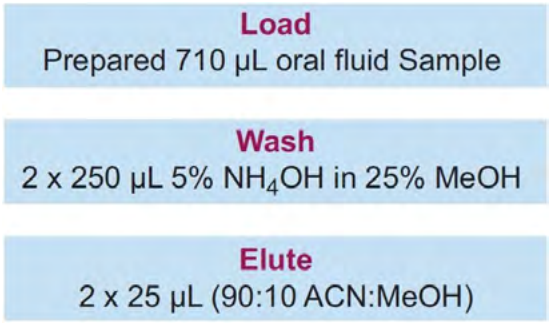

Figure 1. Oasis PRiME HLB extraction methodology for THC, COOH-THC, and OH-THC from oral fluid. With no conditioning and equilibration, sample extraction is simplified to just three steps.

Analyte recovery was calculated according to the following equation:

$$\% \text{ Recovery} = \left(\frac{\text{Area A}}{\text{Area B}}\right) \text{x } 100\%$$

Where A equals the peak area of an extracted sample and B equals the peak area of an extracted blank matrix sample in which the compounds were added post-extraction.

$$\text{Matrix Effects} = \left(\left(\frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}}\right)\right) \text{x } 100\%$$

The peak area in the presence of matrix refers to the peak area of an extracted matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to analytes in a neat solvent solution.

Results and Discussion

Chromatography

A representative UPLC chromatogram of the three cannabinoids from an extracted calibrator at 1 ng/mL is shown in Figure 2. Using a CORTECS UPLC C_{18} Column, all compounds eluted in 3 minutes. Peak shape was excellent for all compounds with all peak widths were under 1.8 seconds at 5% of baseline.

Table 2 lists the UPLC separation retention time and individualized MS parameters of the cannabinoids and their stable isotope labelled internal standards, including MRM transitions, cone voltage, and collision energy. Two MRM transitions were used for each compound, a primary (listed first) and a confirmatory transition (listed second).

Recovery and matrix effects

Extraction recoveries were high and consistent. As Figure 3 shows, recovery for all analytes was at least 75% with all %RSDs within 6% demonstrating the reproducibility of Oasis PRiME HLB. Matrix effects were minimal, at less than 10% for all compounds. Once again, the very low standard deviations (6% or less) demonstrate the consistency of extraction and cleanup seen with Oasis PRiME HLB. All recovery and matrix effect data are summarized in Table 3. The SPE wash step required optimization to eliminate suppression from the oral fluid matrix. The addition of 5% strong ammonia to the wash solution minimized the suppression, resulting in the near complete elimination of matrix effects.

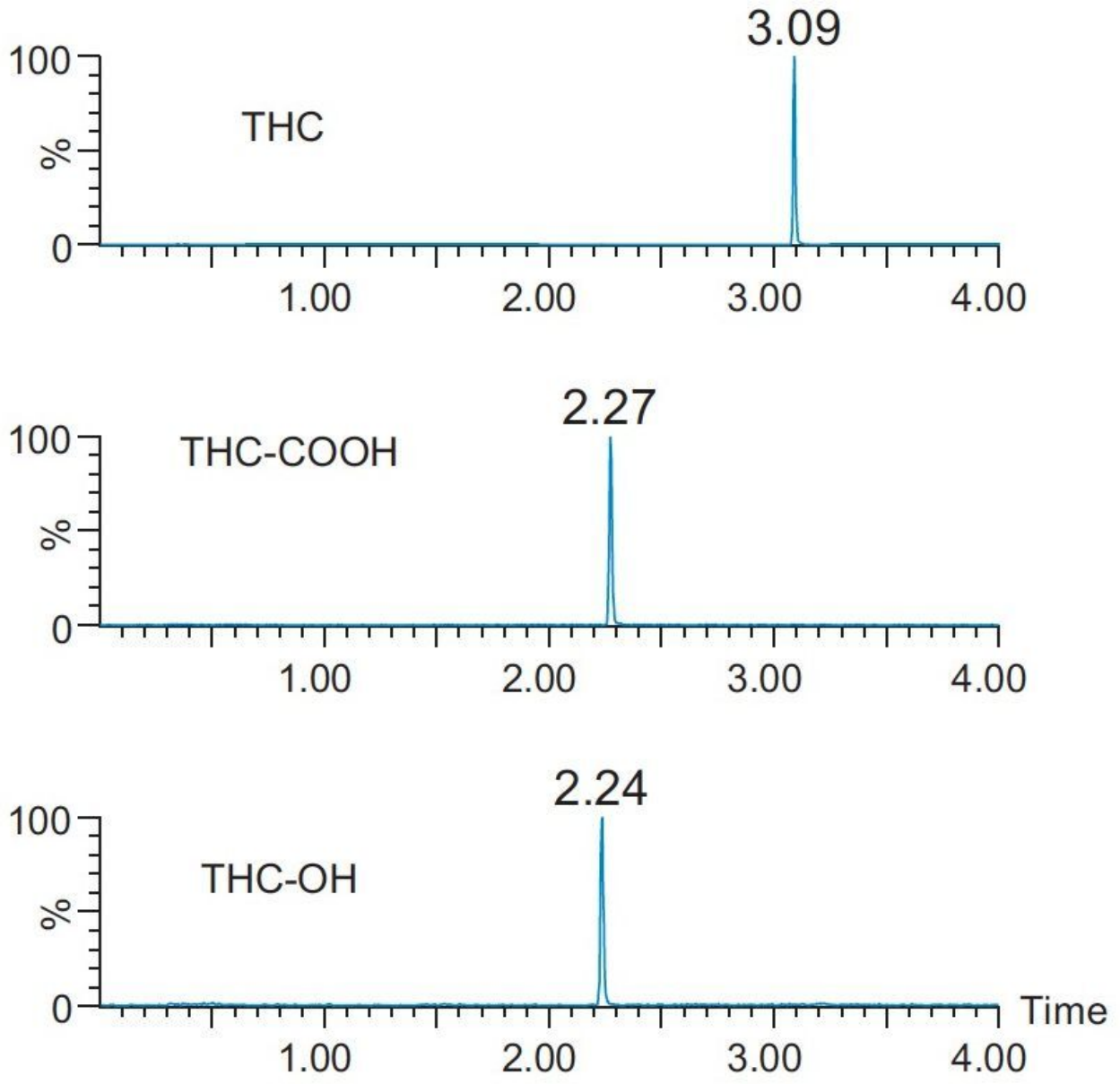

Figure 2. Chromatography of THC-OH, THC-COOH, and THC from extracted oral fluid samples at 1ng/mL of each analytes.

Analyte	RT (min)	MRM transitions (*m/z*)	Cone voltage (V)	Collision energy (eV)
THC-OH	2.24	331.3>313.1 331.3>193.1	40 40	18 30
THC-OH-d3	2.24	334.3>316.1	40	18
THC-COOH	2.27	345.3>327.3 345.3>299.3	50 50	20 25
THC_COOH-d3	2.27	348.3>330.3	50	20
THC	3.09	315.1>193.2 315.1>135.1	40 40	25 25
THC-d3	3.09	318.1>196.2	40	25

Table 2. Mass spectral parameters for all analytes and internal standards.

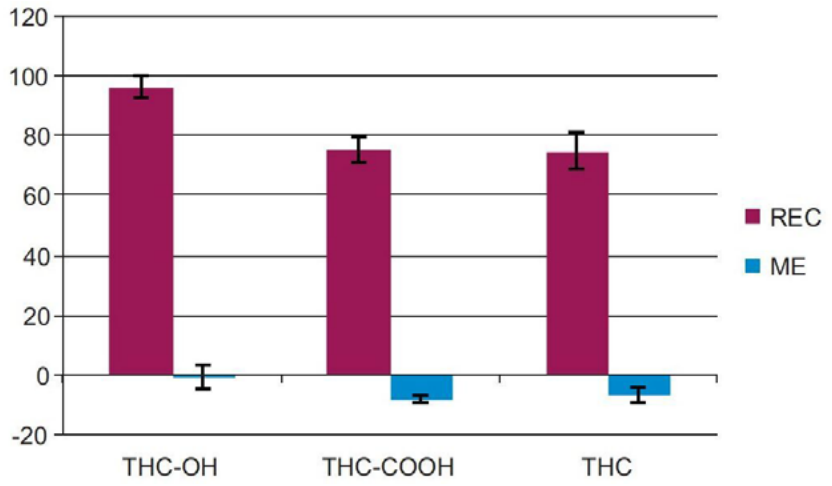

Figure 3. % Recovery and matrix effects of THC-OH, THC-COOH, and THC after extraction using the Oasis PRiME HLB µElution Plate. Error bars indicate standard deviations. %RSDs for extraction recovery were less than 8% for all compounds. Matrix effects were all under 10%.

	Recovery		Matrix Effects	
	Mean	%RSD	Mean	%RSD
THC-OH	96	4	-1	4
THC-COOH	75	4	-8	2
THC	75	6	-7	3

Table 3. Recovery and Matrix effects for THC and its metabolites (N=4 for all tests)

Quantitative results

Calibration and quality control samples were prepared as previously described in the materials and methods section. Calibration ranges were from 0.1–100 ng/mL for THC-OH and THC-COOH, and 0.05–100 ng/mL for THC. Quality control samples were prepared at low, medium, and high concentrations as appropriate for the calibration ranges.

Calibration and quality control (QC) results indicate that this method is linear, accurate and precise. All compounds had linear responses over the entire calibration range with R^2 values of 0.999 or greater using 1/x weighting. Figure 4 shows the calibration curves and Table 4 summarizes the data from these curves for all the compounds. Lower limits of quantification (LLOQ) were 0.1 ng/mL for THC-OH and THC-COOH and 0.05 ng/mL for THC. In each case, all FDA recommendations for accuracy, precision, linearity and analytical sensitivity were met for validated methods.[4]

Quality control samples prepared at 0.375, 1.75, 7.5, and 37.5 ng/mL were accurate and precise. All QC values were within 10% of their target values, and most were within 5%. This data can be seen in Table 5. This demonstrates that the method is linear, accurate and precise over a calibration range that includes the entire scope of expected values of samples. The method was also proved to be both selective and sensitive enough to routinely measure THC in oral fluid well below 2 ng/mL cut off level. This was exemplified by the excellent accuracy and precision at the 0.375 ng/mL QC sample level, where calculated concentrations of all six replicates were within an average of 6% of expected.

	R^2	Mean % dev.	Range (ng/mL)	Curve type
THC-OH	0.9992	3.7%	0.1–100	Linear
THC-COOH	0.9994	2.5%	0.1–100	Linear
THC	0.9995	1.8%	0.05–100	Linear

Table 4. Calibration Curve Summary for THC and its metabolites with 1/x fit weighting.

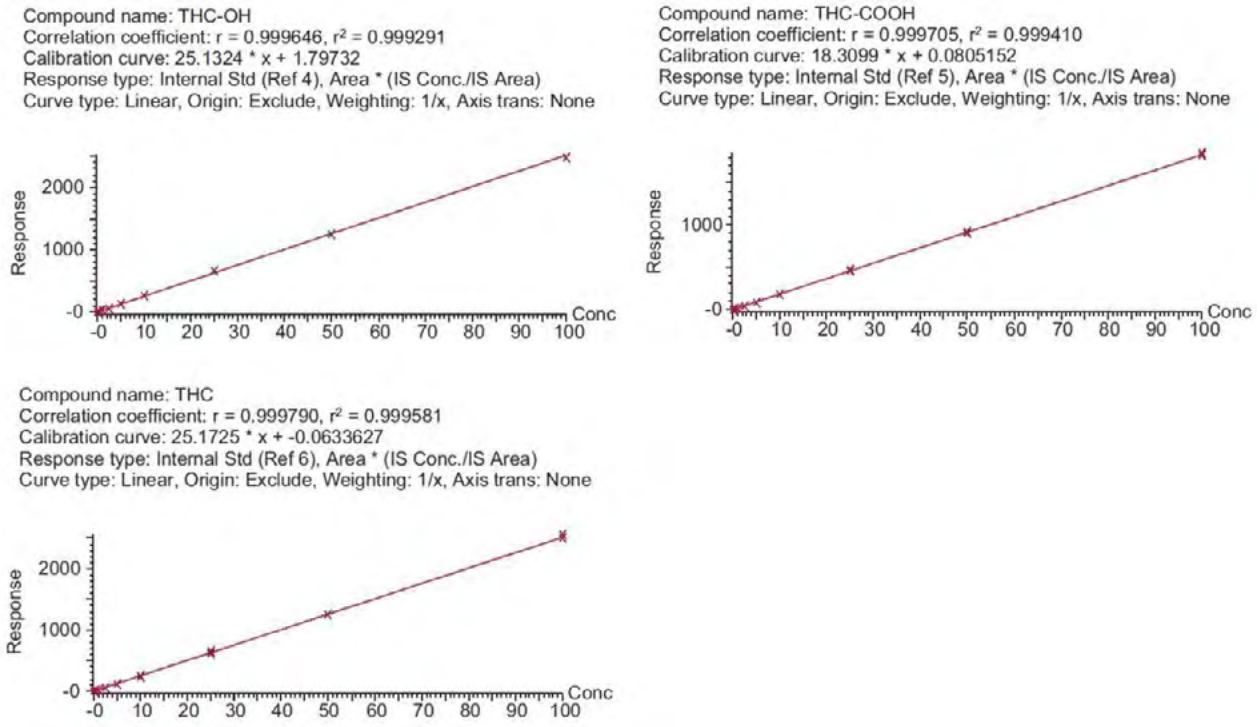

Figure 4. Calibration curves for THC and its metabolites, R^2> 0.999, fit – linear with 1/x weighting.

Accuracy and precision									
N=6	THC-OH			THC-COOH			THC		
QC Level (ng/mL)	Mean (ng/mL)	% Acc.	%RSD	Mean (ng/mL)	% Acc.	%RSD	Mean (ng/mL)	% Acc.	%RSD
0.375	0.36	96.6	8.3%	0.35	93.8	7.1%	0.39	105.2	5.7%
1.75	1.77	101.1	3.4%	1.65	94.3	2.7%	1.69	96.6	3.2%
7.5	7.57	101.0	2.7%	6.94	92.5	3.9%	7.12	94.9	2.4%
37.5	36.88	98.3	1.9%	37.77	100.7	1.4%	36.34	96.9	0.8%
Mean		100	4%		96	3%		96.1	3%

Table 5. Quality control results from extracted oral fluid samples. (N=6 for each compound at all three levels). Mean values at the bottom indicate averages of all compounds at particular concentrations.

Conclusion

This application note details the extraction of THC-OH, THC-COOH, and THC from oral fluid samples using a novel SPE sorbent, Oasis PRiME HLB, in a µElution format for forensic toxicology applications. This sorbent enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. In addition, the µElution format enabled the direct injection of extracts without evaporation or reconstitution, minimizing the risk of nonspecific binding and sample losses. The unique nature of oral fluid resulted in some ion suppression that was not seen in other matrices. In order to overcome this signal suppression, a CORTECS C_{18} Column was utilized for high efficiency and the SPE wash step was optimized with the addition of 5% strong ammonia. These changes resulted in a method with negligible matrix effects (<10%) and calibration curves R^2 value all greater than 0.999.

Recoveries were very consistent, with recoveries >75%, with RSDs under 6%, and minimal matrix effects for all compounds. Linearity, accuracy, precision and analytical sensitivities were excellent for all compounds. All accuracies were within 10% of target concentrations with average %RSDs less than 3% for QC samples, demonstrating the high reproducibility arising from the combination of this sorbent and the UPLC-MS/MS method. In conclusion, Oasis PRiME HLB has been successfully used to achieve consistent recoveries with minimal matrix effects as well as accurate quantification 4 orders of magnitude from oral fluid samples.

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Application Note

UPLC-MS/MS Method for Drug Detection in Exhaled Breath

Beck Olof, Sigurd Hermansson, Stefan Lierheimer, Jbeily M, Böttcher M

Waters Corporation

For forensic toxicology use only.

Abstract

This application brief demonstrates that, using exhaled breath as specimen for drug testing the Xevo TQ-XS instrument provides the needed sensitivity and is a robust and suitable system for routine application.

Benefits

- A safe and convenient specimen to collect
- Detection time related to time of impairment
- Easy procedure for sample preparation
- Method covers large number of analytes
- Screening and identification at the same time

Introduction

Drug testing with urine as the specimen has been in clinical and forensic use ever since the development of analytical technologies based on immunochemistry in the 1970's allowed for cost-effective screening of common drugs of abuse and gas chromatography-mass spectrometry methods for evidential confirmation of positive outcomes. Developments in mass spectrometry technology have now made it possible to avoid immunoassay screening and directly make an analytical investigation with evidential mass spectrometry methods.[1]

Interest in alternative matrices to urine can be traced back to the early days of drug testing, but it is not until more recent times that these alternative specimens became used in routine applications, ***e.g.*** oral fluid. This can to a large extent be credited to the development of more powerful technologies for bioanalysis based on hyphenated liquid chromatography and mass spectrometry instruments that have set a new standard of method performance.[2]

One such alternative specimen is exhaled breath, which is non-invasive and readily available.[3] The specimen can be collected by convenient procedures which are easy to supervise without any need for intrusion of privacy. Apart from volatiles, exhaled breath contains aerosol particles that carry non-volatile components from deeper parts of the lung.[4]

In 2010, the finding that amphetamine and methamphetamine are detectable using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in exhaled breath from illicit drug users following recovery from intoxication many hours after intake were published.[5] This initiated renewed interest and a new series of work aimed at exploring the possibility of using exhaled breath for drug testing with modern day analytical technology.

Experimental

LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS
Column:	ACQUITY UPLC BEH Phenyl (150 mm x 2.1 mm I.D. 1.7 µm)
Mobile phase A:	Water containing 20 mM ammonium formate + 0.1% formic acid (pH=3)

Mobile phase B:	Methanol containing 0.1% formic acid
Wash solvent:	Methanol:acetonitrile:2-propanol:water containing 0.2% formic acid (25:25:25:25 v/v)
Purge solvent:	Water:methanol (80:20 v/v)
Injection volume:	3 µL
Gradient elution :	Table 1

Time (min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.5	85.0	15.0	Initial
3.00	0.5	45.0	55.0	8
4.00	0.5	45.0	55.0	6
5.00	0.5	0.0	100.0	6
6.00	0.5	0.0	100.0	6
6.50	0.5	85.0	15.0	1

Table 1. Gradient conditions. The cycle time injection to injection was 7.5 minutes, giving the column adequate time to re-equilibrate.

MS Conditions

MS system:	Xevo TQ-XS
Data acquisition and processing:	MassLynx with TargetLynx
Ionization mode:	UniSpray, positive mode
Impactor voltage:	2.2 kV
Acquisition mode:	Multiple reaction monitoring (MRM) Table 2

All analytes were detected in positive ionization mode using UniSpray ionization and the MRM transitions stated in Table 2.

Compound	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone voltage (V)	Collision energy (eV)	Retention time (min)	QC 20 pg CV (%)
Alpha-PVP	232.1	126.1/105.1	5	24/24	3.53	7.6
Amphetamine	137.1/136.1	92.1/119.1	8	14/8	2.20	14
Benzoylecgonine	290.1	168.1/105.1	30	18/28	3.27	9.0
Cocaine	304.1	182.1/82.1	30	18/26	3.59	8.7
Delta-9-THC	315.1	193.1/123.1	5	24/32	5.76	20
Gabapentin	172.1	137.1/95.1	18	14/22	1.94	6.5
Ketamine	238.1	125.1/207.1	20	24/12	3.34	6.3
MDA	180.1	105.1/133.1	10	20/15	2.35	8.9
MDMA	194.1	163.1/133.1	18	11/19	2.58	4.8
Methadone	311.1	266.1/105.1	20	14/26	4.92	3.1
Methamphetamine	151.1/150.1	92.1/119.1	15	14/8	2.47	5.6
Methylphenidate	234.1	84.1/56.1	30	20/37	3.52	6.1
Oxazepam	287.1	241.1/269.1	10	21/14	4.98	6.5
Oxycodone	316.1	241.1/256.1	25	281/24	2.43	8.1
Pentedrone	192.1	132.1/161.1	5	16/11	3.30	5.8
Pregabalin	160.1	55.1/83.1	18	20/15	1.84	11
Temazepam	301.1	255.1/177.1	25	22/37	5.18	3.5
Tramadol	264.1/265.1	58.1/58.1	20	14/14	3.49	7.3
Zopiclone	389.1	245.1/217.1	10	18/32	3.65	4.4

†47 analytes were included in the complete method, Table 2 includes conditions for the analytes that were found positive in the study.

Table 2. Analytical parameters used for MRM monitoring and results from the lowest quality control specimen (n=21). Quantifier ions and parameters are indicated in bold font.

The internal standards; alpha-PVP-d8, amphetamine-d5, benzoylecgonine-d3, cocaine-d3, delta-9-THC-d3, gabapentin-(13C)3, ketamine-d4, MDA-d5, MDMA-d5, methadone-d9, methamphetamine-d5, methylphenidate-d9, oxazepam-d5, oxycodone-d3, pregabalin-(13C)3, temazepam-d5, tramadol-13C-d3, and zopiclone-d4 were analyzed with parameters corresponding to their individual analytes' according to Table 2. but with *m/z* adjusted to the number of stable isotopes respectively. For pentedrone, MBDB-d5 was used as internal standard, monitoring the transition 213.1>136.1.

Collection of breath particles

Particles in exhaled breath are collected by impaction technology. A simple commercial device is available for this. The BreathExplor device was validated for collecting methadone and characteristic lung lipids.[6]

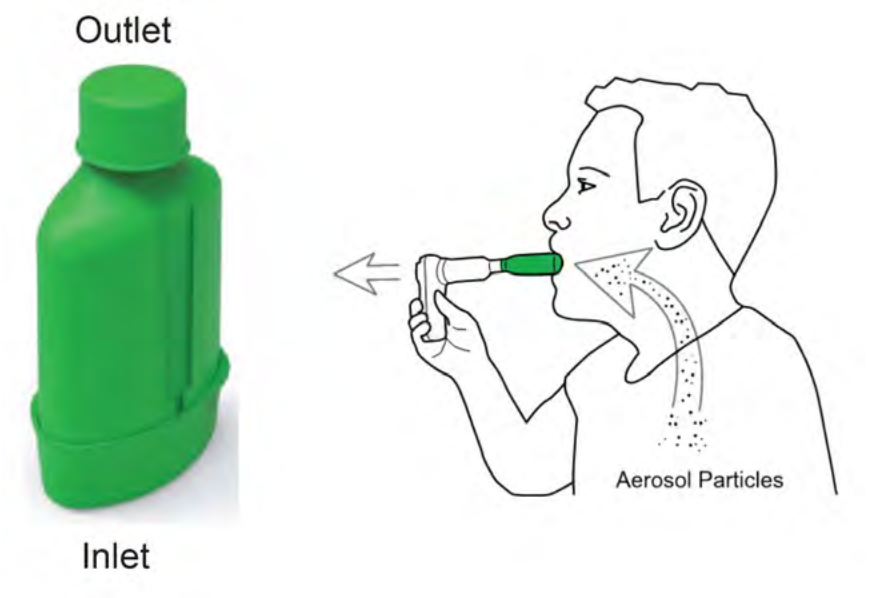

Figure 1. The BreathExplor sampling device and sampling procedure. The sampling can be documented using a spirometer.

Particles from twelve breaths are collected. The collection device contains three parallel collectors, providing three subsamples. After sampling the device is capped, labelled, and sent to the lab.

Sample preparation

The device is taken apart and one collector is used for analysis, while two are being stored.

The collector is put in a test tube and 2 mL of methanol containing internal standards and 10 µL ethylene glycol is added.

The test tube is vortexed for 10 seconds and the collector is taken out.

The methanol is evaporated using a vacuum centrifuge and the residue is dissolved in 60 µL 50% methanol and transferred to autosampler vial and centrifuged.

Standards and controls were prepared by fortifying blank collectors.

Application

The method was applied in studies where exhaled breath specimens were collected at music events, nightclubs, and festivals. In total, 1204 unknown samples were investigated for 47 analytes.

Results and Discussion

During a period of 4 months 21 batches of unknowns were analyzed without any major problems. The fluctuation of response and retention time was small over time and is shown in Figure 2 for cocaine. The extracts proved to be clean and no indication of matrix effects on response was observed. The same column was used for about 2000 injections and no increase in back pressure was observed over time. The data for QC samples supported the stability and good performance of the method (Table 2). Example chromatograms from authentic specimens are shown in Figure 3.

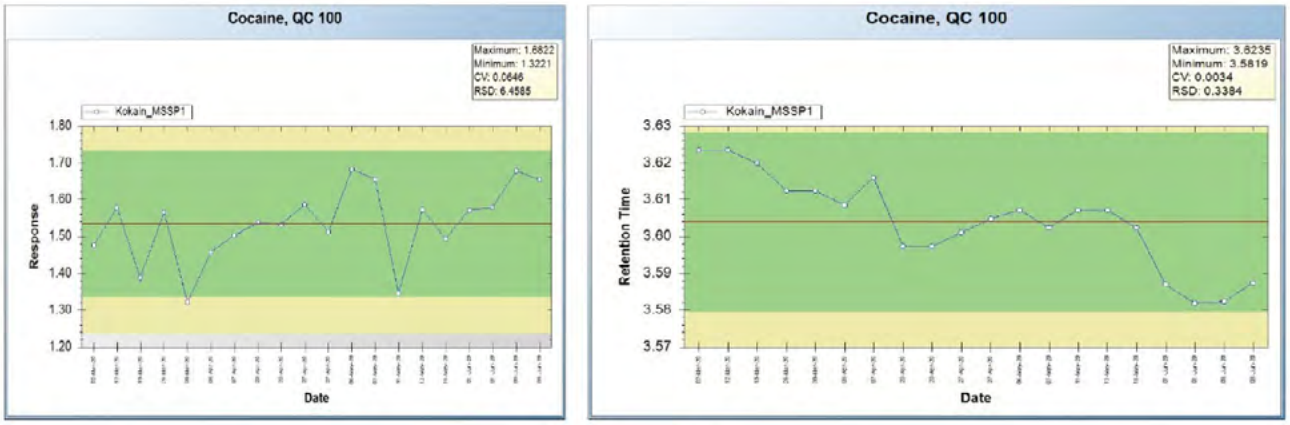

Figure 2. Fluctuation of response and retention time over time.

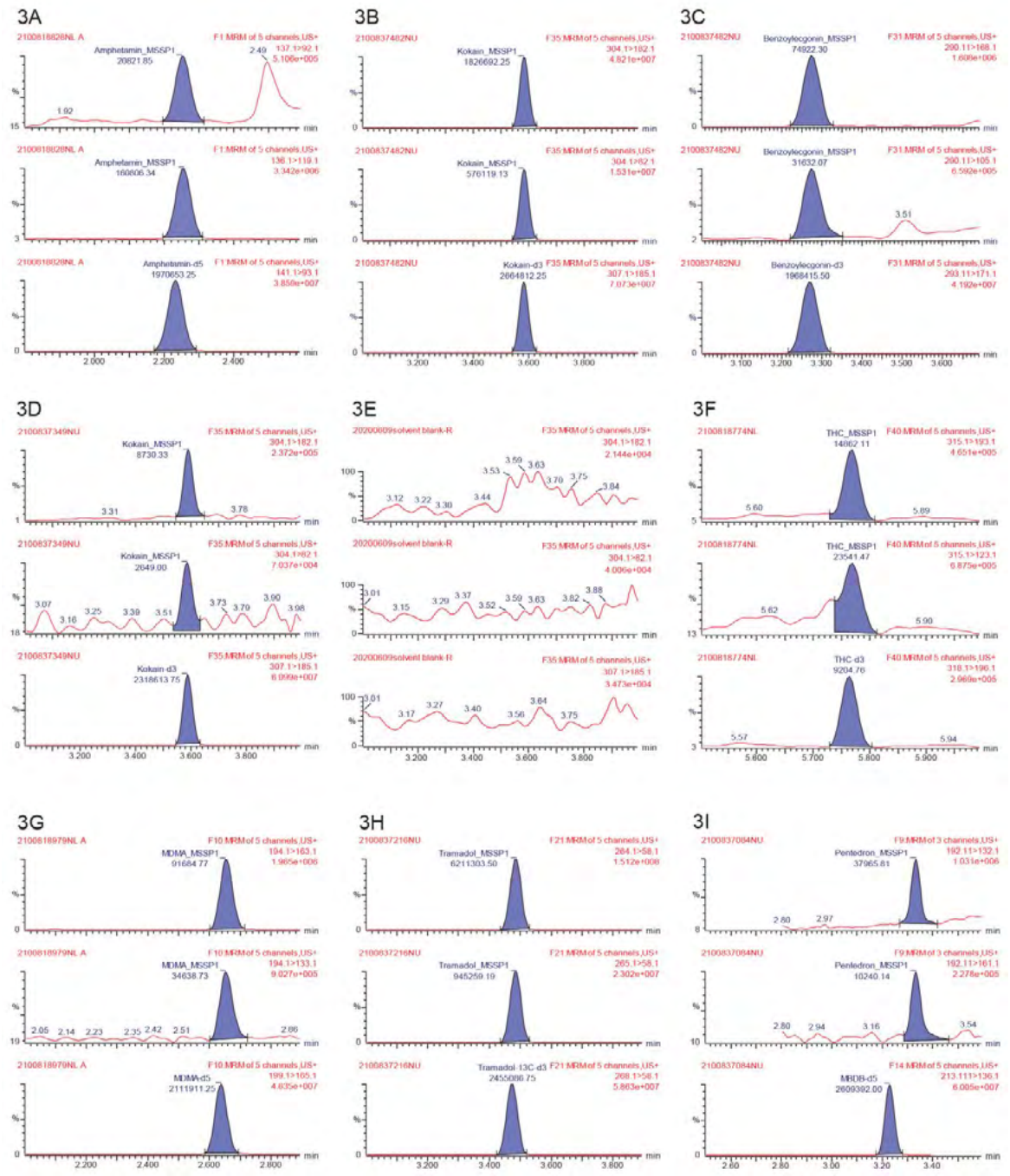

Figure 3. Chromatograms selected from the study samples.

a) Amphetamine at 20 pg/collector

a) Amphetamine at 20 pg/collector

b) Cocaine at 38 pg/collector

c) Benzoyl ecgonine from same sample as b)

d) Cocaine response from a negative study sample

e) Cocaine response in a system blank

f) THC at 102 pg/collector

g) MDMA at 2.1 pg/collector

h) Tramadol at 163 pg/collector

i) Pentedrone at 3.1 pg/collector

Out of the 47 analytes monitored in the method 20 was identified in the study samples (Table 3).

Substance	Number of positive samples	%	Lowest concentration pg/collector	Highest concentration pg/collector
Cocaine*	77	6.4	1.0	375
Amphetamine	46	3.8	1.1	2040
MDMA	22	1.8	1.2	117
THC	10	0.8	1.9	630
Methylphenidate	7	0.6	1.0	88
Tramadol**	5	0.4	1.2	163
Pentedrone	2	0.2	1.0	3.0
Alpha-PVP	2	0.2	1.0	2.0
Temazepam	2	0.2	1.0	1.1
Pregabalin	2	0.2	4.2	5.4
Ketamine	2	0.2	45	58
Methamphetamine	2	0.2	1.0	3.0
MDA	1	0.1	1.0	
Oxazepam	1	0.1	1.0	
Gabapentin	1	0.1	3.9	
Oxycodone	1	0.1	1.0	
Zopiclone	1	0.1	2.0	
Methadone	1	0.1	3.5	

* Benzoylecgonine was sometimes also detected
** O-desmethyl-tramadol was sometimes also detected

Table 3. Analytical findings in 1204 exhaled breath samples.

The use of exhaled breath as specimen made it possible to perform a study with biological sampling in the nightlife scene and provided new insight into drug use in this setting and a valuable complement to self-reports.

Cocaine was the most prevalent finding. It was noted that peaks for cocaine were present in all samples, but at a very low concentration. The background peaks fulfilled the criteria for identification but was lower that 10% of the applied LLOQ of 1 pg per collector. For comparison a system blank is also shown. This observation may agree

with the presence of cocaine in bank notes, sewage, and drinking water, as well as in the free air in major cities.[7] The breath samples were collected in an environment where cocaine users were present, and this might explain the observed background of cocaine.

Conclusion

The collection procedure for aerosol particles in exhaled breath is rapid, simple, and safe. There is no inconvenience for the individual being tested. The sampling procedure can be performed without any need of special facility and is well suited for situations outside the health care system, ***e.g.*** workplaces, roadside.

For using exhaled breath as specimen for drug testing the Xevo TQ-XS instrument provides the needed sensitivity and is a robust and suitable system for routine application.

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720007522, February 2022

Drugs of Abuse Testing

Application Notes

Application Note

LC-MS/MS Analysis of Urinary Benzodiazepines and Z-drugs via a Simplified, Mixed-Mode Sample Preparation Strategy

Jonathan P. Danaceau, Erin E. Chambers

Waters Corporation

For forensic toxicology use only.

Abstract

This application note describes a rapid and simplified solid phase extraction protocol and LC-MS/MS method for the analysis of urinary benzodiazepines and metabolites.

The unique water wettable nature of the Oasis MCX Sorbent enables the elimination of the common conditioning and equilibration steps without any loss in recovery or reproducibility. This property of Oasis also enables the entire hydrolysis step to be conducted within the wells of the Oasis MCX µElution Plate, eliminating time consuming and error-prone transfer steps, reducing the total number of post-incubation steps from 9 to 5. Combining this extraction procedure with the chromatography of the CORTECS C_{18}+ Column, and the sensitive and reproducible quantification of the Xevo TQ-S micro mass spectrometer, results in a rapid and efficient analysis method that is also exceptionally accurate. This method is simpler, faster, and easier, than liquid-liquid extraction and cleaner than reversed-phase SPE while providing excellent sensitivity, accuracy and precision for the analysis of this important class of compounds.

Benefits

- Rapid, simplified sample preparation of urinary benzodiazapines

Benefits

- Rapid, simplified sample preparation of urinary benzodiazapines
- Significant savings in solvent usage and disposal vs. liquid-liquid extraction
- Consistent recovery for all compounds
- Excellent accuracy and reproducibility
- All sample pretreatment and extraction performed in-well, eliminating transfer steps
- Reduced matrix effects vs. reversed-phase SPE

Introduction

Benzodiazepines are frequently prescribed drugs used for their sedative, anxiolytic, and hypnotic properties.[1] They work by potentiating the inhibitory neurotransmitter gamma-amino butyric acid (GABA). Nationally, overdose deaths from benzodiazepines have risen 600% from under 1,600/year in 2001 to 8,000 in 2014, greater than any other drug class with the exception of heroin.[2] So-called "Z-drugs" (zolpidem and zopiclone) are commonly used sleep aids that act in a similar manner to benzodiazepines.[1] While the use of LC-MS/MS for benzodiazepine analysis has increased in recent years, many published techniques still rely on labor intensive liquid-liquid extraction techniques.[3-5] Some of the drawbacks of these techniques include the need to process individual samples one by one, the use of toxic solvents, and the need to evaporate and reconstitute samples after extraction.

This application note details a sample preparation and LC-MS/MS analysis strategy for a comprehensive panel of benzodiazepines, metabolites, and Z-drugs for forensic toxicology use. Using an abbreviated, modified solid phase extraction (SPE) method, Waters Oasis MCX µElution Plates were used to rapidly extract this panel of drugs and metabolites from urine samples. All sample preparation steps, including enzymatic hydrolysis, were performed within the wells of the Oasis MCX µElution Plates, and the extraction method was simplified by eliminating conditioning and equilibration steps. This enabled a streamlined workflow that minimized sample transfer steps while still achieving excellent and reproducible quantitative results. Chromatographic separation was achieved using a CORTECS UPLC C_{18}+ Column while a Xevo TQ-S micro Mass Spectrometer was used for detection. Extraction recovery was efficient, averaging 91%, and the use of the mixed-mode sorbent reduced matrix effects compared to reversed-phase SPE. The CORTECS UPLC C_{18}+ Column enabled the baseline

separation of all target analytes from internal standards with identical nominal masses. This eliminated the risk of chromatographic interference between the labeled internal standards and the native compounds. All within and between batch quality control samples had mean accuracies within 5% of nominal values.

This method was also performed at HPLC scale using a CORTECS UPLC C_{18}+ 2.7 µm Column (3.0 x 100 mm) (p/n 186007372). The same efficient separation was seen as with the 1.6 µm column (p/n 186007402), with backpressures that remained under 4000 psi and a separation time that was increased by only 30%.

Experimental

All standards were obtained from Cerilliant (Round Rock, TX). Deuterated internal standards were used for all compounds with the exception of flurazepam. Stock solutions were prepared in methanol. Working standards were prepared daily by diluting stock standards in 80:20 water:methanol. Calibrators and QC samples were prepared in urine from working standards. All analytes are listed in Table 1, along with retention times and MS transitions and parameters.

	Compound	RT	M+H+	MRM product ions	Cone voltage	Collision energy
1	N-desmethyl zopiclone	1.07	375.1	245.0 331.0	6 6	14 8
2	Zopiclone	1.13	389.1	245.0 111.9	8 8	12 58
3	Zolpidem	1.62	308.1	235.1 92.0	34 34	32 52
4	7-aminoclonazepam	1.92	286.1	121.0 222.1	50 50	26 30
5	Flurazepam	2.32	388.2	315.1 100.0	40 40	26 28
6	7-aminoflunitrazepam	2.36	284.1	135.0 226.9	34 34	26 22
7	Chlordiazepoxide	2.35	300.0	227.0 283.0	34 34	20 12
8	Midazolam	2.53	326.0	291.0 222.9	16 16	36 24
9	α-OH-midazolam	2.91	342.0	203.0 168.0	2 2	24 40
10	α-OH-triazolam	3.78	359.0	176.0 140.8	28 28	24 38
11	α-OH-alprazolam	3.77	325.1	297.1 243.1	50 50	25 30
12	Oxazepam[1]	3.84	289.0	103.9 243.0	50 50	30 20
13	Nitrazepam	3.87	282.1	180.1 236.0	50 50	36 20
14	Lorazepam	4.01	321.0	277.0 229.0	50 50	20 30
15	Clonazepam	4.10	316.0	214.1 241.1	54 54	42 40
16	Alprazolam	4.35	309.1	205.0 281.1	50 50	40 26
17	Nordiazepam	4.36	271.0	140.0 165.0	50 50	30 28
18	Flunitrazepam	4.41	314.1	239.2 268.1	50 50	30 25
19	Temazepam	4.45	301.1	177.0 255.1	36 50	46 20
20	Triazolam	4.47	343.0	308.0 239.0	28 28	24 38
21	Diazepam	5.14	285.1	154.0 193.1	50 50	26 30

Table 1. Analyte list, retention times, and MS parameters for benzodiazepines and metabolites analyzed in this application.

[1]Oxazepam's parent ion was set at 289 to avoid interference with Nitrazepam-d5 seen with m/z 287.

Sample pretreatment

200 µL of urine was added to individual wells of an Oasis MCX µElution Plate, along with 20 µL of internal standard solution (250 ng/mL), and 200 µL of 0.5 M ammonium acetate buffer (pH 5.0) containing 10 µL of β-glucuronidase enzyme/mL of buffer (Sigma Aldrich, P. vulgate, 85k units/mL). The entire plate was incubated at 50 °C for 1 hr. and then quenched with 200 µL of 4% H_3PO_4.

SPE extraction

Pretreated samples were drawn into the sorbent bed by vacuum. All samples were subsequently washed with 200 µL of 0.02 N HCl, followed by 200 µL of 20% MeOH. After washing, the plate was dried under high vacuum (~15 inch Hg) for 30 seconds. Samples were eluted with 2 x 25 µL of 60:40 ACN:MeOH containing 5% strong ammonia solution (Fisher, 28–30%). All samples were then diluted with 100 µL of sample diluent (2% ACN:1% formic acid in MilliQ water). A graphical workflow of the extraction procedure is shown in Figure 1.

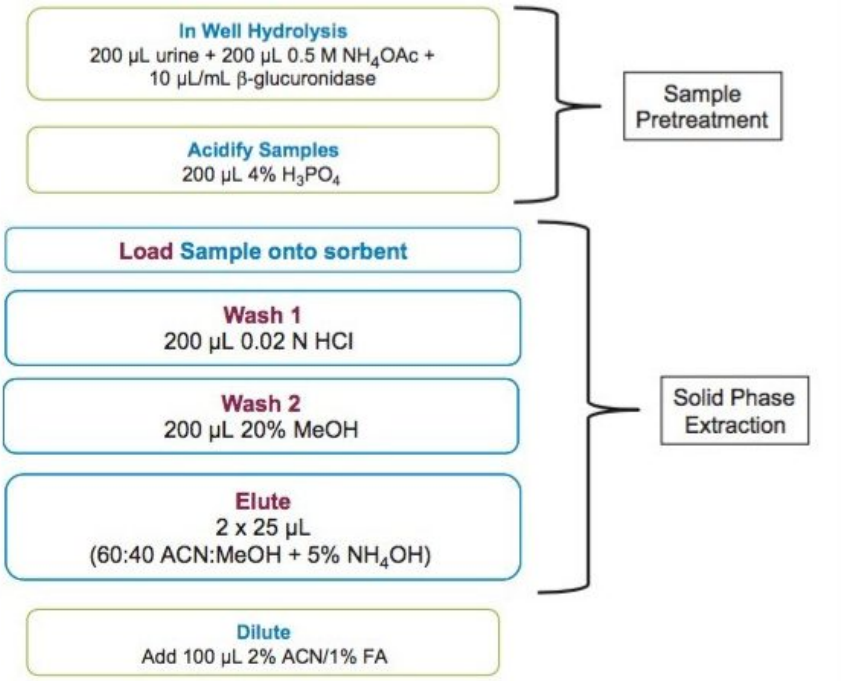

Figure 1. Details of the extraction method for the analysis of urinary benzodiazepines using Oasis MCX µElution Plates. Enzymatic hydrolysis and sample pretreatment are performed in the wells of the extraction plate, minimizing transfer steps. Conditioning and equilibration steps are eliminated, significantly simplifying the procedure.

Method conditions

LC conditions

System:	ACQUITY UPLC I-Class (FL)
Column:	CORTECS UPLC C_{18}+ 1.6 µm, 2.1 x 100 mm (p/n: 186007402)
Column temp.:	30 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.5 mL/min
Mobile phase A (MPA):	0.01% Formic acid in MilliQ water
Mobile phase B (MPB):	0.01% Formic acid in acetonitrile (ACN)
Gradient:	Initial conditions were 90:10 MPA:MPB. The percentage of MPB was increased to 50% over five minutes, ramped up to 95% by 5.25 minutes, held at 95% for 0.75 minutes and returned to 10% over 0.1 minute.

MS conditions

System:	Xevo TQ-S micro
Ionization mode:	ESI+

Detection:	MRM (transitions optimized for individual compounds, Table 1)
Capillary voltage:	0.5 kV
Collision energy:	Optimized for individual compounds (See Table 1)
Cone voltage:	Optimized for individual compounds (See Table 1)

Data management

MassLynx Software with TargetLynx Application Manager

Analyte recovery was calculated according to the following equation:

$$\%Recovery = \left(\frac{Area\ A}{Area\ B}\right) x100\%$$

Where A = the peak area of an extracted sample and B = the peak area of an extracted matrix sample in which the compounds were added post-extraction.

Results and Discussion

Chromatography

All test compounds are listed in Table 1, and their chromatography is shown in Figure 2. Table 1 also lists the retention times and MS conditions of all compounds. Several columns were evaluated for this application, but the selectivity of the CORTECS UPLC C_{18}+ Column enables the baseline separation of all potentially interfering peaks. Two key pairs are shown in Figure 3. While clonazepam-d4 (R.T. 4.08) generates a slight contribution to the primary lorazepam MRM (323>277), the two peaks are baseline separated. Even at the LLOQ (0.5 ng/mL), the clonazepam IS does not interfere with lorazepam and does not affect quantification of the peak. Another

critical pair is alprazolam-d5 and flunitrazepam. In this case, flunitrazepam makes a contribution that can be seen in the MRM trace of alprazolam-d5 (314.1>210.1). However, the baseline separation of these peaks ensures that even at the ULOQ (500 ng/mL) the baseline separation prevents flunitrazepam from affecting the integration and quantification if the alprazolam IS.

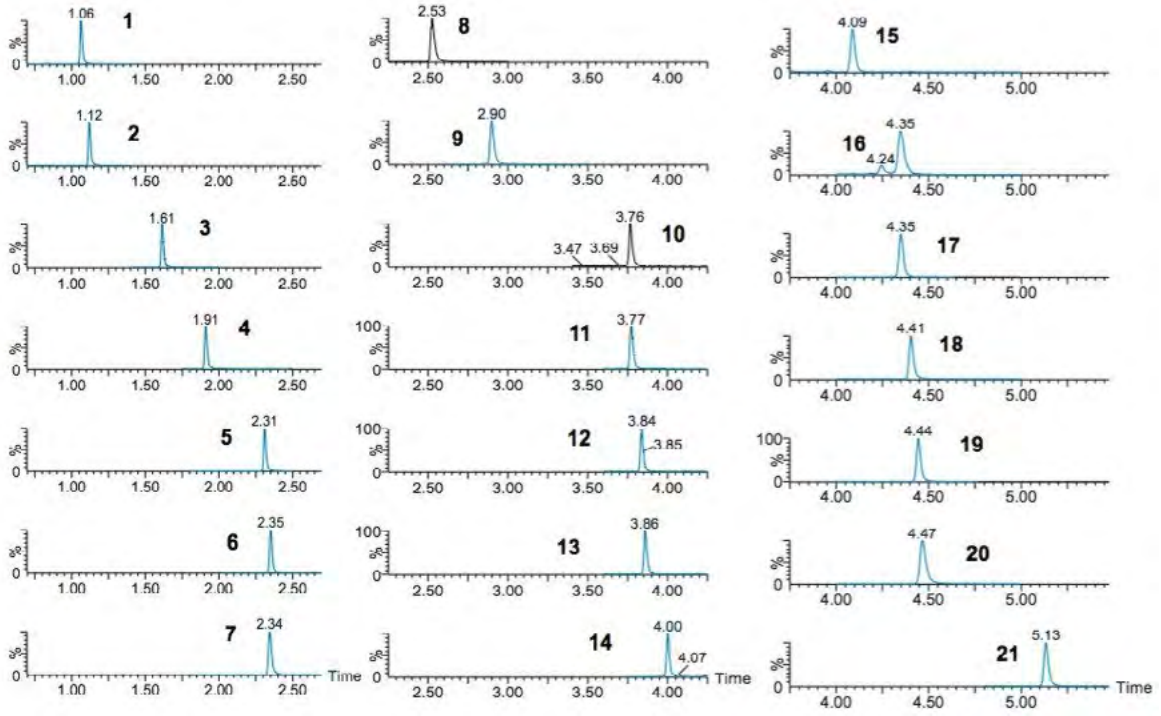

Figure 2. Chromatography of benzodiazepines analyzed in this application. See Table 1 for compound key. Column: CORTECS UPLC C_{18}+ 1.6 µm, 2.1 x 100 mm.

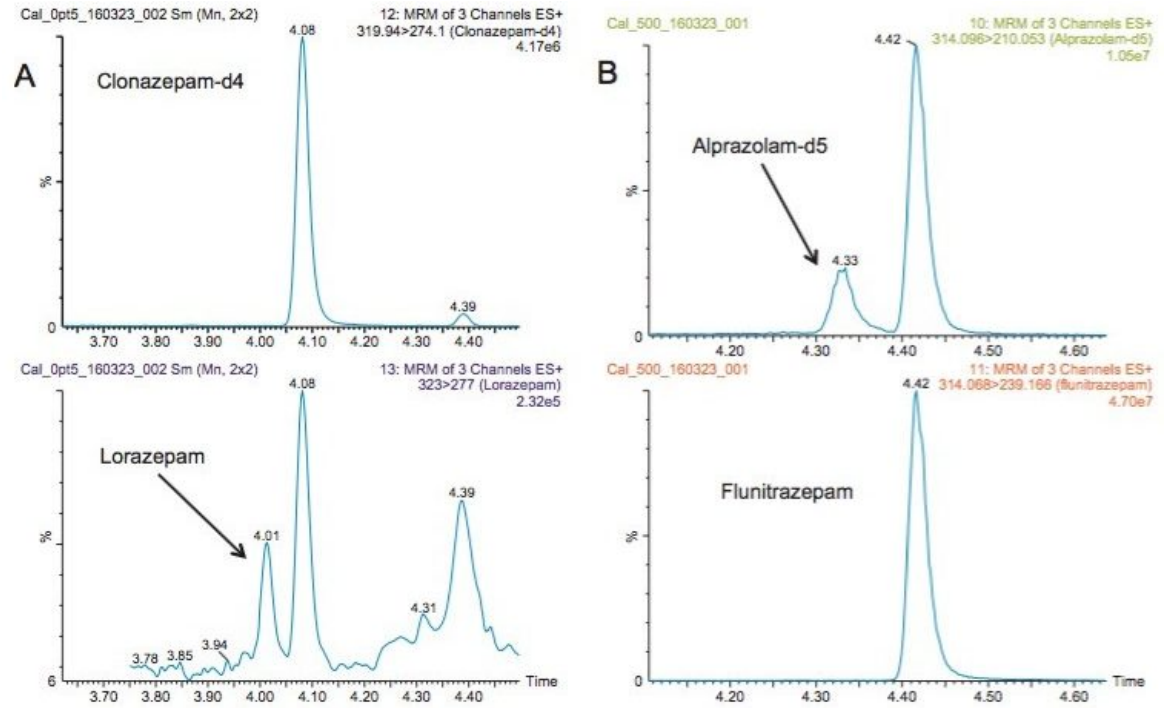

Figure 3. Chromatographic separation of key analyte pairs on the CORTECS UPLC C_{18}+ 1.6 µm Column. A. Clonazepam-d4 contributes to the lorazepam MRM but is baseline separated on this column. B. Alprazolam-d5 at 4.33 minutes is baseline separated from flunitrazepam at 4.42 minutes.

This panel was also analyzed on an HPLC scale using a CORTECS UPLC C_{18}+ 2.7 µm Column (3.0 x 100 mm) and an ACQUITY UPLC H-Class System. Table 2 compares the retention times of the UPLC and HPLC methods. All critical separations were maintained under HPLC conditions. The maximum system pressure stayed below 4000 psi. The retention time of diazepam, the latest eluting peak, only increased from 5.14 to 6.69, a 30% increase, and the solvent ramp duration increased from seven to nine minutes. The increase in retention time was likely due to the decreased linear velocity of the mobile phase resulting from the larger interior diameter of the HPLC column (3.0 mm vs 2.1 mm) and the decrease in the slope of the solvent ramp. If run on a traditional HPLC system, the increase in dwell volume would likely result in an increase in peak width. Nevertheless, the scalability of the CORTECS UPLC C_{18}+ Column should make this adjustment straightforward. While ACQUITY UPLC will provide the fastest and most efficient separation, this enables the method to be performed on HPLC instrumentation if necessary.

Recovery and matrix effects

Figure 4 shows the composite extraction recoveries of the entire panel of compounds from four separate experiments. Recoveries ranged from 76 to 102% with an average of 91%, demonstrating excellent extraction efficiency. The recoveries were consistent as well, with coefficients of variation (%CVs) ranging from 5.2% to 15%, with a mean of 8.6%. The extraction method was modified from a traditional MCX method for basic compounds. The first wash step was modified from aqueous 2% formic acid to 0.02 N HCl to account for the low pKas of compounds such as clonazepam, flunitrazepam, and alprazolam and ensure ion-exchange retention on the MCX sorbent. A series of experiments performed during method development revealed that more than 20% methanol in the wash step resulted in loss of the acidic benzodiazapines, such as oxazepam, lorazepam, and temazepam. Thus, the second wash step consisted of 20% methanol, the strongest organic wash possible that did not result in analyte loss during the wash step. These modifications maximized reversed-phase and ion-exchange retention and enabled the highly efficient and most selective extraction of the entire panel of benzodiazepines.

	Compound	RT-UPLC	RT-HPLC
1	N-desmethyl zopiclone	1.07	1.98
2	Zopiclone	1.13	2.05
3	Zolpidem	1.62	2.58
4	7-aminoclonazepam	1.92	3.05
5	Flurazepam	2.32	3.37
6	7-aminoflunitrazepam	2.36	3.55
7	Chlordiazepoxide	2.35	3.39
8	Midazolam	2.53	3.57
9	α–OH-midazolam	2.91	3.98
10	α–OH-triazolam	3.78	4.95
11	α–OH-alprazolam	3.77	4.93
12	Oxazepam[1]	3.84	5.16
13	Nitrazepam	3.87	5.28
14	Lorazepam	4.01	5.32
15	Clonazepam	4.10	5.51
16	Alprazolam	4.35	5.52
17	Nordiazepam	4.36	5.78
18	Flunitrazepam	4.41	5.90
19	Temazepam	4.45	5.89
20	Triazolam	4.47	5.65
21	Diazepam	5.14	6.69

Table 2. UPLC and HPLC retention times for benzodiazepines and z-drugs.

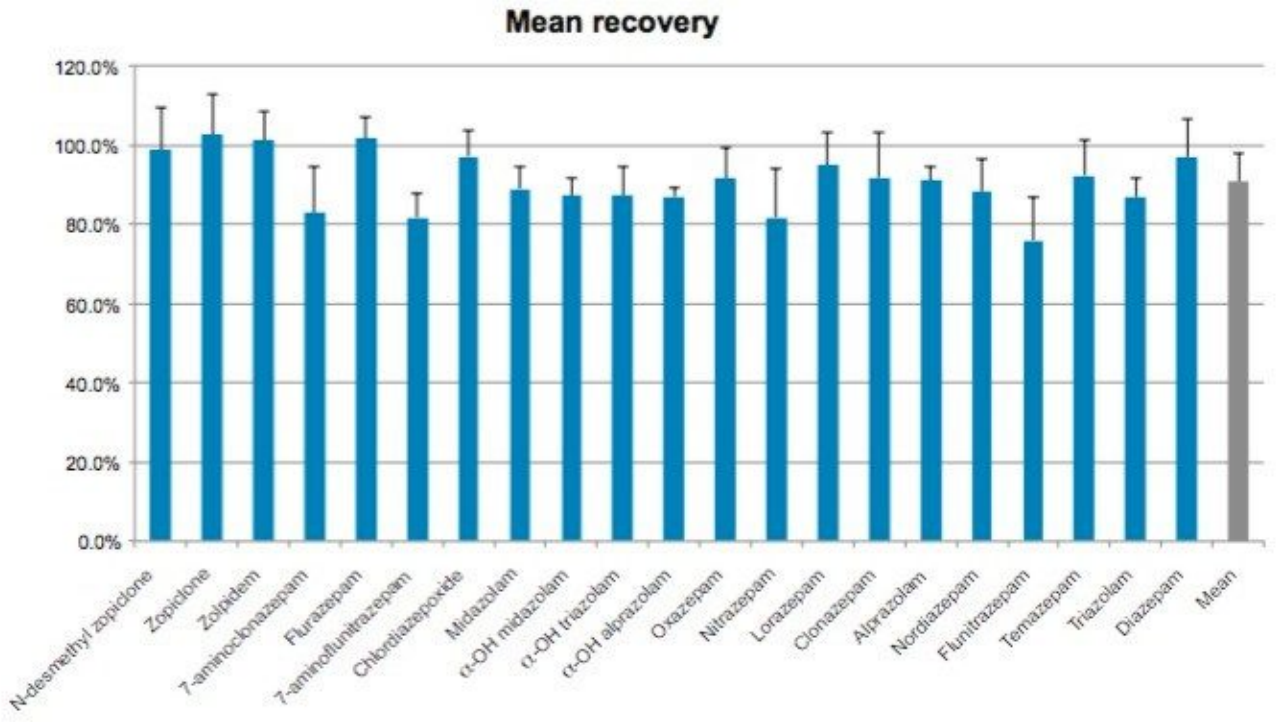

Figure 4. Extraction recovery for the compounds in this application. Values represent the mean of four individual extractions. Recoveries ranged from 76%–102.5% with an average recovery of 91%. Direct loading of the sorbent, without conditioning and equilibration had no impact on analyte recovery.

Two key benefits of this method take advantage of the water-wettable nature of the Oasis sorbent, the ability to directly load without conditioning and equilibration, and the ability to conduct all hydrolysis and pretreatment within the well of the SPE plate. The traditional six-step mixed-mode SPE method was simplified into just four steps. This was accomplished by eliminating the conditioning and equilibration steps. This simplification had no effect on the extraction efficiency of the method (data not shown), and is consistent with the water wettable nature of the Oasis sorbent. This also enables all sample hydrolysis and pretreatment to be performed within the wells of the 96-well plate, eliminating the need to transfer the sample from an incubation vessel to the SPE plate, a step that can be time consuming and error prone. After incubation within the wells of the Oasis MCX µElution Plate, the samples were simply mixed with 4% H_3PO_4 to quench the hydrolysis reaction and ionize the basic benzodiazepines, which were then drawn directly onto the sorbent. No leakage or well blockages were seen in any of the method development or validation experiments. Overall, this method reduces the number of post-incubation steps from nine to five by eliminating conditioning, equilibration, the transfer of samples to the SPE device, and sample evaporation compared to a traditional SPE workflow.

Matrix effects are shown in Figure 5. As with analyte recoveries, matrix effects were equivalent between the direct loaded samples and those in which the sorbent was conditioned and equilibrated. Matrix effects were also compared to traditional reversed phase extraction with Oasis PRiME HLB. Absolute matrix effects were 17.7% for Oasis MCX µElution plate prepared samples vs. 25.3% for Oasis PRiME HLB prepared samples (data not shown), demonstrating the superior cleanup of mixed-mode SPE for this group of analytes.

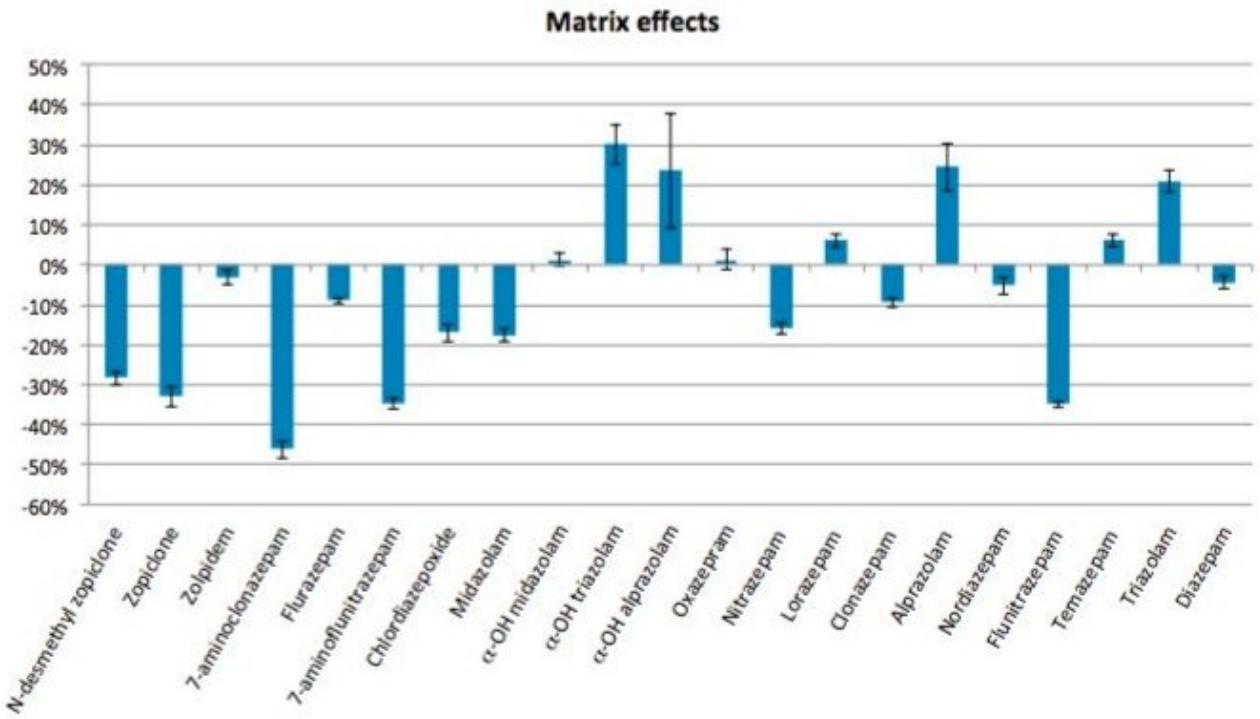

Figure 5. Matrix effects for benzodiazepines. Absolute matrix effects were reduced from 25.3% to 17.7% by using Oasis MCX mixed-mode SPE Plates vs. reversed-phase sorbent (Oasis HLB).

Quantitative results

Calibration curves ranged from 0.5 ng/mL through 500 ng/mL for all compounds. All compounds had LOQs of 0.5 ng/mL and ULOQs of 500 ng/mL. Quality control samples were prepared at 1.5, 7.5, 75, and 300 ng/mL. A calibration summary is shown in Table 3. Six of the curves were fitted with a 1/x weighted linear curve, while 15 were best fit with a 1/x weighted quadratic curve. Figure 6 shows examples of compounds best fit with a linear curve (nitrazepam, alprazolam), and a quadratic fit curve (diazepam, 7-aminoclonazepam). Regardless of the

function used, fits were excellent and fit for purpose for the analytical needs of the method. Seventeen compounds had R2 values of 0.999 or greater, and the remaining compounds had R2 values of 0.997 or greater. Table 3 also shows that the mean % deviations for all compounds were less than 10%. Additionally, Tables 4 and 5 show the results of within-batch and between-batch QC results. The within-batch results show both excellent accuracy and precision. The mean accuracies for all compounds at the four QC levels were 107.8%, 98.5%, 97.5%, and 97.5%. For the highest three QC values (7.5, 75, and 300 ng/mL) all individual accuracies were within 10% of target values and all %CVs were less than 10%. The between-batch results shown in Table 5 were, if anything, even better. Mean accuracies were 102.1%, 99.3%, 98.2%, and 96.8% at the four QC levels. Individual CVs ranged from 1.1% to 9.0%. These high levels of accuracy and precision demonstrate the consistency and reliability of the Oasis MCX sorbent and extraction technique, and demonstrate that there is no compromise of result quality, even with the in-well hydrolysis and direct sorbent loading used in this assay. They also show that the quadratic curves used are fit for purpose and meet the needs of the assay.

Name	R^2	Lin/Quad	Mean %Dev
N-desmethyl zopiclone	0.999	L	5.4
Zopiclone	0.998	L	5.4
Zolpidem	0.999	Q	4.1
7-aminoclonazepam	1.000	Q	2.3
Flurazepam	0.998	Q	4.1
7-aminoflunitrazepam	0.997	L	6.2
Chlordiazepoxide	1.000	Q	3.4
Midazolam	1.000	Q	4.8
α-OH midazolam	0.999	Q	4.0
α-OH triazolam	1.000	Q	4.4
α-OH alprazolam	0.999	Q	9.0
Oxazepam	1.000	Q	6.2
Nitrazepam	0.999	L	4.6
Lorazepam	0.999	Q	4.4
Clonazepam	1.000	Q	6.2
Alprazolam	0.998	L	9.9
Nordiazepam	0.999	Q	6.6
Flunitrazepam	0.999	L	3.9
Temazepam	0.999	Q	5.3
Triazolam	0.999	Q	4.1
Diazepam	0.999	Q	3.7

Table 3. Calibration summary for all compounds in this application. The mean %deviation refers to the average of the absolute value of the deviations of all points in the curve.

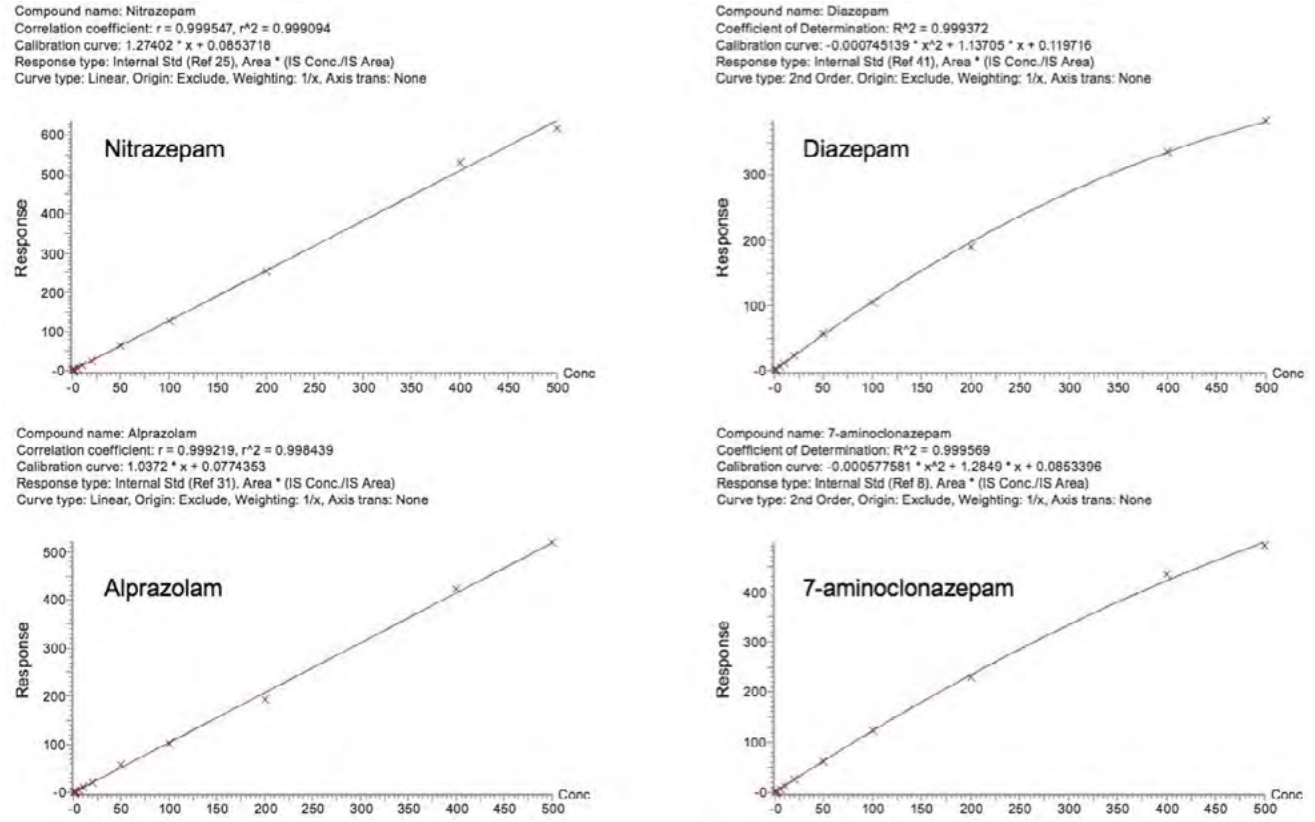

Figure 6. Representative calibration curves of benzodiazepines. Nitraepam and alprazolam were fit with a 1/x linear curve, while diazepam and 7-aminoclonazepam were best fit with a quadratic 1/x weighted curve.

	QC 1.5		QC 7.5		QC 75		QC 300		
Name	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV	Mean
N-desmethyl zopiclone	103.6%	7.3%	99.3%	3.1%	99.8%	2.0%	98.2%	3.9%	**100.2%**
Zopiclone	101.4%	7.4%	100.6%	3.2%	100.9%	2.3%	98.1%	4.0%	**100.3%**
Zolpidem	102.7%	6.7%	100.1%	2.5%	96.8%	0.9%	93.4%	4.2%	**98.2%**
7-aminoclonazepam	102.3%	8.1%	96.5%	2.6%	95.4%	1.0%	96.8%	3.5%	**97.8%**
Flurazepam	111.0%	9.0%	95.8%	4.5%	96.0%	2.1%	99.2%	4.7%	**100.5%**
7-aminoflunitrazepam	101.9%	10.9%	95.8%	4.5%	98.4%	1.7%	97.5%	3.6%	**98.4%**
Chlordiazepoxide	100.7%	9.5%	97.8%	4.2%	98.5%	1.0%	100.3%	6.1%	**99.3%**
Midazolam	107.0%	9.9%	98.3%	2.3%	98.6%	2.3%	99.4%	2.8%	**100.8%**
α-OH midazolam	107.4%	8.1%	99.5%	2.3%	99.0%	1.6%	101.1%	3.8%	**101.8%**
α-OH triazolam	109.9%	9.2%	95.1%	2.3%	93.1%	1.6%	94.5%	5.4%	**98.1%**
α-OH alprazolam	114.5%	12.6%	98.9%	5.2%	94.1%	4.5%	95.4%	8.3%	**100.7%**
Oxazepam	105.4%	6.3%	94.6%	3.2%	96.9%	1.4%	95.6%	3.1%	**98.1%**
Nitrazepam	108.8%	7.7%	96.8%	2.6%	97.0%	0.8%	98.2%	3.5%	**100.2%**
Lorazepam	107.0%	7.2%	95.5%	2.0%	96.1%	2.0%	97.4%	4.0%	**99.0%**
Clonazepam	106.7%	10.6%	97.2%	3.0%	95.4%	2.0%	94.6%	3.8%	**98.4%**
Alprazolam	116.8%	10.0%	99.3%	5.7%	98.7%	4.4%	101.3%	6.1%	**104.0%**
Nordiazepam	110.9%	10.1%	103.2%	2.4%	99.2%	1.6%	96.3%	3.0%	**102.4%**
Flunitrazepam	111.1%	8.2%	101.4%	2.4%	97.2%	1.9%	100.7%	4.3%	**102.6%**
Temazepam	110.6%	8.0%	102.8%	2.7%	98.5%	1.4%	95.4%	6.0%	**101.8%**
Triazolam	113.6%	8.4%	103.4%	2.5%	101.1%	2.4%	99.4%	1.8%	**104.4%**
Diazepam	110.3%	7.9%	101.5%	2.3%	97.3%	0.8%	95.3%	3.6%	**101.1%**
Mean	**107.8%**		**98.7%**		**97.5%**		**97.5%**		

Table 4. Within-batch QC results. N=6. Mean values show the average for each compound and the average for all compounds at each QC level.

	QC 1.5		QC 7.5		QC 75		QC 300		
Name	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV	Mean
N-desmethyl zopiclone	99.2%	3.8%	96.7%	2.4%	96.6%	2.9%	97.1%	4.7%	**97.4%**
Zopiclone	97.7%	3.2%	96.7%	3.4%	98.0%	2.8%	96.2%	3.5%	**97.2%**
Zolpidem	99.4%	3.4%	98.8%	1.5%	95.8%	1.1%	91.7%	1.6%	**96.4%**
7-aminoclonazepam	100.4%	1.9%	95.6%	1.0%	93.8%	2.4%	95.1%	2.0%	**96.2%**
Flurazepam	103.6%	7.1%	97.6%	4.3%	99.3%	7.4%	97.6%	5.0%	**99.5%**
7-aminoflunitrazepam	99.3%	2.3%	93.7%	2.3%	96.1%	4.7%	97.0%	3.2%	**96.5%**
Chlordiazepoxide	100.5%	1.1%	100.3%	2.1%	99.3%	1.5%	98.4%	3.2%	**99.6%**
Midazolam	103.7%	4.4%	104.2%	5.4%	102.1%	3.1%	98.9%	2.0%	**102.2%**
α-OH midazolam	103.4%	4.3%	102.5%	4.7%	100.8%	5.0%	99.1%	2.5%	**101.4%**
α-OH triazolam	101.5%	8.4%	98.8%	4.9%	98.3%	4.9%	95.1%	2.6%	**98.4%**
α-OH alprazolam	104.4%	9.6%	101.4%	2.2%	99.1%	5.9%	97.7%	2.4%	**100.7%**
Oxazepam	100.4%	4.3%	98.5%	4.1%	98.2%	4.7%	97.6%	4.6%	**98.7%**
Nitrazepam	102.0%	6.2%	95.8%	1.3%	95.7%	2.4%	98.1%	1.8%	**97.9%**
Lorazepam	100.3%	6.9%	100.2%	4.2%	100.8%	5.4%	98.7%	4.9%	**100.0%**
Clonazepam	102.0%	4.9%	98.2%	3.0%	97.5%	3.3%	95.2%	4.5%	**98.2%**
Alprazolam	107.0%	8.7%	94.6%	4.7%	95.0%	4.6%	98.8%	4.5%	**98.9%**
Nordiazepam	106.1%	9.0%	106.7%	3.7%	101.7%	4.6%	95.4%	5.2%	**102.5%**
Flunitrazepam	101.8%	8.1%	98.2%	2.8%	96.3%	2.6%	96.3%	7.8%	**98.1%**
Temazepam	102.9%	7.3%	101.6%	1.2%	97.5%	2.8%	94.7%	1.8%	**99.2%**
Triazolam	104.4%	8.4%	102.4%	2.3%	99.9%	3.2%	98.2%	3.4%	**101.2%**
Diazepam	104.3%	6.5%	103.8%	2.1%	99.6%	4.1%	94.9%	7.6%	**100.6%**
Mean	**102.1%**		**99.3%**		**98.2%**		**96.8%**		

Table 5. Between-batch QC results. Values represent the mean and %CV of four separate extraction batches. Mean values show the average for each compound and the average for all compounds at each QC level.

Conclusion

This application note describes a rapid and simplified solid phase extraction protocol and LC-MS/MS method for the analysis of urinary benzodiazepines and metabolites for forensic toxicology use. The unique water wettable nature of the Oasis MCX sorbent enables the elimination of the common conditioning and equilibration steps without any loss in recovery or reproducibility. This property of Oasis also enables the entire hydrolysis step to be conducted within the wells of the Oasis MCX µElution plate, eliminating time consuming and error-prone transfer steps, reducing the total number of post-incubation steps from nine to five. This extraction procedure combining the chromatography of the CORTECS UPLC C_{18}+ Column and the sensitive and reproducible quantification of the Xevo TQ-S micro results in a rapid and efficient analysis method that is also exceptionally accurate. This method is simpler, faster, and easier than liquid-liquid extraction. It is also cleaner than reversed-phase SPE while providing excellent sensitivity, accuracy, and precision for the analysis of this important class of compounds.

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Application Note

Determination of Drugs of Abuse in Whole Blood Using the Ostro™ Pass-through Sample Preparation Plate

Michelle Wood, Emily Lee

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

The use of whole blood, as a biological matrix for forensic testing, has been popular for many years. As whole blood is considered a complex matrix, any analytical workflow should include a robust sample preparation method. Here we describe a simple pass-through sample preparation method. The method has been applied to a large panel of drug substances which covers differing chemical classes and will support a range of forensic toxicology testing scenarios.

Benefits

- Single generic sample preparation protocol using an Ostro Pass-Through Sample Preparation Plate
- Precipitation, filtration, and sample clean-up in one device
- Multiple drug classes separated on either a BEH™ C_{18} ACQUITY™ UPLC or HSS C_{18} ACQUITY Column using an ACQUITY UPLC I-Class
- Excellent sensitivity of the Xevo™ TQ-S micro mass spectrometer allows for the analytes to be detected at toxicologically relevant concentrations

Introduction

Forensic toxicology deals with the investigation of drugs or toxic substances within a biological specimen. To this end, laboratories require reliable methods that can detect a wide variety of these toxicants in postmortem and antemortem specimens.

The use of whole blood as a biological matrix for forensic testing has been popular for many years. For example, it is the matrix of choice for investigations into probable cause of death, where analytical protocols commonly involve the application of general unknown screening procedures (also known as 'systematic toxicological analysis') that are designed to screen for potentially hundreds of xenobiotics. These may be banned or illicit drugs, emerging psychoactive substances, or prescribed and over-the-counter medications.

Blood is also the most frequently used specimen for confirmatory analysis where recent drug ingestion is suspected, for example, in investigations into driving under the influence (DUID), as the quantitative measurement of drugs in blood is considered to provide the most accurate picture of the current effects of drugs on the body as some blood concentrations are known to correlate well with impairment. As driving under the influence is a significant concern globally, most countries around the world now have legislation in place to improve road safety and to decrease motor vehicle accidents. Typically, this involves monitoring a defined panel of specific drug substances. For example, in England and Wales, analytical methods currently target a panel of 17 specific drug substances to support the requirements of Section 5A of the Road Traffic Act 1988,[1] while in the United States the National Safety Council's Drugs and Impairment Division recommends a panel of compounds that it considers crucial for monitoring in drivers (Tier 1 compounds) as these substances are frequently found in arrests associated with impaired driving.[2] However, as there is an awareness that there are many other drug substances, or combinations of drugs, that can pose the risk of impairment, a much wider panel of substances is also of interest for more comprehensive testing as countries continue to adapt their own testing protocols and recommendations for monitoring drivers, depending on the drugs used/prescribed in their specific region and their prevalence of use.

Forensically relevant substances can be quite diverse, covering different chemical classes and ranging from polar bases, such as opiates and amphetamines to non-polar acids, such as non-steroidal anti-inflammatory drugs, therefore when monitoring these substances the laboratory will need to employ a generic sample preparation method that is capable of extracting all of these different chemical classes from the whole blood, while still providing some clean-up of the sample prior to the use of sensitive analytical procedures such as UPLC™-MS/MS.

Previously we have described a simple clean-up protocol for whole blood based on Waters Ostro Pass-Through Sample Preparation Plate.[3] Ostro combines removal of proteins and phospholipids and filtration in one device. The method was applied in combination with UPLC-MS/MS methods for the analysis of the specific panel of 17 drugs to support the specific requirements of the Section 5A of the England and Wales Road Traffic Act 1988. In this study we apply the same protocol to much wider panel of drug substances that will support a broader range of forensic testing scenarios.

Experimental

Control human whole blood (K2 EDTA, pooled) was supplied by Bio-IVT (Burgess Hill, West Sussex, UK).

Reference material for toxicologically relevant substances were obtained from Merck (Poole, Dorset, UK), LGC (Teddington, London, UK). These were supplied as individual 1mg/mL solutions in either methanol or acetonitrile. Analytes were combined to produce several mixed-drug spiking solutions in methanol.

Control whole blood was spiked with a range of drugs that covered the following groups at the following concentrations: the analytes which ionize in electrospray positive mode (ESI+) were spiked at 100 ng/mL, those which ionize in electrospray negative (ESI-) mode were spiked at 200 ng/mL and the cannabinoids were spiked at 10 ng/mL. Aliquots of the blood was prepared as previously described.[3] In brief, an aliquot (100 µL) of control or spiked blood was added to 100 µL zinc sulphate/ammonium acetate solution in the well of an Ostro Sample Preparation 96-well Plate (p/n: 186005518 <https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186005518-ostro-protein-precipitation--phospholipid-removal-plate-25-mg-1-.html>) and briefly mixed. Elution solvent (600 µL of 0.5% formic acid in acetonitrile) was added to the wells and the plate vortex-mixed for three minutes. The plate was placed onto a vacuum manifold and the elution solvent was drawn into a Waters 2 mL Square-well Collection Plate (p/n: 186002482 <https://www.waters.com/nextgen/global/shop/vials-containers--collection-plates/186002482-96-well-sample-collection-plate-2-ml-square-well-50-pk.html>). Three

separate aliquots (3 x 150 µL) of the eluant were transferred to a Round-well Collection Plate (p/n: 186002481 < https://www.waters.com/nextgen/global/shop/vials-containers--collection-plates/186002481-96-well-sample-collection-plate-800--l-round-well-50-pk.html>) and taken to dryness using an Ultravap Mistral microplate evaporator (Porvair). Dried wells were reconstituted by addition of 50 µL of the appropriate solvent for the specific UPLC-MS/MS method used to analyze the samples, as detailed below.

The drugs that ionize in ESI+ mode were analyzed by a UPLC-MS/MS method using an ACQUITY HSS C_{18} Column (2.1 x 150 mm, 1.8 µm, p/n: 186003534 < https://www.waters.com/nextgen/global/shop/columns/186003534-acquity-uplc-hss-c18-column-100a-18--m-21-mm-x-150-mm-1-pk.html>) with mobile phases containing 0.1% formic acid in water and acetonitrile. The reconstitution solvent for this assay was 5% acetonitrile in 0.1% formic acid.

The drugs that ionize in ESI- mode were analyzed on the same ACQUITY HSS C_{18} Column but with mobile phases containing 0.001% formic acid in water and 0.001% formic acid in acetonitrile. The reconstitution solvent for this assay was 10% acetonitrile in 0.001% formic acid.

A separate method, for the measurement of cannabinoids was based on the ACQUITY BEH C_{18} Column (2.1 x 100 mm, 1.7 µm; p/n: 186002352 <https://www.waters.com/nextgen/global/shop/columns/186002352-acquity-uplc-beh-c18-column-130a-17--m-21-mm-x-100-mm-1-pk.html>) with mobile phases containing 0.05% formic acid in water (mobile phase A) and 0.05% formic acid in acetonitrile (mobile phase B). The reconstitution solvent for this assay was 0.05% formic acid in acetonitrile.

For each UPLC-MS/MS method 2 MRM transitions (where possible) were monitored for each analyte.

Results and Discussion

In total 155 analytes were investigated using the developed sample preparation procedure, these included 110 substances that were analyzed using a broad MRM screening method in ESI+, 40 analyzed using a broad screen MRM in ESI- and 5 cannabinoids detected using a dedicated MRM with switching between ESI+/ESI-.

All substances investigated in this study were detected at the investigated concentrations and are listed in Table 1.

6-MAM	Clozapine, Desmethyl	Lorazepam	Normeperidine	Temazepam	Felodipine	Carboxy THC
7-Aminoclonazepam	Cocaethylene	Lormetazepam	Nortryptyline	Thioridazine	Fenoprofen	Cannabinol
Acetylcodeine	Cocaine	LSD	Oxazepam	Timolol	Flufenamic acid	Cannabidiol
Alprazolam	Codeine	Maprotilline	Oxazepam glucuronide	Tramadol	Fluvastatin	Hydroxy THC
Alprenolol	Codeine glucuronide	MDA	Oxprenolol	Trazadone	Furosemide	THC
Amiodorone	Diazepam	MDEA	Oxycodone	Triprolidine	Hydrochlorothiazide	
Amitriptyline	Dihydrocodeine	MDMA	Oxymorphone	Venlafaxine	Hydroflumethiazide	
Amphetamine	Dihydrocodeine glucuronide	Meperidine	Paracetamol	Verapamil	Ibuprofen	
Atenolol	Doxapram	Mephedrone	Paroxetine	Zolpidem	Indomethacin	
Atropine	Doxepine	Meprobamate	PCP	Zopiclone	Ketoprofen	
Brompheniramine	Ecgonine methyl ester	Methadone	Perphenazine	Acetazolamide	Metolazone	
Buprenorphine	EDDP	Methamphetamine	Phenmetrazine	Amobarbital	Naproxen	
Buprenorphine glucuronide	Enalapril	Methylone	PMMA	Barbital	Parecoxib	
Benzoykecgonine	Ephedrine	Midazolam	Primidone	Bendroflumethiazide	Pentobarbital	
Caffeine	Ethylone	Morphine	Procyclidine	Benzthiazide	Phenobarbital	
Carisoprodol	Fentanyl	Morphine glucuronide	Propanolol	Bezafibrate	Phenytoin	
Cetirizine	Flunitrazepam	Nadolol	Propoxyphene	Bumetanide	Piroxicam	
Chlordiazepoxide	Fluoxetine	Naltrexone	Protriptyline	Butabarbital	Pravastatin	
Chloroquine	Flurazepam	Nifedipine	Ramipril	Butalbital	Probenecid	
Chlorpheniramine	Gabapentin	Nitrazepam	Ranitidine	Celecoxib	Rofecoxib	
Citalipram, Desmethyl	Gliclazide	Norbuprenorphine	Risperidone, Hydroxy	Chlorothiazide	Salicylic acid	
Citalopram	Haloperidol	Norbuprenorphine glucuronide	Scopoloamine	Chlorthalidone	Secobarbital	
Clobazam	Hydrocodone	Nordiazepam	Sertraline	Chlorzoxazone	Tenoxicam	
Clonazepam	Hydromorphone	Norfentanyl	Sildenafil	Cyclothiazide	Thiopental	
Clozapine	Ketamine	Norketamine	Sotalol	Etodolac	Tolvaptan	

Table 1. Substances evaluated with the pass-through Ostro procedure; compounds shaded in green were analysed using a MRM screen in ESI+. Substances shaded in blue were analysed using an ESI- screen. Cannabinoids (shaded pink) were analysed using a separate dedicated method with ESI+/ESI- switching).

Figure 1 shows an example of a typical mixture containing twenty substances which were prepared at 10 ng/mL in whole blood, prepared using the pass-through sample preparation method as described and analysed using UPLC-MS/MS in ESI+. Figure 2 shows analysis of blood containing a mixture of negatively ionizing compounds spiked at 200 ng/mL and Figure 3 shows the resultant chromatogram for the cannabinoids spiked in whole blood at 10 ng/mL.

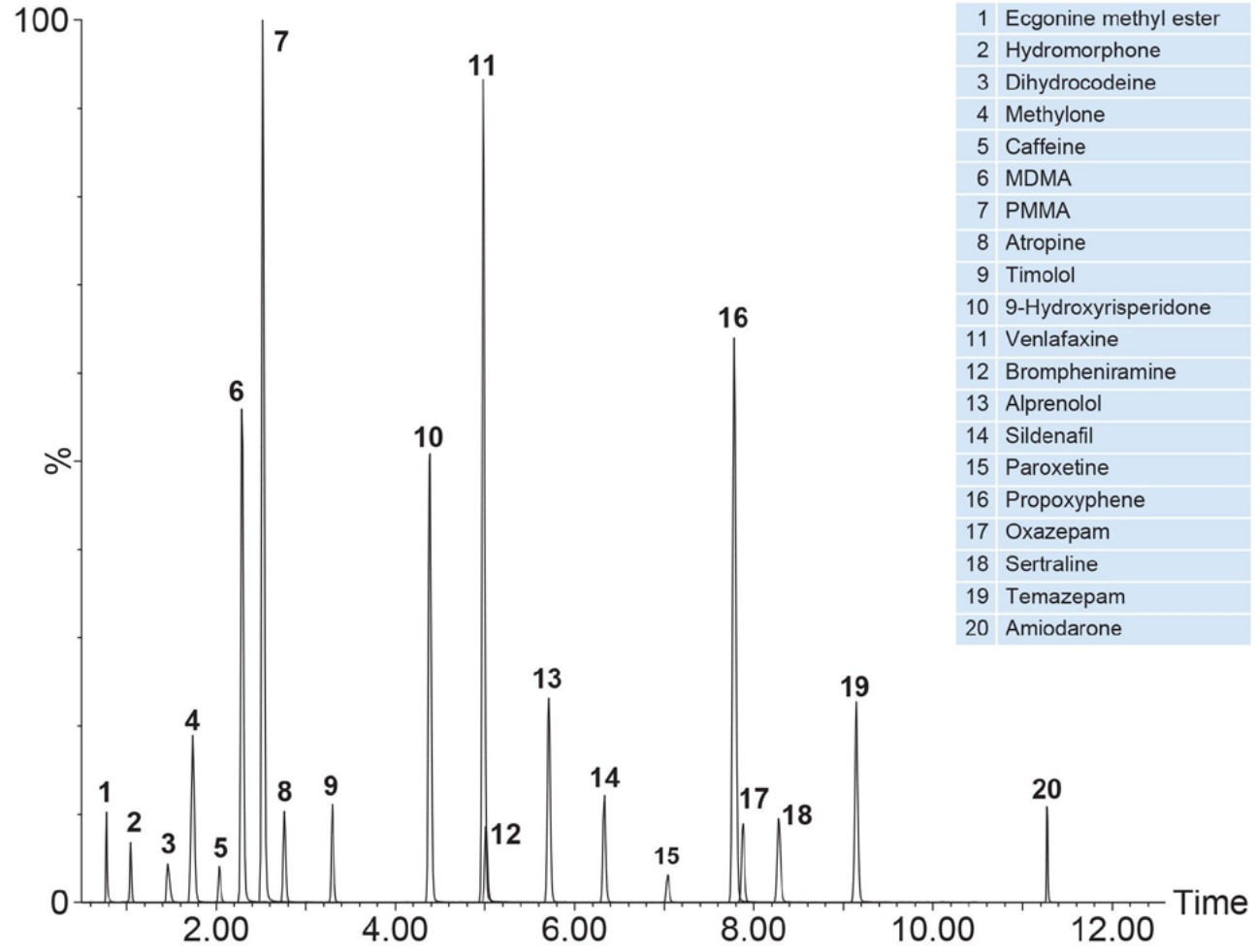

Figure 1. Representative selection of positively ionizing compounds spiked at 10 ng/mL in whole blood and prepared using the described Ostro pass-through procedure. MRM chromatograms are overlaid and responses are normalized to the most abundant.

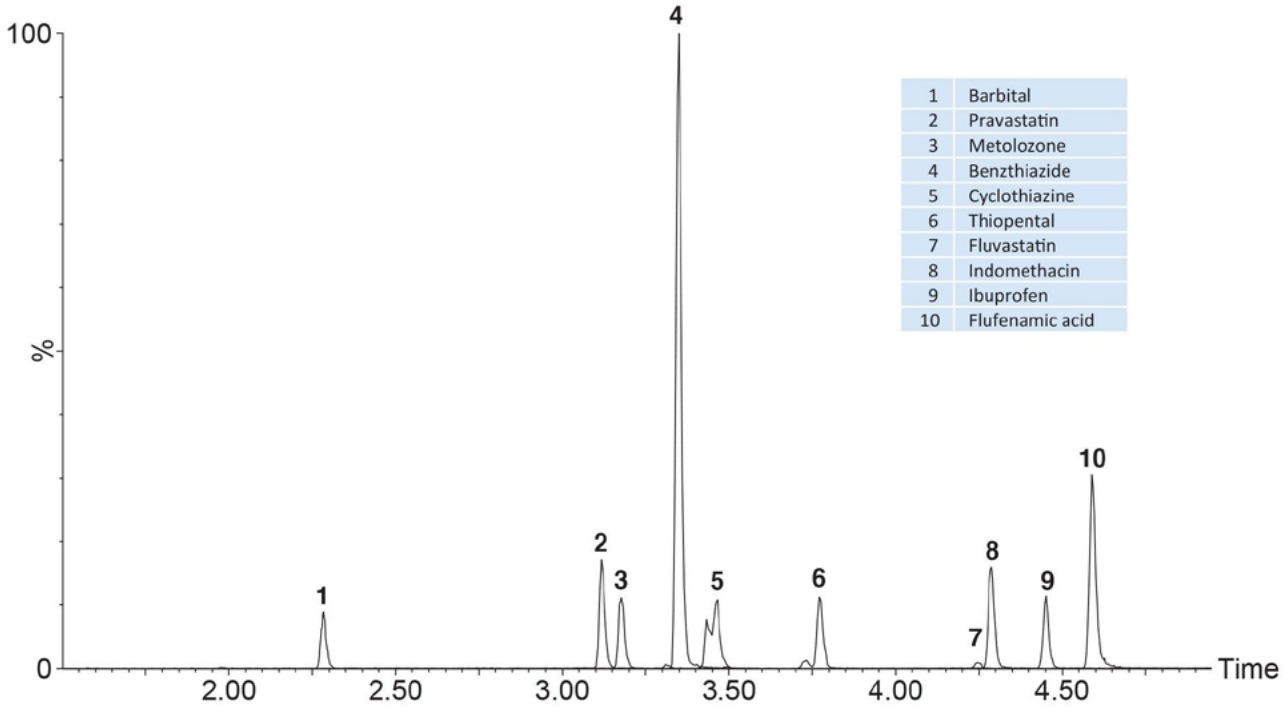

Figure 2. Representative selection of negatively ionizing compounds spiked at 200 ng/mL in whole blood and prepared using Ostro. MRM chromatograms are overlaid and responses are normalized to the most abundant.

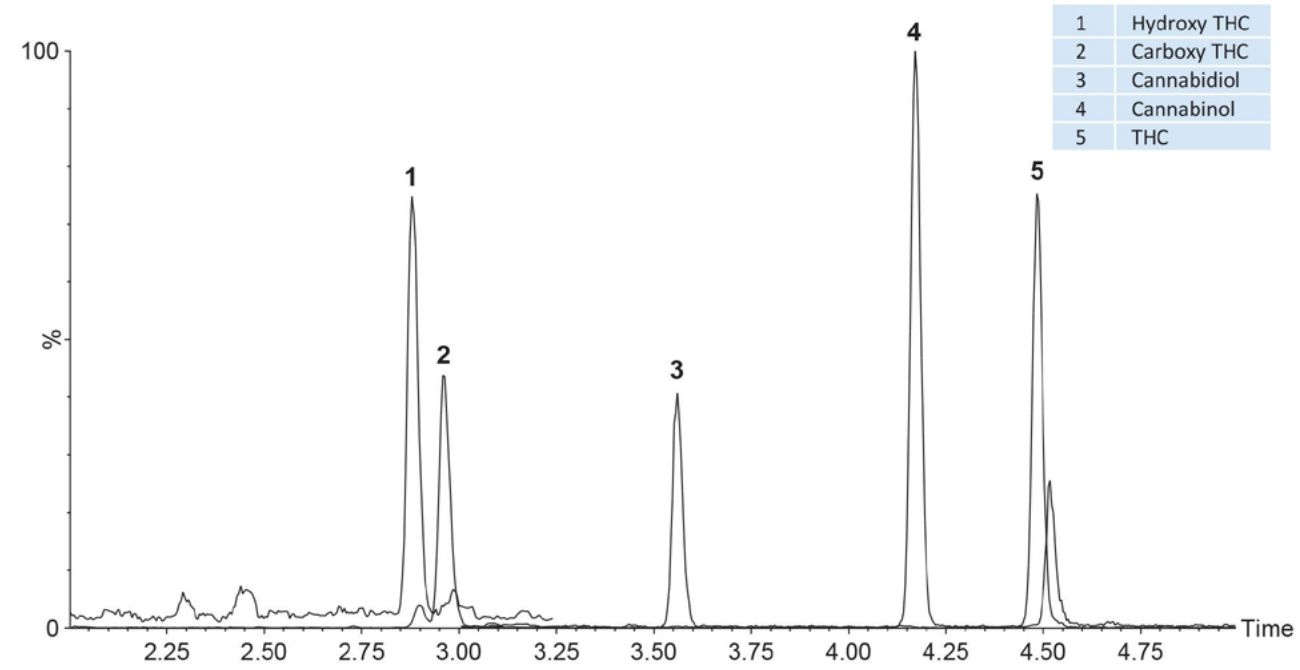

Figure 3. Cannabinoids (10 ng/mL) in whole blood. All analytes detected in ESI+ with the exception of carboxy THC, which was detected in ESI-. MRM chromatograms are overlaid and responses are normalized to the most abundant.

Conclusion

The increased use of whole blood for drug testing has highlighted the need for quick, accurate, reliable, and robust methods to screen for these compounds. This note details a simple, yet robust sample preparation procedure that has been successfully applied to a large panel of chemically diverse drug substances in whole blood. This generic sample preparation method may be useful for a broad range of testing scenarios.

The pass-through sample clean-up procedure is in microplate format and therefore also offers potential for automation if high sample throughput is required.

References

1. Drug Driving (Specified Limits) (England and Wales) Regulations 2014 and the Drug Driving (Specified Limits) (England and Wales) (Amendment) Regulations 2015.
2. Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities—2021 Update. A.L. D'Orazio ***et al.***, ***J. Anal. Toxicol.***, 45: 529-536 (2021).
3. M. Wood and R. Lee. Analysis of Drugs in Blood to Support the Section 5A Driving Under the Influence of Drugs Act. Application Note, 720007451, 2021.

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Application Note

Analysis of Drugs in Blood to Support the UK Section 5 Driving Under the Influence of Drugs Act

Michelle Wood, Robert Lee

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This note details the sample preparation and UPLC-MS/MS methods developed to meet the requirements of the UK Section 5A of the Road Traffic Act 1988.[1]

A single, robust sample preparation method using the Waters Ostro Pass-Through Sample Preparation Plate was developed. This preparation method allows for all analytes detailed in the legislation to be quantified, using one of two UPLC-MS/MS methods, at concentrations lower than those stated in the act. The UPLC-MS/MS methods highlight how the chromatographic resolution of the ACQUITY UPLC I-Class coupled with the sensitivity of the Xevo TQ-S micro Mass Spectrometer provides a simple, robust platform for this analysis.

Benefits

- Single sample preparation protocol using Ostro Pass-Through Sample Preparation Plate (p/n: 186005518 < https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186005518-ostro-protein-precipitation--phospholipid-removal-plate-25-mg-1-.html>)

- Procedure is based on just 100 µL of blood which is advantageous as the amount of specimen available can be limited
- Multiple drug classes separated on a Waters ACQUITY UPLC BEH C_{18} Column (p/n: 186002352 < https://www.waters.com/nextgen/global/shop/columns/186002352-acquity-uplc-beh-c18-column-130a-17--m-21-mm-x-100-mm-1-pk.html>) using an ACQUITY UPLC I-Class with Flow-Through-Needle (FTN)
- Excellent sensitivity of the Xevo TQ-S micro Mass Spectrometer allows for the analytes to be detected at concentrations relevant to the legislation

Introduction

Many illicit and prescribed medications have been reported to impair a driver's control of their vehicle, and to increase the potential for road traffic accidents.[2,3] Since March 2015, changes to the existing legislation were introduced which made it an offence for a vehicle driver to have certain drugs, including legal medication, at blood concentrations above specified limits (Table 1).[1] The compounds fit broadly into two groups: the first group contains prescribed drugs (medicines) where the concentration in blood (threshold) to be detected is relatively high so as not to discourage patients from taking their prescriptions whilst still driving. The second group of compounds are the illicit compounds; drugs of abuse where a zero-tolerance approach has been applied to setting their thresholds.

Prescribed drugs	Blood threshold (ng/mL)	Illicit drugs	Blood threshold (ng/mL)
Clonazepam	50	LSD	1
Morphine	80	Delta-9-THC (THC)	2
Lorazepam	100	6-Monoacetylmorphine (6-MAM)	5
Oxazepam	300	Cocaine	10
Flunitrazepam	300	MDMA	10
Methadone	500	Methylamphetamine	10
Diazepam	550	Ketamine	20
Temazepam	1000	Benzoylecgonine	50
		Amphetamine	250

Table 1. Specified controlled drugs and specified limits for the purposes of Section 5A of the Road Traffic Act 1988.

Analytical testing to support this legislation requires the quantitation of a panel of drugs from differing drug classes, and as such, involves a range of chemical properties, from polar substances such as morphine, to the non-polar THC. This diversity in chemical properties can present some analytical challenges if one is to achieve a simple workflow to detect all relevant molecules optimally, and at their specified concentrations.

Experimental

Control human whole blood was supplied by Bio-IVT (Burgess Hill, West Sussex, UK).

Reference material for the 17 analytes of interest were obtained from Merck (Poole, Dorset, UK) or LGC (Teddington, London, UK). These were supplied as individual 1 mg/mL solutions in either methanol or acetonitrile. The analytes were combined to prepare a mixed-drug spiking solution; further dilutions were prepared using methanol. Stable-labelled internal standards for the analytes were also obtained from the same suppliers at a concentration of 0.1 mg/mL. These internal standards were combined to yield a mixed deuterated internal standard solution (ISTD). All standard solutions were stored at -20 °C.

Whole blood was spiked with the mixed-drug solution to provide a range of concentrations.

Control or spiked blood (100 µL) was added to 100 µL of a 0.1 M zinc sulphate/ammonium acetate solution in the wells of the Ostro Sample Preparation Plate. Internal standards were added and the samples briefly vortex-mixed. Elution solvent (600 µL of 0.5% formic acid in acetonitrile) was added to the samples and the plate further vortex-mixed for 3 minutes. The plate was placed onto a vacuum manifold and the elution solvent was drawn into a Waters 2 mL Square-Well Collection Plate (p/n: 186002482 < https://www.waters.com/nextgen/global/shop/vials-containers--collection-plates/186002482-96-well-sample-collection-plate-2-ml-square-well-50-pk.html>) under full vacuum.

Two separate aliquots (2 x 150 µL) of the Ostro Eluant were transferred to a 1 mL Round-Well Collection Plate (p/n: 186002481 <https://www.waters.com/nextgen/global/shop/vials-containers--collection-plates/186002481-96-well-sample-collection-plate-800--l-round-well-50-pk.html>) and taken to dryness using an Ultravap Mistral Evaporator (Porvair Sciences).

One dried aliquot, for analysis of THC, was reconstituted in 50 µL of 50% acetonitrile in 0.05% formic acid. The other dried aliquot, for the analysis of all other drugs, was reconstituted in 50 µL of 10% acetonitrile in 0.05% formic acid. The samples were quantified using one of the UPLC-MS/MS methods detailed below.

Both UPLC-MS/MS methods employed the same column *i.e.,* an ACQUITY BEH C_{18} (2.1 x 100 mm, 1.7 µm) and the same mobile phases, 0.05% formic acid in water (mobile phase A) and 0.05% formic acid in acetonitrile

(mobile phase B), however, each method employed a different chromatographic gradient. For the analysis of THC the initial starting condition was 50% mobile phase B, while the initial starting conditions for the method to quantify all other drugs was 2% mobile phase B. The Xevo TQ-S micro was operated in electrospray positive (ESI+) mode for both methods with 2 MRM transitions monitored for each analyte and a single MRM transition monitored for the internal standards.

Results and Discussion

To solve the challenges of efficient reconstitution for THC and acceptable chromatography for morphine and the other early eluting basic compounds, two aliquots of the Ostro Eluant were dried. The first aliquot was reconstituted in a solvent suitable for the analysis of THC and the other aliquot was reconstituted in a solvent suitable for all the other drugs. To ensure high throughput, two UPLC-MS/MS methods were developed using the same column and mobile phases. The first, to quantify THC and the other, for the quantitation of all the other analytes.

Figure 1 shows a chromatogram for whole blood spiked with THC at 1 ng/mL. Note the described THC protocol is also suitable for the analysis of several other cannabinoids ***i.e.,*** hydroxy-THC, carboxy-THC, cannabinol and cannabidiol. Figure 2 shows the data for a whole blood sample spiked with all remaining illicit drugs and prescribed medications at a concentration of 10 ng/mL.

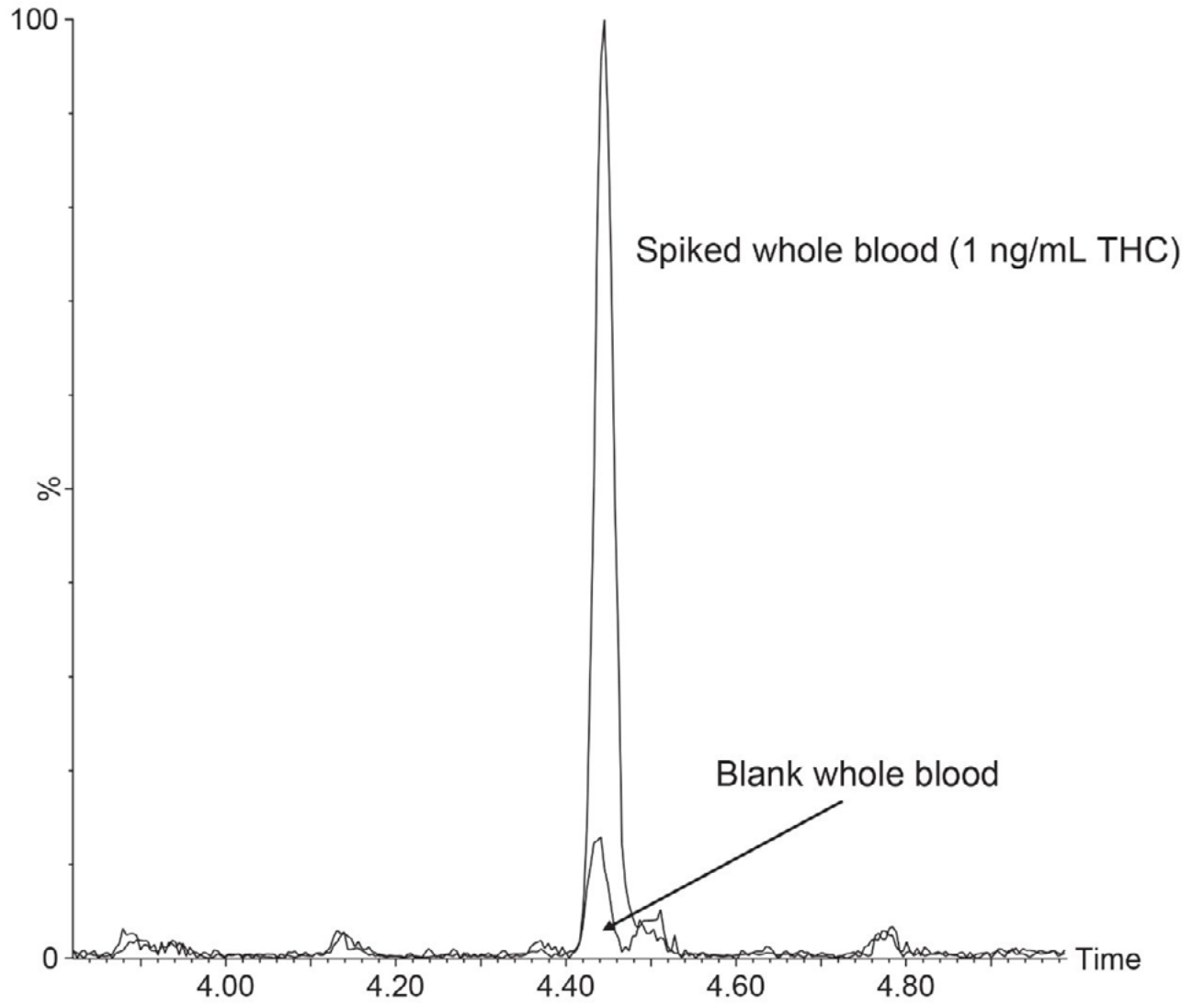

Figure 1. Chromatogram for the quantifier trace for THC in spiked whole blood. The response for the quantifier trace for the blank whole blood sample is also displayed for comparative purposes.

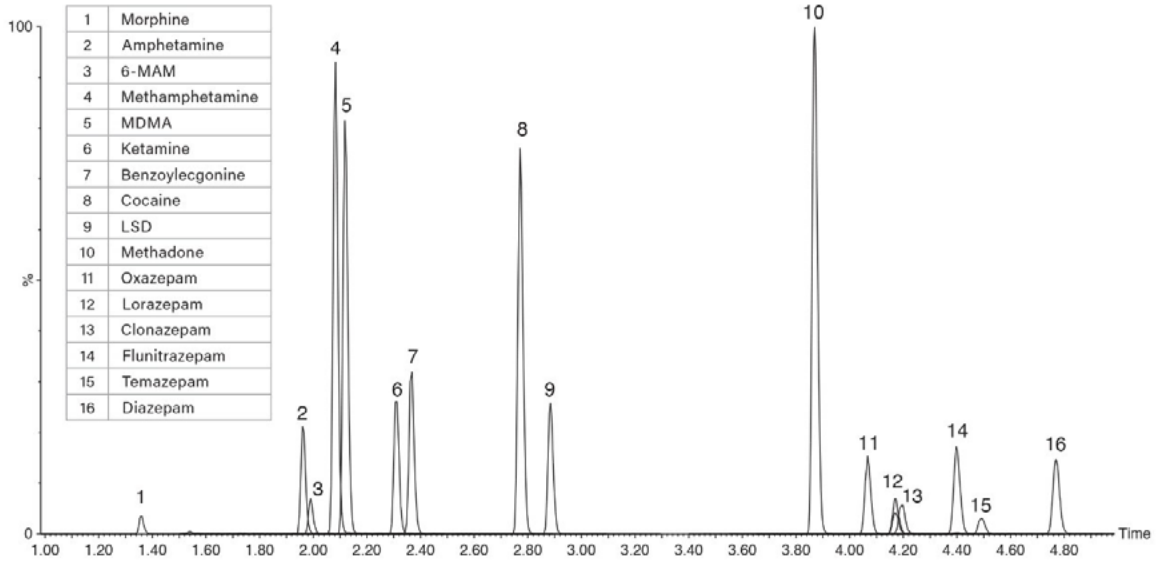

Figure 2. Chromatographic separation of a whole blood sample spiked with a range of illicit drugs and prescribed medications at a concentration of 10 ng/mL. The data is scaled to the most intense peak in the chromatogram.

Conclusion

The increased testing for drugs in drivers has highlighted the need for quick, accurate, reliable, and robust methods to quantify these compounds. This note details a complete workflow that can be used to determine a large panel of drugs from whole blood using the Ostro Pass-Through Sample Preparation Plate. The described procedure utilizes just 100 µL of blood which is beneficial as the amount of available sample can be limited, particularly if the sample requires multiple analyses.

The excellent sensitivity of the Xevo TQ-S micro Mass Spectrometer allows for the analytes to be detected at concentrations relevant to the legislation, while the use of the Ostro Plate allows the sample preparation protocol to be automated for any laboratories requiring higher sample throughput.

References

1. The Drug Driving (Specified Limits) (England and Wales) Regulations 2014 (as amended, Road Traffic Act, England and Wales, 2015 No.2015. (2014)
2. Honkanen, R *et al*. Role of Drugs in Traffic Accidents. *British Medical Journal*, 281, 1309–1312 (1980).
3. Drummer, OH *et al*. The Involvement of Drugs in Drivers of Motor Vehicles Killed in Australian Road Traffic Crashes. *Accident; Analysis and Prevention*, 36, 239–248 (2004).

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720007451, December 2021

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Application Note

A Comprehensive Method for the Analysis of Pain Management Drugs and Drugs of Abuse Incorporating Simplified, Rapid Mixed-Mode SPE with UPLC-MS/MS for Forensic Toxicology

Jonathan P. Danaceau, Scott Freeto, Lisa J. Calton

Waters Corporation

For forensic toxicology use only.

Abstract

This application note describes a complete method for the solid phase extraction and UPLC-MS/MS analysis of pain management drugs and drugs of abuse for frensic toxicology.

A number of advantages are highlighted.

Sample preparation is optimized to efficiently extract all analytes with a simplified procedure that reduces the number of manual steps. The water wettable nature of the sorbent enables in-well sample pretreatment and direct loading without conditioning and equilibration, eliminating sample transfer and potential transcription errors. The efficient and reproducible extraction is evident in the high recoveries, consistent matrix effects, and accurate and precise quantitative data.

The use of the ACQUITY UPLC BEH C_{18} Column results in rapid analysis of a large panel while maintaining all required baseline separations for accurate quantification.

The Waters Xevo TQ-S micro, with features such as StepWave Technology and XDR Detector ensures extremely rapid and accurate quantification of all compounds over wide dynamic ranges. This enables the simultaneous quantification of 6-MAM at 2 ng/mL and methamphetamine at 2500 ng/mL.

This combination of sample preparation, UPLC separation, and MS/MS detection optimizes the workflow and results in a rapid, accurate, and precise method.

Benefits

- Rapid, simplified sample preparation of a comprehensive drug panel
- Efficient and consistent recovery for all analytes
- Consistent matrix effects
- All sample pretreatment and extraction performed in-well, eliminating transfer steps
- LC-MS/MS analysis of 80 compounds in four minutes
- Accurate and precise quantitative data for all compounds

Introduction

Analyte panels for use in forensic toxicology analysis typically include illicit drugs and common drugs of abuse. Often, multiple methods are used to obtain a comprehensive view of the multiple drug classes. These methods may include immunoassay, GC-MS, LC-MS/MS, or a combination of methods. Waters has developed a method for the quantification of a comprehensive drug panel to achieve the appropriate analytical sensitivity, selectivity, and accuracy for unambiguous identification for forensic toxicology.

This method employs a simple sample extraction procedure using Oasis MCX µElution Plates coupled with a rapid and reproducible chromatographic method using an ACQUITY UPLC BEH C_{18} Column that achieves baseline separation for all critical pairs of potentially interfering analytes. A Waters Xevo TQ-S micro with Xtended Dynamic Range (XDR) capablilities provided the analytical sensitivity and dynamic range capabilities required for this diverse group of compounds.

Experimental

All standards were obtained from Cerilliant (Round Rock, TX) and Cayman Chemical (Ann Arbor, MI). A mixed stock solution was prepared in methanol at concentrations of 2, 10, and 25 µg/mL, depending upon the analyte. An internal standard stock solution was prepared in methanol at a concentration of 1 µg/mL. Stable isotope labeled internal standards were used for all compounds except naltrexone, methedrone, dehydronorketamine, m-OH-benzoylecgonine, α-Pyrrolidinovalerophenone (alpha-PVP) metabolite 1, meprobamate, flurazepam, norpropoxyphene, and clonazepam. In those cases, either the internal standard interfered with the quantification of one of the other analytes (naltrexone and clonazepam) or the stable labeled IS was not readily available. Samples were prepared by diluting stock solutions into pooled, blank urine. External quality control material was obtained from UTAK Laboratories (Valencia, CA). All analytes, along with their retention times and calibration ranges are listed in Table 1.

Name	RT	Concentration range (ng/mL)	Name	RT	Concentration range (ng/mL)
Morphine	0.86	25–2500	Tapentadol	1.71	10–1000
Oxymorphone	0.91	25–2500	alpha-PVP	1.77	10–1000
Hydromorphone	0.98	25–2500	7-aminoflunitrazepam	1.69	10–1000
Dihydrocodeine	1.15	10–1000	Cocaine	1.81	10–1000
Naloxone	1.15	10–1000	Normeperidine	1.82	10–1000
Codeine	1.17	25–2500	Meperidine	1.83	10–1000
Pregabalin	1.20	10–1000	Zolpidem	1.85	10–1000
Gabapentin	1.20	10–1000	alpha-PVP Metabolite 1	1.88	10–1000
Methylone	1.21	10–1000	Norbuprenorphine	1.90	2–200
Noroxycodone	1.25	10–1000	Chlordiazepoxide	1.93	10–1000
6-beta Naltrexol	1.26	10–1000	Trazodone	1.99	10–1000
Naltrexone	1.26	10–1000	Cocaethylene	2.01	10–1000
Amphetamine	1.28	25–2500	Fenfluramine	2.03	10–1000
Oxycodone	1.28	25–2500	PCP	2.09	10–1000
6-MAM	1.28	2–200	Meprobamate	1.96	10–1000
MDA	1.30	25–2500	Fentanyl	2.15	2–200
Norhydrocodone	1.31	10–1000	alpha-OH Midazolam	2.13	10–1000
Ethylone	1.32	10–1000	Midazolam	2.17	10–1000
O-desmethyl Tramadol	1.32	10–1000	Flurazepam	2.23	10–1000
Methedrone	1.33	10–1000	Buprenorphine	2.27	2–200
Hydrocodone	1.34	25–2500	EDDP	2.29	10–1000
Dehydronorketamine	1.33	10–1000	Norprpoxyphene	2.51	25–2500
Methamphetamine	1.36	25–2500	Verapamil	2.52	10–1000
MDMA	1.37	25–2500	Propoxyphene	2.56	10–1000
m-OH BZE	1.34	10–1000	Methadone	2.60	10–1000
Butylone	1.41	10–1000	alpha-OH Alprazolam	2.51	10–1000
Phentermine	1.43	25–2500	alpha-OH Triazolam	2.51	10–1000
Mephedrone	1.47	10–1000	Nitrazepam	2.52	10–1000
Norketamine	1.47	10–1000	Oxazepam	2.59	10–1000
MDEA	1.48	25–2500	Clonazepam	2.65	10–1000
Ritalinic Acid	1.48	25–2500	Lorazepam	2.66	10–1000
Ketamine	1.52	10–1000	Carisoprodol	2.67	10–1000
Norfentanyl	1.54	2–200	Alprazolam	2.68	10–1000
BZE	1.52	10–1000	2-OH Ethyl Flurazepam	2.68	10–1000
7-aminoclonazepam	1.51	10–1000	Nordiazepam	2.68	10–1000
N-desmethyl Zopiclone	1.58	10–1000	Triazolam	2.73	10–1000
Zopiclone	1.61	10–1000	Desalkylflurazepam	2.78	10–1000
Tramadol	1.68	10–1000	Flunitrazepam	2.77	10–1000
N-desmethyl Tramadol	1.69	10–1000	Temazepam	2.87	10–1000
Methylphenidate	1.70	25–2500	Diazepam	3.05	10–1000

Table 1. Retention times and calibration ranges of all compounds.

LC conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC BEH $C_{18,}$ 1.7 µm, 2.1 x 100 mm
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.6 mL/min.
Mobile phase A (MPA):	0.1% Formic acid in MilliQ water
Mobile phase B (MPB):	0.1% Formic acid in acetonitrile (ACN)
Purge solvent:	50:50 MeOH:H_2O
Wash solvent:	25:25:25:25 MeOH:H_2O:IPA:ACN

UPLC Gradient Program:

Time (min)	Flow (mL/min)	% MPA	% MPB
0	0.6	98	2
3.33	0.6	33	67
3.5	0.6	10	90
3.6	0.6	98	2
4	0.6	98	2

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI positive
Desolvation temp.:	500 °C
Desolvation gas flow:	1000 L/hr
Cone gas flow:	150 L/hr
Acquisition range:	MRM transitions optimized for individual compounds
Capillary voltage:	1.0 kV
Collision energy:	Optimized for individual compounds (See Appendix 1)
Cone voltage:	Optimized for individual compounds (See Appendix 1)
Data management:	MS software: MassLynx Quantification software: TargetLynx XS

Analyte recoveries and matrix effects were calculated as described previously.[1] Internal standard corrected matrix effects were calculated using the response factor of the analyte.

SPE Extraction

100 µL of urine was added to individual wells of an Oasis MCX µElution Plate, followed by 100 µL of a solution containing hydrolysis buffer, 10 µg/mL of β-glucuronidase enzyme, and 100 ng/mL internal standards and mixed by several aspirations. After incubation, 200 µL of 4% H_3PO_4 was added and mixed by several aspirations. All samples were drawn directly into the sorbent bed by vacuum and subsequently washed with 200 µL of 80:20 H_2O:MeOH. The plate was dried under high vacuum (~15 inch Hg) for one minute to remove as much of the wash solution as possible. Samples were eluted using 2 x 25 µL of 50:50 ACN:MeOH

containing 5% strong ammonia solution (Fisher, 28–30%). All samples were diluted with 150 µL of sample diluent (2% ACN:1% formic acid in MilliQ water) prior to LC-MS/MS analysis. A graphical workflow of the extraction procedure is shown in Figure 1.

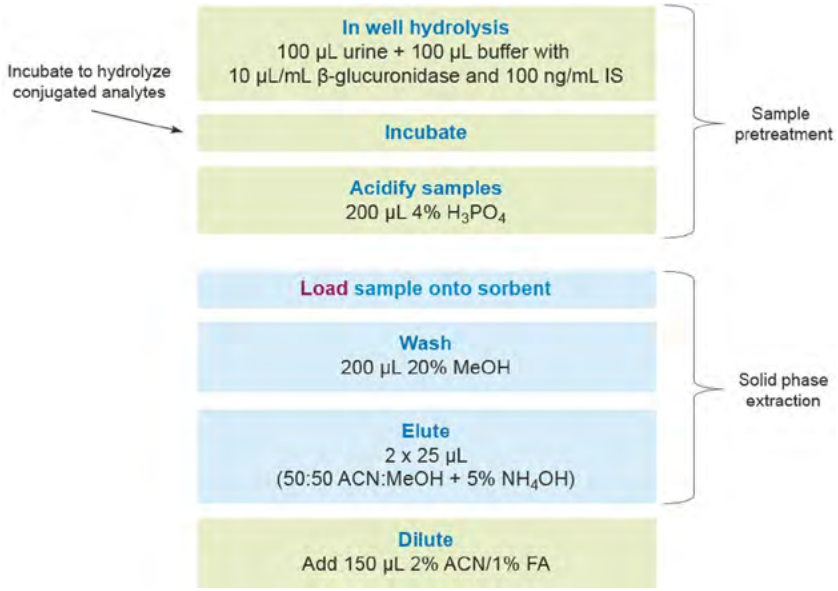

Figure 1. Details of the extraction method for the analysis of a comprehensive drug panel using Oasis MCX µElution Plates. Enzymatic hydrolysis and sample pretreatment are performed in the wells of the extraction plate, minimizing transfer steps. Conditioning and equilibration steps are eliminated and a single wash step is used instead of two, significantly simplifying the procedure.

Results and Discussion

Chromatography

All test compounds are listed in Table 1, along with their retention times and calibration ranges. Figure 2 shows the chromatography of all compounds included in the panel on the ACQUITY UPLC BEH C_{18} Column. Meprobamate and norpropoxyphene were included in the panel but were only monitored qualitatively, as they are not fully compatible with the sample preparation procedures. As with any multi-analyte panel, care must be taken to ensure that compounds and internal standards do not interfere with each other. Figures 3A and 3B highlight the chromatography of several groups of analytes with the potential to interfere with each other. In each case, either baseline separation is achieved (see naloxone vs. 6-MAM, Figure 3B) or the MRMs

do not interfere with each other (see dehydronorketamine and ethylone, Figure 3A). In some cases, certain internal standards were not used.

For example, clonazepam-d4 was not used as it interfered with the quantification of lorazepam. The high efficiency of the UPLC Column enabled all compounds to elute in just over three minutes, without any compromise in resolution for this large panel, with a total run time of four minutes.

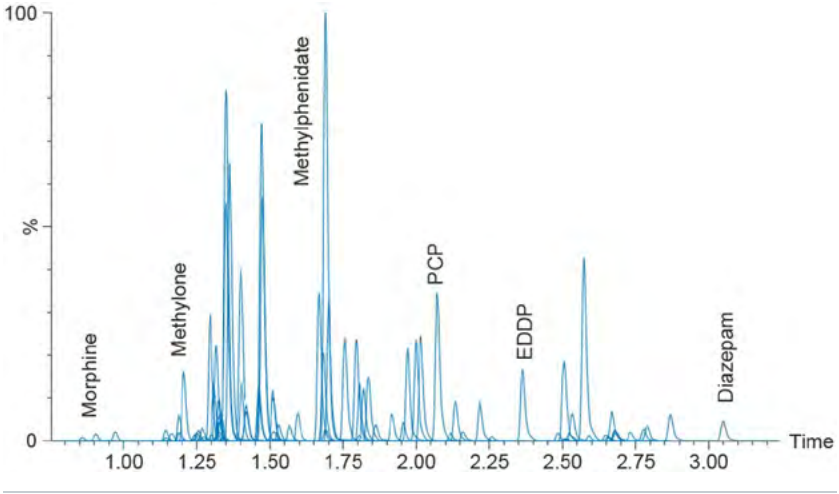

Figure 2. Chromatography of all compounds on the ACQUITY UPLC BEH C_{18} Column. The earliest eluting compound is morphine at 0.86 minutes and the latest eluting compound is diazepam at 3.05 minutes.

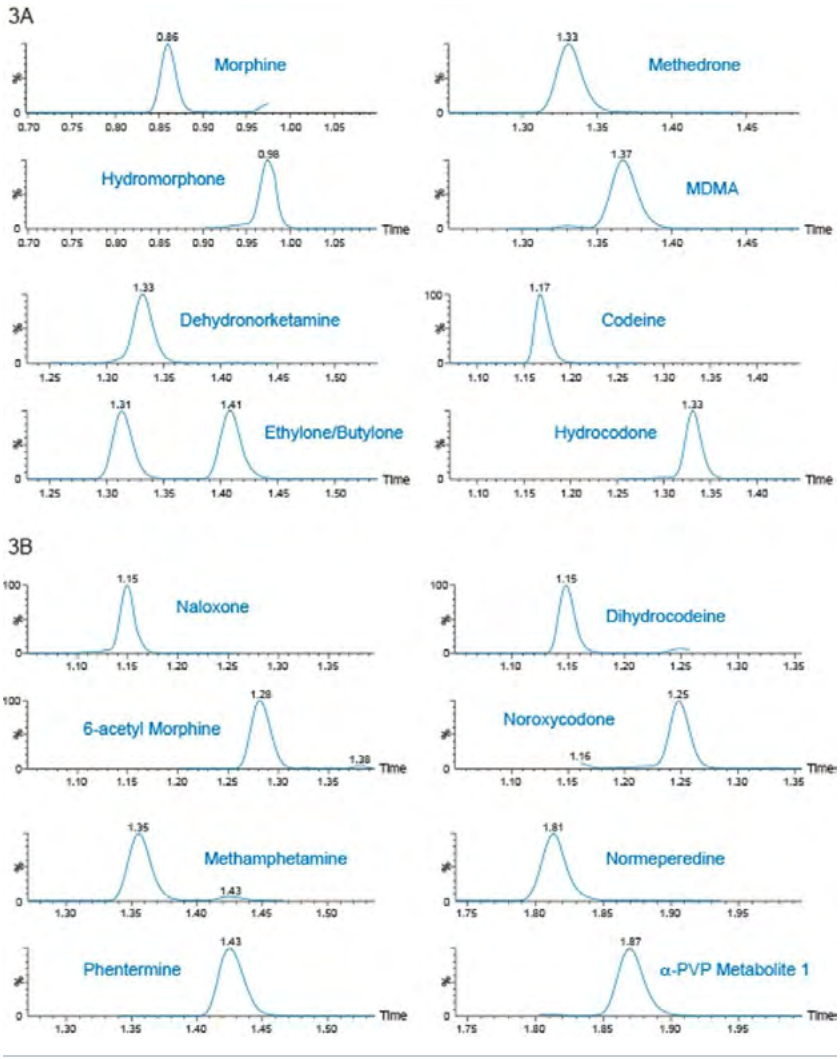

Figure 3A and 3B. Selected chromatography of compounds with the potential to interfere with each other. In each case, compounds are either baseline separated or else did not contain any product ions that caused interference. Column: ACQUITY UPLC BEH C_{18} , 1.7 µm, 2.1 x 100 mm.

Recovery and Matrix Effects

The goal of any extraction technique is to achieve efficient and reproducible recovery for all relevant analytes. As in previous work, the wash protocol was modified from the traditional MCX technique to accommodate the benzodiazepines.[2] Figure 4 shows the mean extraction recoveries of the entire panel of compounds from six different lots of urine. With the exception of meprobamate and norpropoxyphene, all compounds but two (MDMA and EDDP) had recoveries greater that 70%. Extraction efficiencies were also consistent. Coefficients

of variation (%CV) were less than 10% for all quantitative compounds. Recovery data for individual batches followed the same pattern. These highly efficient recoveries across different matrix lots demonstrate the robustness of the extraction technique and are important for quantification of these compounds in samples from different sources.

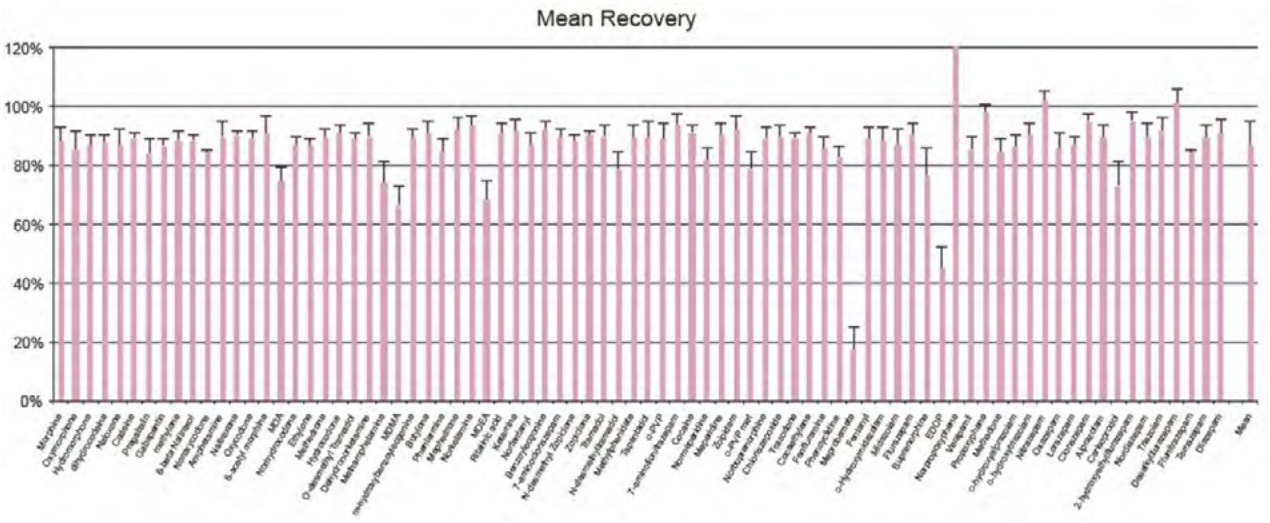

Figure 4. Mean extraction recovery for the compounds in this application. Values represent the mean of six different lots of urine. N = 4 for each lot.

Matrix effects were also evaluated using multiple lots of urine. As with recovery, consistent matrix effects are essential for accurate quantification. Figure 5A shows the aggregate matrix effects from six lots of urine. Ion suppression was observed for the majority of analytes, with up to 60% ion suppression was observed for morphine and hydroxymorphone. However, with only two exceptions (m-OH BZE and α-OH midazolam) standard deviations from matrix effects were less than 20% indicating consistent matrix lot to lot performance. Figure 5B shows the matrix effects when corrected using the internal standards. In this case 75/78 of the corrected matrix effects were less than 20%.

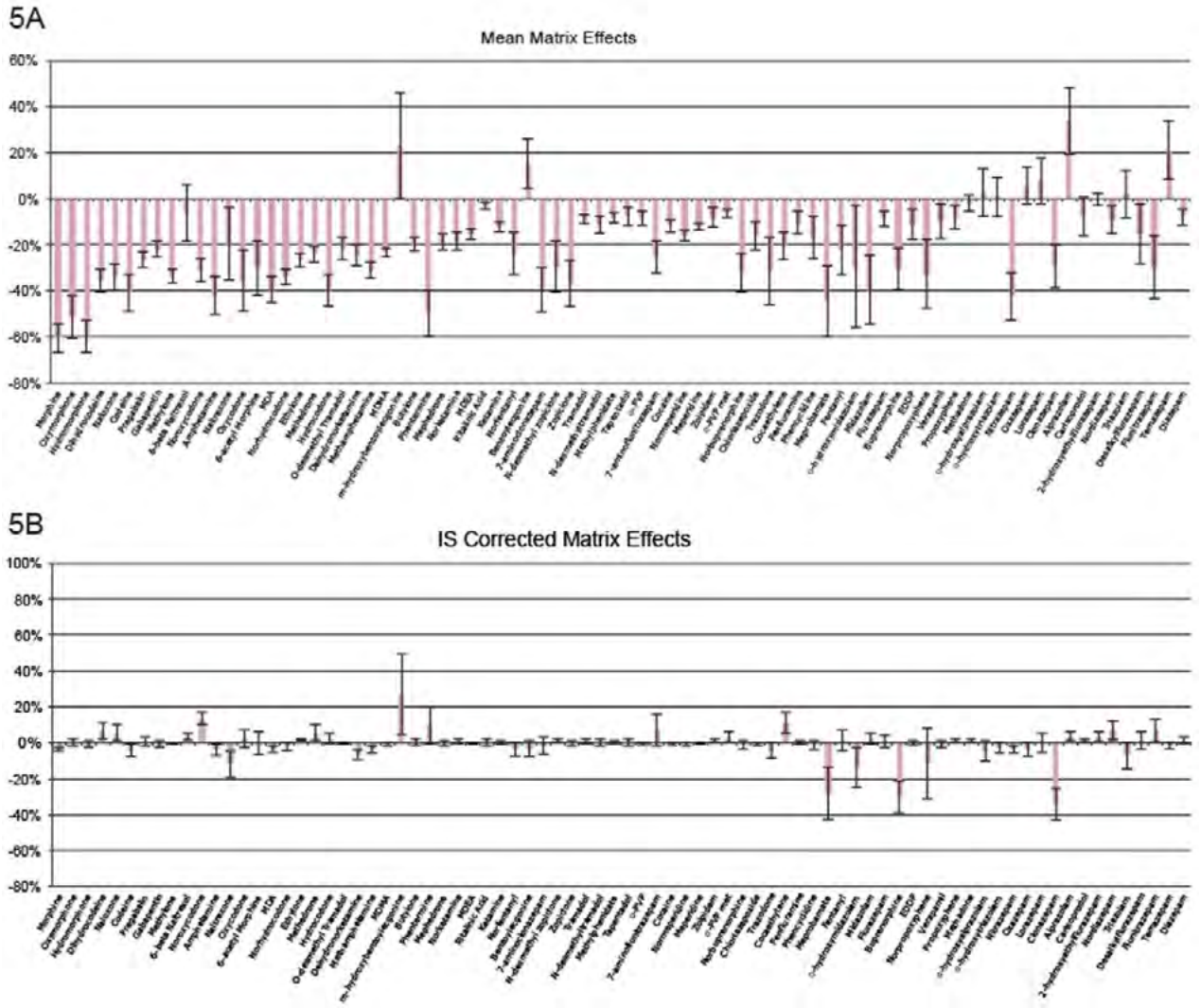

Figure 5A. Mean matrix effects from all compounds from six lots of urine. Bars indicate mean matrix effects and errors indicate standard deviations. Figure 5B. Internal standard corrected matrix effects from six lots of urine. In this graph the matrix effects from figure 5A have been corrected using the internal standards. Of all compounds assessed, only two had standard deviations exceeding 20% and only three of the quantitatively assessed compounds had corrected matrix effects greater than 20%.

Quantitative Analysis

Seven point calibration curves were extracted across the concentration ranges shown in Table 1. Calibration ranges were tailored to reflect the expected concentrations of various compounds. Quality control samples were prepared at 4 concentrations spanning the range of the calibrators, with the lowest at 1.5x the lowest calibrator and the highest at 75% of the highest calibrator. For most compounds, these QC levels were 15, 75, 250, and 750 ng/mL. The compounds at the lower concentrations had QC levels at 3, 15, 50, and 150 ng/mL

and the analytes at the higher concentration range had QC levels at 37.5, 187.5, 625, and 1875 ng/mL. Quantitative method validation involved extracting full curves and QC samples over five different days. Calibration curves were extracted in duplicate and six replicates of QC samples were prepared each day. Control limits for individual calibrators and QC samples were ±15% of target values, with the exception of the lowest points, which were required to be within 20%. Precision limits for QC samples were 20% for the lowest QC point and 15% for the other points. Meprobamate and norpropoxyphene were assessed qualitatively only and were not subject to these controls. A summary of the five independent extractions and analyses met all of these criteria and can be seen in Appendix 2. The majority of compounds were within 10% of their target values with %CVs under 10%. For within batch results, all compounds met the accuracy criteria, and the only compound that had precision results greater than 15% was the high amphetamine QC at 18%.

All calibration curves conformed to FDA bioanalytical method validation requirements,[3] which dictate that all calibrators be within 15% of target values except the lowest point, which must be within 20% of its target value and that 75% of calibrators meet this criteria. All compounds met these criteria and all curves had R^2 values of 0.99 or greater.

Limits of quantification were defined as those points in which the signal was 5X greater than that of an extracted matrix blank, signal to noise ratios were >10, and both bias and %CV were both less than 20%. To evaluate this, six replicates of the lowest calibrator were extracted in one of the validation batches. All compounds met these criteria.

On instrument stability was also assessed. A single batch was extracted and analyzed five times over an eight day period. Through four days, all compounds met the quantitative validation criteria described above.

In order to assess accuracy, external quality control samples from UTAK Laboratories were evaluated. These results can be seen in Tables 2A–2D. Analytes assessed using external quality control samples included opioids, benzodiazepines, stimulants, and synthetic cathinones. These results show that 91/98 (93%) of the results were within 20% of the target value. The larger deviations for analytes such as fentanyl, norfentanyl, and buprenorphine could be a result of slight errors in the preparation of the master stock mix, as these compounds were spiked using low volumes (20 µL of stock solution). In addition, 7-aminoclonazepam may have stability issues in the urine matrix which could account for its low bias. All results had %RSD values <10%.

Name	Mean (ng/mL)	Acc.	%RSD	Mean (ng/mL)	Acc.	%RSD
Morphine	55.0	110.0%	2.4%	404.5	101.1%	0.4%
Oxymorphone	50.0	100.0%	1.9%	405.1	101.3%	1.2%
Hydromorphone	49.8	99.6%	3.6%	405.1	101.3%	1.2%
Codeine	52.0	104.1%	9.8%	411.5	102.9%	3.4%
Oxycodone	48.5	97.0%	8.3%	419.9	105.0%	6.8%
6-AM	5.4	109.0%	7.7%	43.5	108.7%	2.5%
Norhydrocodone	52.0	104.1%	4.6%	384.6	96.1%	4.9%
Hydrocodone	44.0	88.0%	2.9%	336.7	84.2%	3.1%
O-desmethyl-tram	49.1	98.1%	2.0%	375.5	93.9%	2.3%
Norfentanyl	6.1	121.4%	4.1%	45.8	114.4%	2.5%
Tramadol	53.8	107.5%	2.7%	396.1	99.0%	1.3%
Tapentadol	49.3	98.6%	2.7%	388.8	97.2%	1.4%
Normeperidine	53.4	106.8%	2.6%	385.0	96.3%	1.3%
Meperidine	48.7	97.4%	2.8%	372.7	93.2%	1.5%
Norbuprenorphine	55.1	110.2%	5.2%	392.4	98.1%	3.0%
Fentanyl	6.6	131.5%	2.2%	49.4	123.4%	1.0%
Buprenorphine	71.4	142.8%	1.9%	389.5	97.4%	3.0%
EDDP	50.6	101.3%	2.6%	391.5	97.9%	1.1%
Methadone	54.6	109.2%	1.5%	399.3	99.8%	2.0%

Table 2A. Opioid results from external quality control material. Each sample was analyzed in replicates of four. Highlighted cells represent bias values >20%.

Name	Mean (ng/mL)	Acc.	%RSD	Mean (ng/mL)	Acc.	%RSD
Amphetamine	321.7	91.9%	3.3%	678.9	97.0%	0.8%
MDA	319.7	91.3%	1.5%	664.9	95.0%	3.8%
Methamp	331.1	94.6%	1.4%	656.4	93.8%	3.7%
MDMA	313.2	89.5%	0.6%	667.5	95.4%	2.3%
Phentermine	300.7	85.9%	2.0%	638.8	91.3%	5.1%
MDEA	309.4	88.4%	1.8%	593.2	84.7%	3.0%

Table 2B. Amine stimulant results for external quality control samples. Each sample was analyzed in replicates of four.

Name	Mean (ng/mL)	Acc.	%RSD	Mean (ng/mL)	Acc.	%RSD
7-aminoclonazepam	70.1	70.1%	3.6%	317.5	79.4%	1.6%
7-aminoflunitrazepam	85.9	85.9%	2.8%	353.0	88.2%	1.7%
Zolpidem	93.9	93.9%	2.6%	372.1	93.0%	0.6%
Chlordiazepoxide	87.2	87.2%	2.1%	352.3	88.1%	1.6%
a-OH-midazolam	128.3	128.3%	3.0%	471.2	117.8%	3.2%
Midazolam	92.0	92.0%	1.0%	371.0	92.7%	1.5%
Flurazepam	107.1	107.1%	4.1%	402.8	100.7%	3.7%
alpha-OH Alprazolam	96.4	96.4%	4.4%	366.7	91.7%	3.8%
a-OH-triazolam	108.5	108.5%	8.9%	395.8	99.0%	1.7%
Nitrazepam	95.8	95.8%	4.9%	366.7	91.7%	0.7%
Oxazepam	98.7	98.7%	2.7%	398.2	99.5%	0.7%
Lorazepam	102.5	102.5%	4.4%	382.1	95.5%	2.2%
Clonazepam	96.2	96.2%	1.1%	379.5	94.9%	1.5%
Alprazolam	103.0	103.0%	4.5%	464.8	116.2%	4.8%
2-OH Ethyl Flurazepam	100.6	100.6%	4.3%	364.2	91.0%	1.4%
Nordiazepam	99.9	99.9%	2.5%	379.5	94.9%	4.2%
Triazolam	96.8	96.8%	3.1%	382.4	95.6%	2.4%
Desalkylflurazepam	89.2	89.2%	2.2%	393.6	98.4%	2.6%
Flunitrazepam	98.5	98.5%	2.5%	390.5	97.6%	1.7%
Temazepam	100.0	100.0%	1.4%	383.9	96.0%	1.2%
Diazepam	88.7	88.7%	2.4%	379.6	94.9%	2.7%

Table 2C. Benzodiazepine results for external quality control samples. Each sample was analyzed in replicates of four. Highlighted cells represent bias values >20%.

Name	Mean (ng/mL)	Acc.	%RSD
Methylone	16.9	112.5%	2.8%
Ethylone	15.6	103.9%	2.7%
Methedrone	16.7	111.2%	2.2%
Butylone	16.2	107.9%	1.5%
Mephedrone	17.7	117.9%	2.4%
alpha-PVP	16.2	107.7%	2.7%

Table 2D. Synthetic cathinone results for external quality control samples. Each sample was analyzed in replicates of four.

Conclusion

This application note describes a complete method for the solid phase extraction and UPLC-MS/MS analysis of illicit drugs and drugs of abuse for forensic toxicology. A number of advantages are highlighted.

- Sample preparation is optimized to efficiently extract all analytes with a simplified procedure that reduces the number of manual steps. The water wettable nature of the sorbent enables in-well sample pretreatment and direct loading without conditioning and equilibration, eliminating sample transfer and potential transcription errors. The efficient and reproducible extraction is evident in the high recoveries, consistent matrix effects, and accurate and precise quantitative data.
- The use of the ACQUITY UPLC BEH C_{18} Column results in rapid analysis of a large panel while maintaining all required baseline separations for accurate quantification.
- The Waters Xevo TQ-S micro, with features such as StepWave Technology and XDR Detector ensures extremely rapid and accurate quantification of all compounds over wide dynamic ranges. This enables the simultaneous quantification of 6-MAM at 2 ng/mL and methamphetamine at 2500 ng/mL.

This combination of sample preparation, UPLC separation, and MS/MS detection optimizes the workflow and results in a rapid, accurate, and precise method.

References

1. Zhang, X., J.P. Danaceau, E. Chambers: Quantitative analysis of THC and its metabolites in whole blood using LC-MS/MS for Toxicology and Forensic Laboratories. Waters Application Note (720005769EN), 1–7 (2016).
2. Danaceau, J.P., E. Chambers: LC-MS/MS analysis of urinary benzodiazepines and Z-drugs via a simplified, mixed-mode sample preparation strategy. Waters Application Note (720005973EN), 1–9 (2017).
3. Bansal, S., A. DeStefano: Key elements of bioanalytical method validation for small molecules. *The AAPS Journal* 9(1), E109-E114 (2007).

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MassLynx Software <https://www.waters.com/513164>

TargetLynx Application Manager <https://www.waters.com/513791>

720006187, March 2019

Appendix 1

Name	RT	M+H+	MRM product ions	Cone voltage	Collision energy
Morphine	0.86	286.1	201.1 165.1	25 25	25 35
Oxymorphone	0.91	302.1	227.1 242.1	25 25	25 25
Hydromorphone	0.98	286.1	185.1 157.1	25 25	30 40
Dihydrocodeine	1.15	302.2	199.1 128.1	25 25	30 60
Naloxone	1.15	328.2	253.1 212.1	25 25	28 38
Codeine	1.17	300.2	215.1 165.1	25 25	25 40
Pregabalin	1.20	160.1	125.1 107.1	25 25	12 15
Gabapentin	1.20	172.1	137.1 95.0	25 25	15 20
Methylone	1.21	208.1	160.1 132.1	25 25	15 25
Noroxycodone	1.25	302.1	187.1 227.1	25 25	22 28
6-beta Naltrexol	1.26	344.2	308.2 254.1	10 10	26 30
Naltrexone	1.26	342.2	324.2 270.1	25 25	18 26
Amphetamine	1.28	136.1	119.1 91.1	25 25	15 40
Oxycodone	1.28	316.2	241.1 256.2	25 25	25 25
6-MAM	1.28	328.2	165.1 211.1	25 25	45 30
MDA	1.30	180.1	163.1 105.1	22 22	8 20
Norhydrocodone	1.31	286.1	199.1 128.1	25 25	25 50
Ethylone	1.32	222.2	174.1 146.1	25 25	15 25
O-desmethyl Tramadol	1.32	250.2	58.1	25	15
Methedrone	1.33	194.1	161.1 146.1	25 25	15 30
Hydrocodone	1.34	300.2	199.1 171.1	20 20	28 36
Dehydronorketamine	1.33	222.1	142.1 177.1	25 25	25 15
Methamphetamine	1.36	150.1	119.1 91.1	24 24	9 15
MDMA	1.37	194.1	163.1 105.1	26 26	10 22
m-OH BZE	1.34	306.1	168.1 121.1	25 25	20 25
Butylone	1.41	222.1	174.1 146.1	25 25	15 25
Phentermine	1.43	150.1	133.1 91.1	24 24	9 15
Mephedrone	1.47	178.1	145.1 91.1	25 25	15 30
Norketamine	1.47	224.1	125.0 179.1	25 25	20 15
MDEA	1.48	208.1	163.1 105.1	26 26	10 24
Ritalinic Acid	1.48	220.1	84.0 56.0	25 25	40 40
Ketamine	1.52	238.1	125.0 179.1	25 25	25 15
Norfentanyl	1.54	233.2	84.1 177.1	25 25	15 15

*MS Parameters for all analytes. *Chlorine isotopes were used for the precursor icons for Clonazepam and Lorazepam.*

Name	RT	M+H+	MRM product ions	Cone voltage	Collision energy
BZE	1.52	290.1	168.1 105	36 36	18 32
7-aminoclonazepam	1.51	286.1	121.1 222.1	25 25	30 26
N-desmethyl Zopiclone	1.58	375.1	245.0 331.0	8 8	12 8
Zopiclone	1.61	389.1	245.0 112.0	6 6	14 58
Tramadol	1.68	264.2	58.1	25	15
N-desmethyl Tramadol	1.69	250.2	44.0 232.2	25 25	10 7
Methylphenidate	1.70	234.2	84.1 91.1	25 25	15 40
Tapentadol	1.71	222.2	121.1 107.1	25 25	20 25
alpha-PVP	1.77	232.2	91.1 126.1	25 25	20 25
7-aminoflunitrazepam	1.69	284.1	135.1 227.1	34 34	26 22
Cocaine	1.81	304.2	182.2 82.1	25 25	34 20
Normeperidine	1.82	234.1	160.1 131	25 25	15 28
Meperidine	1.83	248.2	174.1 220.2	25 25	20 20
Zolpidem	1.85	308.2	235.1 92.1	34 34	32 52
alpha-PVP Metabolite 1	1.88	234.2	117.1 173.1	25 25	25 20
Norbuprenorphine	1.90	414.3	101.3 83.3	20 20	48 48
Chlordiazepoxide	1.93	300.1	227.0 283.1	34 34	20 12
Trazodone	1.99	372.2	176.1 148.1	25 25	20 35
Cocaethylene	2.01	318.2	196.1 105.1	42 42	20 38
Fenfluramine	2.03	232.1	159.0 109.0	25 25	20 40
PCP	2.09	244.2	86.1 159.1	25 25	12 12
Meprobamate	1.96	219.1	158.1 96.9	25 25	5 10
Fentanyl	2.15	337.2	188.1 105.1	25 25	22 35
alpha-OH Midazolam	2.13	342.1	168.1 203.1	20 20	40 24
Midazolam	2.17	326.1	291.1 223.1	16 16	24 36
Flurazepam	2.23	388.2	315.1 100.1	25 25	26 28
Buprenorphine	2.27	468.3	55.1 101.3	25 25	50 40
EDDP	2.29	278.2	234.1 249.2	25 25	30 25
Norprpoxyphene	2.51	326.2	252.2 118.0	10 10	5 5
Verapamil	2.52	455.3	165.1 303.2	25 25	25 25
Propoxyphene	2.56	340.2	266.2 143.1	25 25	7 25
Methadone	2.60	310.2	265.2 105.0	25 25	15 25
alpha-OH Alprazolam	2.51	325.1	297.1 243.1	25 25	25 30
alpha-OH Triazolam	2.51	359.1	176.1 141.0	28 28	24 38
Nitrazepam	2.52	282.1	236.1 180.1	25 25	20 36

*MS Parameters for all analytes. *Chlorine isotopes were used for the precursor icons for Clonazepam and Lo*

Application Note

A Comprehensive Comparison of Solid Phase Extraction (SPE) vs. Solid Liquid Extraction (SLE) vs. Liquid Liquid Extraction (LLE) Sample Prep Techniques in Bioanalysis and Forensic Toxicology Analyses

Jonathan P. Danaceau, Kim Haynes, Erin E. Chambers

Waters Corporation

For forensic toxicology use only.

Abstract

In this application, a comprehensive comparison of sample preparation techniques including SPE, SLE, and LLE was conducted in plasma and urine, using a wide variety of compounds found in bioanalysis and forensic toxicology.

Oasis PRiME HLB also demonstrated superior recoveries and matrix effects for a variety of tested drugs without any additional method development. SLE and LLE required additional method development or multiple extraction protocols to achieve recoveries that were comparable to Oasis PRiME HLB for all of the tested analytes. The unique nature of Oasis PRiME HLB enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. The µElution format enabled the direct injection of extracts without evaporation or reconstitution.

Benefits

- Provide a process and results comparison of different sample preparation techniques for bioanalysis and forensic toxicology

- Significantly faster extraction protocols compared to SLE and LLE
- Oasis PRiME HLB shows higher analyte recoveries and improved matrix effects compared to SLE and LLE in plasma samples
- Oasis PRiME HLB resulted in higher analyte recoveries for polar bases than SLE and LLE in urine samples
- Flexible sample capacity options with Oasis PRiME HLB compared with SLE (rigid sample amount on specific SLE plate)

Introduction

Solid-phase extraction (SPE) is a sample preparation technique by which compounds that are dissolved or suspended in a liquid matrix are extracted according to their physical and chemical properties. Reversed phase SPE sorbents can be either polymeric or silica based. In both cases, compounds are retained on the sorbent mainly by hydrophobic interactions. A washing step helps to remove matrix interferences. The analyte(s) can be eluted with an organic solvent, which disrupts the interaction of the analyte and the sorbent.[1,2] Waters Oasis PRiME HLB is a novel reversed phase SPE sorbent developed to enable simpler and faster SPE protocols, while at the same time generating cleaner extracts than other sample preparation methods with a simple, generic three step protocol.

Liquid-liquid extraction (LLE) employs water-immiscible solvents to extract analytes from aqueous solutions. This is usually accomplished by shaking and collecting the solvent layer containing the analytes of interest.

Supported liquid extraction (SLE, aka, solid supported liquid extractionSSLE) is analogous to traditional liquid-liquid extraction (LLE) and utilizes the same water-immiscible solvent systems for analyte extraction from aqueous solutions. Instead of shaking the two immiscible phases together as in LLE, in SLE, the aqueous sample is immobilized on an inert support, and the organic phase flows through the supported matrix to extract the targeted analytes.[3]

In this application note, a comparison was performed between Oasis PRiME HLB SPE, SLE, and LLE in both plasma and urine matrices for bioanalysis and forensic toxicology. In plasma, 22 commonly analyzed pharmaceuticals, steroids, and drugs of abuse were extracted using the three aforementioned methods and the results were compared. In urine, 23 drugs of abuse representing opioids, stimulants, benzodiazepines, and synthetic cannabinoid metabolites were tested for forensic toxicology analysis.

Key areas of comparison were: procedure simplicity, analyte recoveries, and matrix effects (ME). The mechanisms behind these three techniques and how they affect their respective performances are discussed as well. Oasis PRiME SPE shows very high and consistent recoveries and excellent matrix effects across all of the tested analytes in both matrices. For SLE and LLE, lower recoveries were observed for polar basic analytes in urine samples and acidic analytes in plasma samples. The LLE and SLE methods were then optimized for these specific compounds and improvements in the recoveries of problematic analytes were successfully achieved, but only at the expense of other analytes. Only Oasis PRiME HLB was able to successfully extract all analytes from plasma and urine samples with a single method.

Materials

RCS-4 M10, RCS-4 M11, JWH-073 4-COOH, JWH-073 4-OH, and JWH-018 5-COOH were purchased from Cayman Chemical (Ann Arbor, MI). All other compounds and metabolites were purchased from Cerilliant (Round Rock, TX).

Individual stocks (1 mg/mL) were prepared in methanol, DMSO, or 50:50 DMSO:methanol. A combined stock solution of all compounds (5 µg/mL) was prepared in methanol, except naproxen, which was at 50 µg/mL. Working solutions were prepared daily by spiking standards into matrices (plasma and urine) and performing serial dilutions to achieve the desired concentrations.

In plasma, 22 drugs were analyzed including acids (naproxen), bases (most analytes), and neutrals (phenacetin, 17 α-OH progesterone) used in a variety of application areas. In urine, 23 drugs of abuse representing opioids, stimulants, benzodiazepines, and synthetic cannabinoid metabolites were tested.

Experimental

UPLC Conditions

LC system:	ACQUITY UPLC I-Class, (FL)

Column:	CORTECS $C_{18,}$ 1.6 µm, 2.1 × 100 mm
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile
Flow rate:	0.6 mL/min
Gradient:	See Table 1
Column temp.:	40 °C
Sample temp.:	10 °C
Strong needle wash:	70/30 ACN/water with 2% formic acid
Weak needle wash:	5/95 ACN/water with 1% formic acid
Injection mode:	Partial loop with needle overfill
Injection volume:	2–5 µL

Gradient

Time (min)	Profile %A	Profile %B	Curve
0	95	5	
2	75	25	6
6	50	50	6

Time (min)	Profile %A	Profile %B	Curve
6.1	30	70	6
7	5	95	6
7.5	95	5	6
9	95	5	6

MS Conditions

MS system:	Xevo TQ-S
Ionization mode:	ESI+
Capillary voltage:	3.0 kV
Desolvation temp.:	500 °C
Cone gas flow:	150 L/Hr
Desolvation gas flow:	1000 L/Hr
MRM transition monitored:	See Table 2

Compound	Function	MRM	Cone V	Coll. E	Matrix
Phenacetin	Analgesic, fever reducer	180.1>110.1	26	20	Plasma
Propranolol	□-blocker	260.2>116.2	48	16	Plasma
Cortisol	Corticosteroid	363.2>121.1	42	22	Plasma
Protriptyline	Antidepressant	264.2>155.1	38	26	Plasma
Amirtriptyline	Antidepressant	278.2>91.0	44	22	Plasma
Naproxen	Analgesic, fever reducer	231.1>185.1	20	16	Plasma
17α-OH-progesterone	Steroid	331.2>97.1	58	26	Plasma
Cocaine	Stimulant	304.1>82.0	40	30	Plasma/urine
Fentanyl	Opiate/opioid	337.2 >188.2	48	22	Plasma/urine
Oxazepam	Benzodiazepine	287.0>104.0	44	30	Plasma/urine
Benzoylecgonine (BZE)	Stimulant (Cocaine metabolite)	290.1>168.1	55	19	Plasma/urine
Clonazepam	Benzodiazepine	316.0>214.1	54	42	Plasma/urine
Lorazepam	Benzodiazepine	321.0>229.1	40	28	Plasma/urine
Alprazolam	Benzodiazepine	309.1>205.1	60	42	Plasma/urine
Flunitrazepam	Benzodiazepine	314.1>268.1	50	25	Plasma/urine
Temazepam	Benzodiazepine	301.1>177.1	36	46	Plasma/urine
Diazepam	Benzodiazepine	285.1>154.0	54	26	Plasma/urine
RCS-4, M10	Synthetic cannabinoid	324.2>121.0	40	36	Plasma/urine
RCS-4, M11	Synthetic cannabinoid	322.2>121.0	42	32	Plasma/urine
JWH-073, 4-COOH	Synthetic cannabinoid	358.2>155.1	52	32	Plasma/urine
JWH-073, 4-OH	Synthetic cannabinoid	344.2>155.1	52	32	Plasma/urine
JWH-018, 5-COOH	Synthetic cannabinoid	372.2>155.1	54	32	Plasma/urine
Amphetamine	Amine stimulant	136.0>119.0	22	8	Urine
MDEA	Amine stimulant	208.1>105.0	26	24	Urine
Methamphetamine	Amine stimulant	150.0>91.0	24	20	Urine
MDMA	Amine stimulant	194.1>163.0	26	12	Urine
MDA	Amine stimulant	180.1>163.0	22	11	Urine
Phentermine	Amine stimulant	150.0>91.0	24	20	Urine
Norfentanyl	Fentanyl metabolite	233.2>177.2	30	14	Urine
6-Acetylmorphine	Heroin metabolite	328.2>165.1	60	26	Urine

Table 2. Drug functions, MRM transitions, cone voltages (Cone V), and collision energies (Coll. E) for test analytes.

Sample preparation protocols

In this evaluation, the protocol used with Oasis PRiME HLB was the generic 3-step load-wash-elute protocol. Depending on the matrix, either 400 µL of plasma diluted 1:1 with 4% H_3PO_4 or 400 µL hydrolyzed urine diluted 1:1 with 4% H_3PO_4 was directly loaded onto an Oasis PRiME HLB µElution Plate. No conditioning or equilibration

was needed or performed for either matrix. The samples were then washed with 2 × 200 µL 5% MeOH and eluted with 2 × 25 µL 90:10 ACN:MeOH. The eluate was then diluted with 100 µL water, vortexed, and directly injected into the LC-MS system without evaporation or reconstitution.

For SLE, there are multiple dilution buffers (to dilute the biological sample for loading) and extraction solvents suggested depending on the analytes of interest. Since the aim of this work was to compare one single method targeting all compounds, we evaluated protocols with the highest likelihood of success. The protocols selected for this evaluation were designed for neutral and basic analytes as they are predominant in the mixture. For plasma samples, 400 µL diluted plasma (200 µL rat plasma + 200 µL water) was loaded into an SLE plate (obtained from a competitor, rigidly designed for 400 µL sample load). Loading was initiated by applying gentle vacuum (~ 3 psi) for 2–5 seconds and waiting 5 minutes for the sample to completely absorb onto the support matrix. To begin the extraction of the analytes, 800 µL of extraction solvent (MTBE: Methyl tert-butyl ether) was then applied and allowed to flow over the matrix for 5 minutes under gravity. Vacuum (10 psi) was applied again for 10–30 seconds to complete the elution. The extraction steps were then repeated by adding another 800 µL of MTBE. To ensure compatibility with LC-MS analysis and concentrate the analytes, the extract was evaporated to dryness under N_2 gas flow at 40 °C and then reconstituted in 200 µL of 30% acetonitrile (ACN). For urine samples, two similar pretreatment protocols were used. 200 µL hydrolyzed urine was diluted 1:1 with either water or 0.5 M NH_4OH. Samples were then loaded onto the SLE plate and processed as described above for plasma samples.

For LLE, the experiments were performed using single 2 mL centrifuge tubes. As LLE and SLE share a similar mechanism, similar protocols were applied. 1000 µL MTBE was added to either 400 diluted plasma or hydrolyzed urine for the LLE experiments. As with SLE, plasma samples were diluted with 200 µL water. 200 µL hydrolyzed urine samples were diluted with either 200 µL water or 200 µL of 0.5 M NH_4OH. The samples were then vortexed for 5 min and centrifuged for 5 min at 11000 rcf. The top layer was transferred to a collection plate and evaporated to dryness under N_2 gas flow at 40 °C and reconstituted in 200 µL of 30% acetonitrile (ACN).

Urine hydrolysis for all samples/techniques: 200 µL of spiked urine was mixed with 160 µL of water and 40 µL of β-glucuronidase enzyme (Roche, *E. coli*) at room temperature for 30 minutes to simulate enzymatic hydrolysis.

Recovery and matrix effect calculations

Analyte recovery was calculated according to the following equation:

% Recovery = (Area A/ Area B) x 100%

Where A equals the peak area of an extracted sample and B equals the peak area of an extracted matrix sample in which the compounds were added post-extraction.

Matrix effects were calculated according to the following equation: Matric Effects = ((Peak area in presence of matrix/Peak area in the absence of matric)$^{-1}$) x 100%

The peak area in the presence of matrix refers to the peak area of an extracted matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to analytes in a neat solvent solution.

Results and Discussion

Chromatography

A representative chromatogram of all compounds from a 20 ng/mL extracted plasma sample is shown in Figure 1. The urinary chromatography is shown in Figure 2. Using a CORTECS UPLC C_{18} Column (90Å, 1.6 µm, 2.1 x 100 mm), all analytes were analyzed within 6.5 minutes. Peak shape was excellent for all compounds, with no significant tailing or asymmetries, and all peak widths were under 3 seconds at 5% of baseline. All potentially interfering compounds such as methamphetamine and phentermine, which share an MRM transition (150>91) were baseline separated.

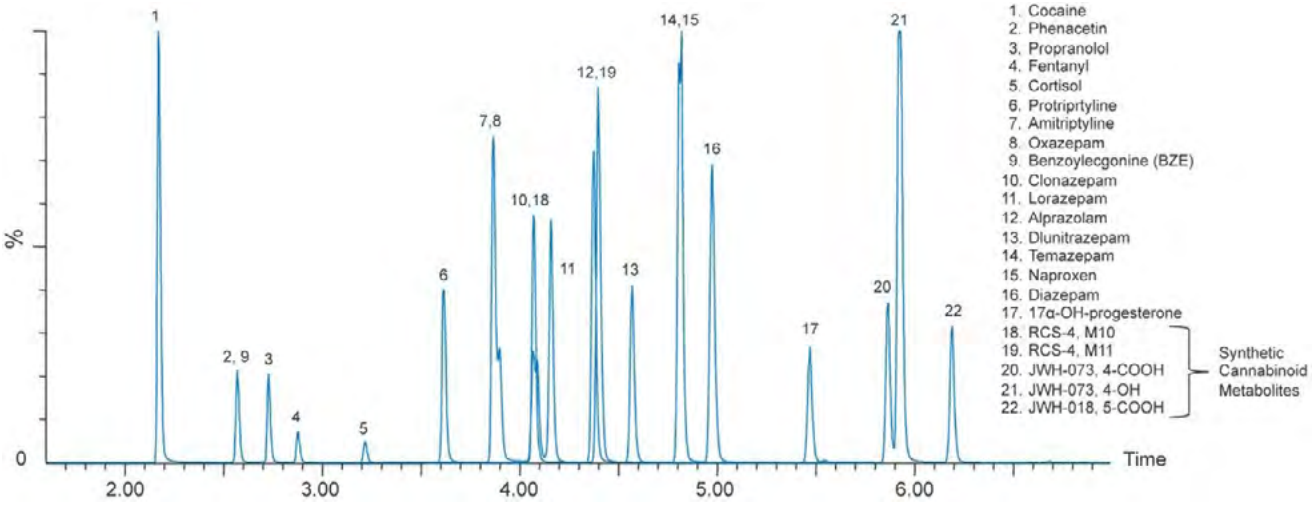

Figure 1. Chromatography of analytes in an extracted plasma sample. The LC gradient is shown in Table 1. MRM transitions for all compounds are listed in Table 2.

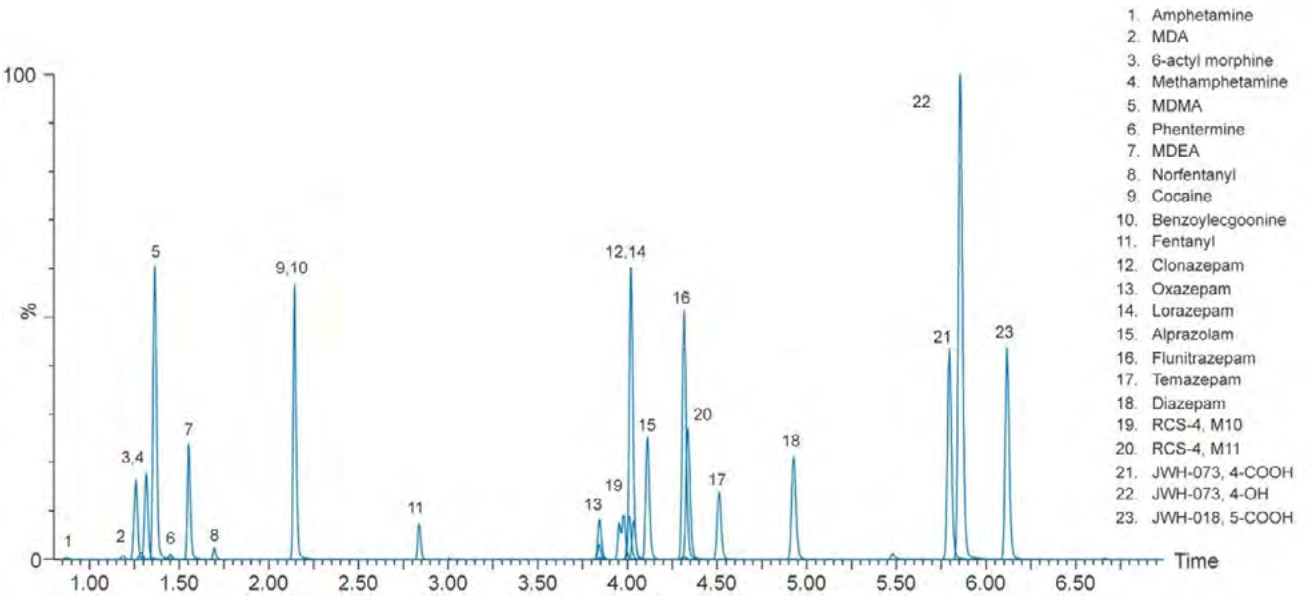

Figure 2. Chromatography of analytes in an extracted urine sample. The LC gradient is shown in Table 1. MRM transitions for all compounds are listed in Table 2.

Figure 3 details the extraction protocols and processing time for SPE, SLE, and LLE. The total time required to prepare 96 plasma samples is 15 minutes for Oasis PRiME HLB, 40 minutes for SLE, and 60 minutes for LLE. Oasis PRiME HLB uses a simple, generic three step SPE technique that removes salt, proteins, and phospholipids without the need for evaporation and reconstitution (in the µElution format), whereas SLE and LLE require method development with different sample pretreatment or extraction solvents for different classes of analytes. SLE requires a 5 minute waiting time after loading to allow the sample to fully adsorb onto the support matrix. In addition, an additional 5 minute waiting time is required after the extraction solvent is applied to allow the analytes to interact with the extraction solvent. Since a water-immiscible solvent is used in extraction step, evaporation and reconstitution are required for LC-MS analysis. In addition, the initiation of the flow in the SLE sample loading step, which is accomplished by applying very gentle vacuum (~3 psi) for 2–5 seconds, is very subtle and takes time and practice to perfect. If the initiation time is too short (shorter than 2–5 seconds) or the pressure is too low, the aqueous sample won't be able to successfully immobilize to the sorbent. If the time is too long or the pressure is too high, the plasma sample will directly elute and result in a cloudy elution solution and higher matrix factors. In SLE and LLE, the use of harsh water-immiscible extraction solvents may also extract impurities from the frits and plates, contaminating the extraction solution. Extraction solvents such as MTBE also have a negative impact on both operators' health and the environment.

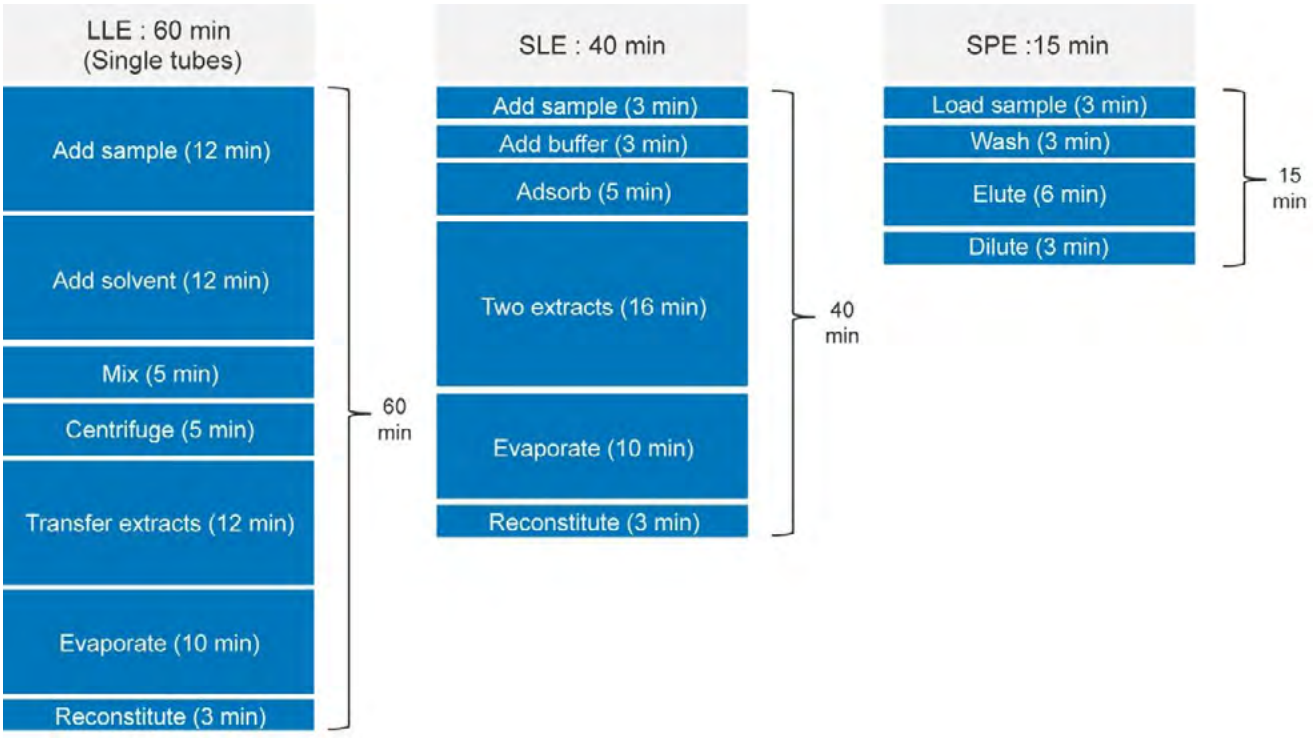

Figure 3. Protocols and processing times for LLE, SLE, and Oasis PRiME HLB extraction protocols.

With the simple three step SPE protocol, Oasis PRiME HLB demonstrates excellent and consistent recoveries across all the tested analytes (Figure 4A) with an average % recovery of 98±8%. All tested recoveries were within 75–110%. SLE showed good recoveries for neutral and basic drugs, but poor recoveries for acidic analytes such as naproxen and the COOH metabolite of the synthetic cannabinoid, JWH-073. Average recoveries were 89±7%. All analyte recoveries for LLE were lower than 80% with an average recovery at 70 ± 10%. Only one extraction was performed during the experiment, which may have resulted in decreased extraction efficiency. A second extraction may have increased recovery, but would also have increased processing time. Previous work has also indicated that additional extractions can contribute to increased matrix effects. For SLE and LLE, the extraction method was selected for neutral and basic analytes. Acidic analytes such as naproxen, JWH-073, 4-COOH, and JWH-018, 5-COOH were not recovered well at all (less than 30% recovery). Further method development or a separate protocol would be required for SLE or LLE to improve acidic analyte recovery such as different sample pretreatment or buffering. However, this could adversely affect the recovery of the basic drugs. Under these conditions, only Oasis PRiME HLB was able to extract the full complement of basic, neutral, and acidic compounds with a single protocol.

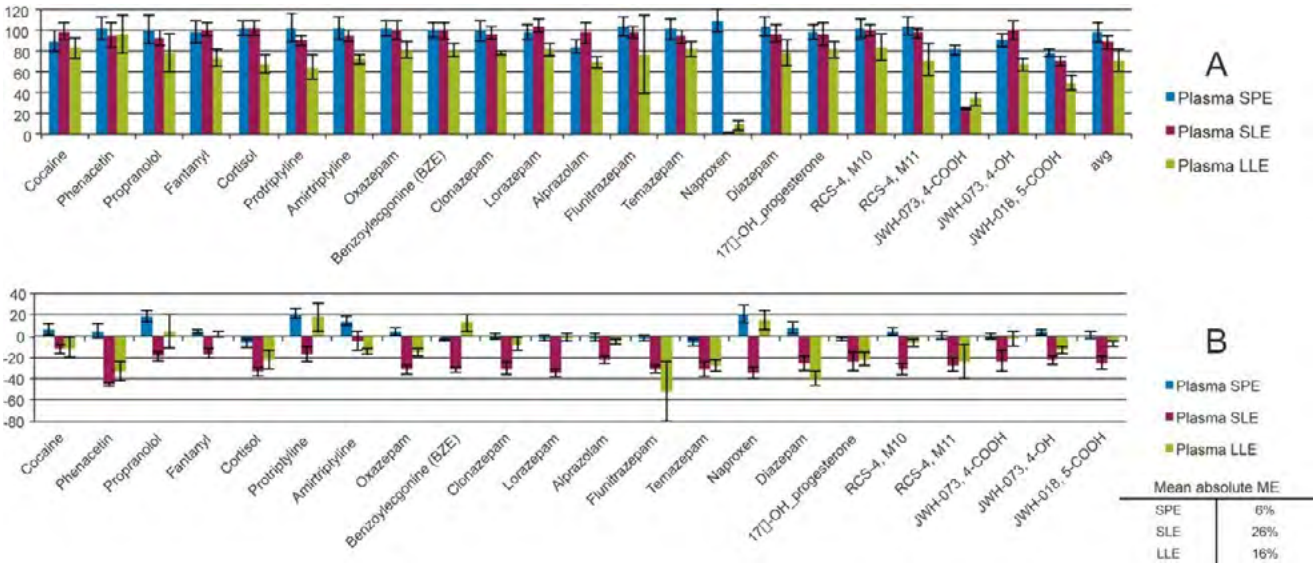

Figure 4A. Extraction recoveries for compounds in plasma samples (N=4). Blue, red, and green bars represent recoveries from Oasis PRiME HLB, SLE, and LLE, respectively. Error bars represent standard deviations. Figure 4B. Matrix effects for compounds extracted from plasma samples. Blue, red, and green bars represent mean matrix effects from Oasis PRiME HLB, SLE, and LLE, respectively. The means of the absolute values of matrix effects are listed on the lower right.

The overall matrix effects for Oasis PRiME HLB were lower than SLE or LLE (Figure 4B). All matrix effects for Oasis PRiME HLB were <20%, while 17/22 drugs from SLE and 7/22 drugs from LLE processing have MEs that are greater than 20%. The average magnitude of matrix effects for Oasis PRiME HLB was only 6%, while SLE was 26% and LLE was 16%. Furthermore, matrix effects for LLE were more variable. Matrix effect standard deviation values ranged from 1.4–8.8% for SPE, 1.9–10.3% for SLE and 2.6–28.3% for LLE. The three step protocol on Oasis PRiME HLB removed salts, proteins, and phospholipids and resulted in very clean final extracts with minimal matrix effects for all 22 different drugs, from several diverse classes. The higher matrix effects seen in SLE extracts may be a result of impurities extracted from the SLE sorbent as this wasn't seen in LLE extracts where the same sample and extraction solvent were used. Alternatively, it could also be simply a result of the more efficient extraction seen with SLE vs. LLE. Since LLE appears to be more effective at extracting the analytes from urine, it may also extract other components that could contribute to ion suppression.

Overall, Oasis PRiME HLB demonstrated superior recovery and minimal matrix effects when the sample matrix contains a wide variety of compounds. In this case, this included acids (naproxen and the synthetic cannabinoid metabolites), bases (most drugs), and neutral compounds of varying polarities. SLE yielded acceptable recoveries for neutral and basic analytes, but with much higher matrix effects. LLE, due to its limited extraction efficiency, had lower recoveries (10–20% lower in recoveries compared to SPE and SLE). LLE also demonstrated higher variability in matrix effects, particularly for compounds such as flunitrazepam and propranolol. Using an SLE or LLE extraction, acidic analytes can't be efficiently recovered with this single procedure, and additional method development would be required to improve acid recoveries.

Urine samples

A wide panel of 23 drugs of abuse which included stimulants, opioids, benzodiazepines, benzoylecgonine (BZE), and synthetic cannabinoid metabolites was hydrolyzed and extracted with SPE, SLE, and LLE. As shown in Figure 5, high and consistent recoveries were obtained using the Oasis PRiME HLB generic 3-step protocol. Recoveries were >75% for 21/23 tested drugs and the overall average recovery was 86% ± 6.6%. Two extraction protocols for SLE and LLE extractions were performed as described in the materials and methods section. When samples were diluted with water (Figure 5A) SLE showed good recoveries for many drugs with the exception of the hydrophilic bases such as most of the amine stimulants and norfentanyl (the recoveries were lower than 60%). LLE exhibited a similar trend to SLE with much lower recoveries. When SLE and LLE extractions were performed after adjusting the pH of the urine samples to 11 with 0.5 M ammonium hydroxide, recoveries of the polar amines improved significantly (Figure 5B). However, this was at the expense of the more acidic compounds such as the carboxy metabolites of JWH-073 and JWH-018. Unlike Oasis PRiME HLB, a single protocol for SLE or LLE was unable to extract all of the analytes from the samples with acceptable recovery.

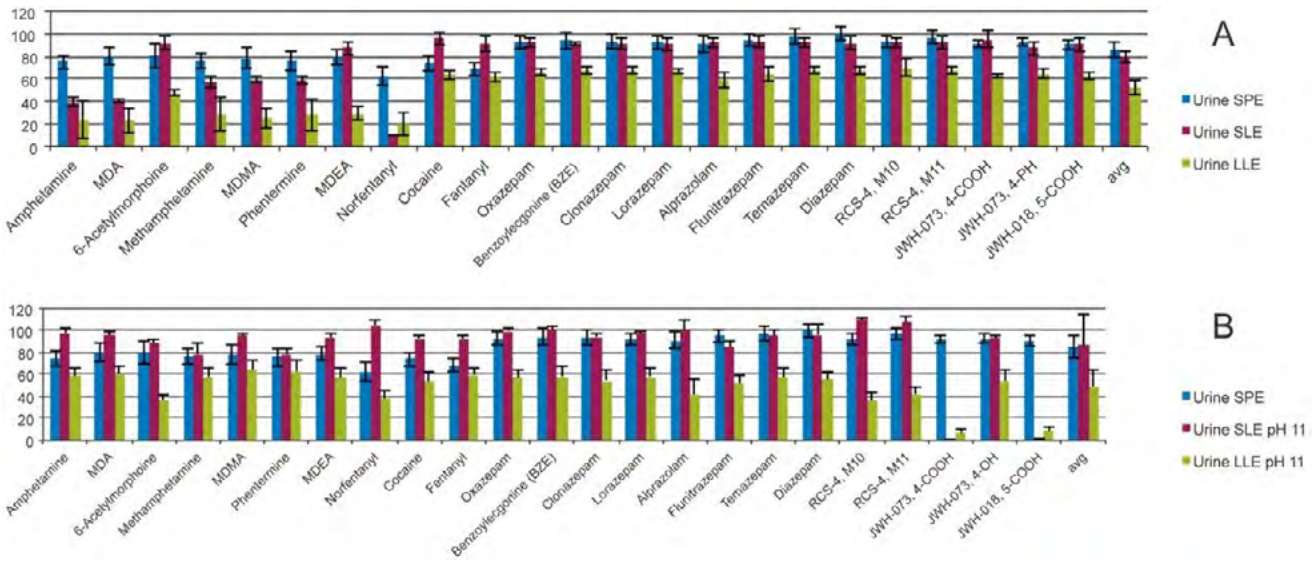

Figure 5A. Extraction recoveries for compounds in urine samples. SLE and LLE processed samples were diluted with water (N=4). Blue, red, and green bars represent recoveries from Oasis PRiME HLB, SLE, and LLE, respectively. Error bars represent standard deviations. Figure 5B. Extraction recoveries for samples extracted from urine. In this case, the LLE and SLE samples were diluted with 0.5 M NH_4OH.

The matrix effects for Oasis PRiME HLB, SLE, and LLE are roughly comparable (Figure 6A). The absolute average of matrix effects for SPE, SLE, and LLE were 12, 12, and 17 respectively, all of which are acceptable. Matrix effects were within 20% for the majority of the compounds using any of the three extraction techniques. When the urine pH was adjusted with ammonium hydroxide (Figure 6B), matrix effects for SLE increased to an average of 25%, while those for LLE remained relatively low, with a mean absolute value of 14%.

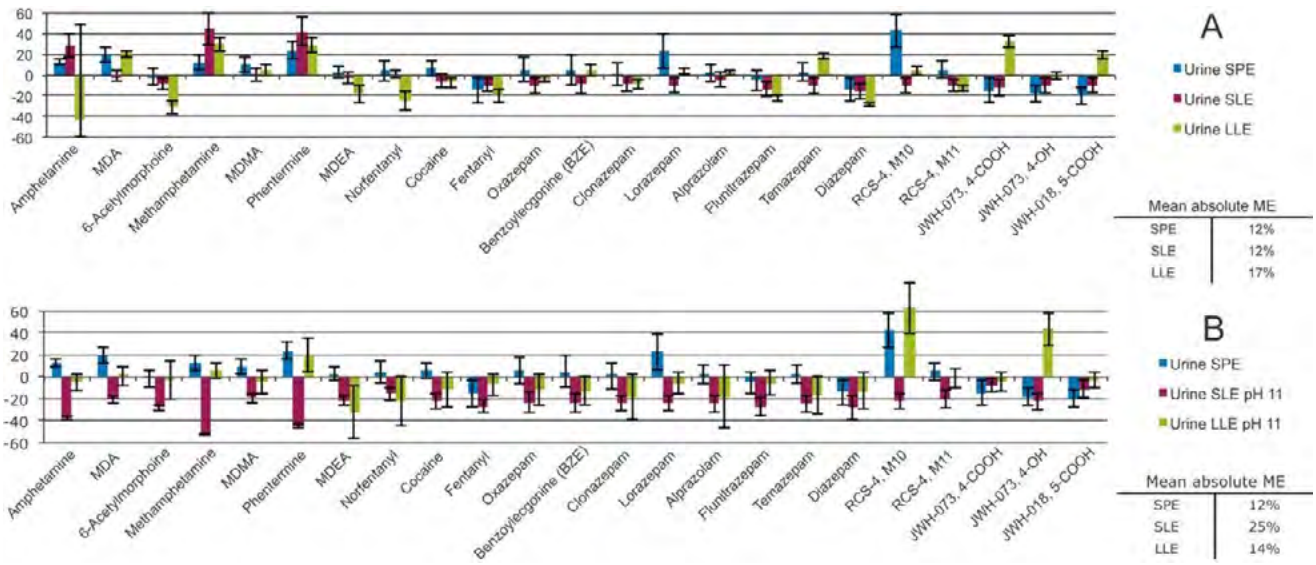

Figure 6A. Matrix effects from urine samples. Samples diluted with water and extracted by SLE or LLE. Figure 6B. Samples were diluted with 0.5 M NH_4OH to pH 11 and extracted by SLE or LLE. The means of the absolute values of matrix effects are listed on the right.

Conclusion

In this application, a comprehensive comparison of sample preparation techniques including SPE, SLE, and LLE was conducted in plasma and urine, using a wide variety of compounds found in bioanalysis and forensic toxicology. Oasis PRiME HLB employed a simple, three step protocol in which reduced extraction time by 60% and 75% compared to SLE, and LLE, respectively, for forensic toxicology. Oasis PRiME HLB also demonstrated superior recoveries and matrix effects for a variety of tested drugs without any additional method development. SLE and LLE required additional method development or multiple extraction protocols to achieve recoveries that were comparable to Oasis PRiME HLB for all of the tested analytes. The unique nature of Oasis PRiME HLB enabled the elimination of conditioning and equilibration steps, simplifying the extraction procedure and speeding up the sample preparation workflow. The µElution format enabled the direct injection of extracts without evaporation or reconstitution.

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Application Note

Tof-MRM for the Confirmation of Fentanyl Analogues for Forensic Toxicology

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For forensic toxicology use only.

Abstract

The objective of this current analytical work was to evaluate the performance of Tof-MRM – a targeted mode of acquisition, which is also available on the same HRMS platform and to develop a confirmatory method for the fentanyl class. A secondary aim was to utilize a previously reported novel technique i.e., Threshold Accurate Calibration (TAC), for matrix normalization without the requirement for deuterated internal standards.

Benefits

- Tof-MRM for superior specificity and enhanced sensitivity of fentanyl analogues
- Novel analytical approach to compensate for matrix effects
- Simplified in-well sample preparation
- Differentiation of structural isomers
- Method is adaptable and can be easily updated as new fentanyl analogues emerge

Introduction

High resolution mass spectrometry (HRMS) using quadrupole time-of-flight (QTof) instrumentation is increasingly used within the field of forensic toxicology as a comprehensive screening technique. Typically it is used in a non-targeted mode of data acquisition i.e., Tof-MS^{E}, which provides a highly specific identification based on a combination of precursor and fragment ions generated under low and high-energy conditions, respectively.[1-4] The Tof-MS^{E} approach has also been applied recently as the confirmatory step in a dual-definitive workflow protocol for commonly analyzed drug substances in forensic urine drug testing.[5]

QTof instruments can also be used in targeted acquisition mode, when the goal is to identify and quantify a more limited panel of analytes of interest.[6] In recent years, the availability and use of illicit fentanyl and, in particular fentanyl analogues, has become increasingly evident in forensic toxicology. Consequently updated methods, which enable the sensitive detection and confirmation of these emerging substances, are urgently required.

The objective of this current analytical work was to evaluate the performance of Tof-MRM – a targeted mode of acquisition, which is also available on the same HRMS platform and to develop a confirmatory method for the fentanyl class. A secondary aim was to utilize a previously reported novel technique i.e., Threshold Accurate Calibration (TAC), for matrix normalization without the requirement for deuterated internal standards.[7-9]

Experimental

Reference analytes

Reference material for the fentanyls (fentanyl, norfentanyl, and all analogues) were obtained from Cerilliant and/or Cayman Chemical at a concentration of 1 mg/mL. A mixed fentanyl stock solution was prepared by dilution in methanol, to give a concentration of 10 µg/mL, and stored at -20 °C until use.

TAC spiking solutions

A TAC spiking solution was prepared by dilution of the mixed fentanyl stock with water to give a concentration of 30 ng/mL. Water was used as a corresponding blank spiking solution.

Calibrators, controls and case samples

Analyte-free urine was enriched with the mixed fentanyl stock solution to yield a single calibrator at a concentration 2 ng/mL. A lower limit of detection (LLD) control was prepared at 0.8 ng/mL urine.

Quality control samples were prepared by enriching analyte-free urine with a mixed stock of fentanyls from a separate source, to yield the following QCs: 1.5, 3.0, 10 ng/mL urine.

Authentic samples were obtained from routine casework.

Sample preparation

Fifty microlitres of each sample (calibrator, control or case urine) were added to duplicate, adjacent wells of a 96-well plate for analysis of 'neat' and 'spiked' samples (Figure 1).

Fifty microlitres of the TAC spiking solution was added to all 'spiked' wells and 50 µL of blank spike was added to corresponding 'neat' wells.

After addition of 500 µL mobile phase (87% MPA:13% MPB) to all wells, the plate was transferred to the ACQUITY UPLC autosampler for the analysis of 5 µL of sample using UPLC-Tof-MRM.

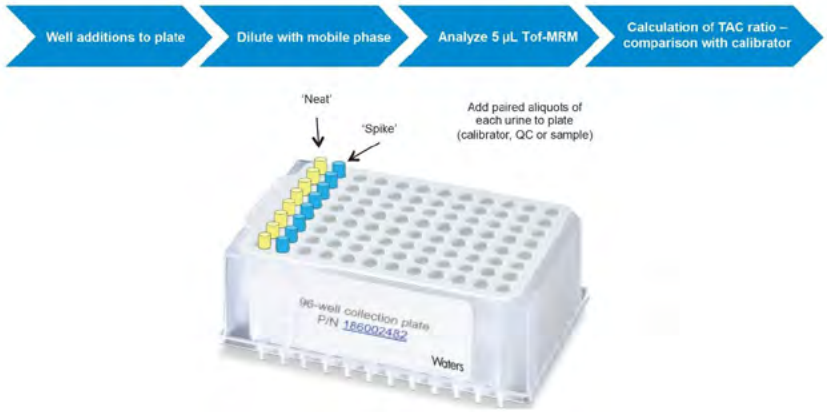

Figure 1. Summary of the analytical procedure: in-well sample preparation followed by Tof-MRM analysis.

LC conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC HSS C_{18} 100 Å, 1.8 µm, 2.1 mm × 150 mm (p/n 186003534)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM Ammonium formate pH 3.0
Mobile phase B:	0.1% Formic acid in acetonitrile
Gradient program:	Table 1

Gradient

UPLC gradien t table			
Time (min)	Flow (mL/min)	% MPA	% MPB
0.0	0.4	87	13
0.5	0.4	87	13
10.0	0.4	50	50
10.75	0.4	5	95
12.25	0.4	5	95
12.5	0.4	87	13
15.0	0.4	87	13

Table 1. UPLC Gradient Program.

MS conditions

MS conditions

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI positive
Capillary voltage:	0.8 kV
Cone voltage:	25 V
Desolvation temp.:	500 °C
Desolvation gas flow:	1000 L/hr
Cone gas:	20 L/Hr
Acquisition mode:	Tof-MRM (Table 2)

Name	RT	Precursor mass (*m/z*)	Product ion 1 quantifier (*m/z*)	CE (eV)	Product ion 2 qualifier (*m/z*)	CE (eV)
4-ANPP	6.16	281.2012	188.1434	15	105.0699	40
Acetyl fentanyl	5.11	323.2118	188.1434	32	105.0699	40
Acryl fentanyl	5.97	335.2118	188.1434	26	105.0699	40
Butyrfentanyl (BF) Isobutyrfentanyl (iBF)	7.21 7.08	351.2431	188.1434	25	132.0808	40
Carfentanil	7.03	395.2329	335.2118	20	246.1489	20
Fentanyl	6.20	337.2274	188.1434	38	105.0699	40
4-Fluorobutyrfentanyl (4-BF) 4-Fluoroisobutyrfentanyl (4-FiBF)	7.53 7.42	369.2337	188.1434	26	105.0699	40
Furanyl fentanyl	6.44	375.2067	188.1434	22	105.0699	40
Beta-hydroxy fentanyl	5.40	353.2224	204.1385	26	335.2118	20
Methoxy acetyl fentanyl	4.81	353.2224	188.1434	22	105.0699	40
3-Methyl fentanyl	7.03	351.2431	204.1385	25	146.0964	30
Norfentanyl	3.22	233.1648	177.1386	28	150.0913	20

Table 2. Retention times (RT) and dual Tof-MRM conditions including optimized collision energy (CE).

Data management

UNIFI Scientific Information System was used for instrument control and data processing.

Innovative analytical approaches: TAC

A previously-described approach (Threshold Accurate Calibration; TAC)[1-3] was employed in this study to prepare the samples and to normalize matrix effects without the use of deuterated internal standards.

Briefly, calibrator, QC and unknown samples were analyzed without ('neat'), and with ('spiked'), addition of a cut-off amount of reference analytes (Figure 1).

The TAC ratio of 'neat' to 'spiked' peak-area response was determined for each specimen and compared with the ratio obtained for the urine calibrator containing drugs at the cut-off threshold concentration (in this assay, 2 ng/mL) for a simplified qualitative presentation of results e.g.:

- Analytes with a TAC ratio equal to, or greater than, the calibrator TAC ratio are POSITIVE
- Analytes with a TAC ratio below that of the calibrator are NEGATIVE

TAC ratio = 'Neat' peak-area response /'Spiked' peak-area response – 'Neat' peak-area response

Enabling technologies: Tof-MRM

Time-of-flight-mass spectrometers (Tof-MS) are typically used in a non-targeted acquisition mode such as MS^E to facilitate broad forensic screening.[4-6] Although this mode already provides a sensitive assay with limits of detection in the low ng/mL range, the same instrument can also be used in an alternative, mode e.g., Tof-MRM (Figure 2), this targeted mode can offer further increases in sensitivity; indeed, enhancements ranging from 2 to 200-fold have been reported.[7] Improved sensitivity can be useful where analytes are likely at very low concentrations and/or where simplified sample preparation techniques such as dilution-only protocols, are being utilized.

In this study, Tof-MRM was applied to diluted urine samples urine samples to analyze fentanyls – due to their high potency, these substances are often encountered in samples at low, or sub-ng/mL concentrations.

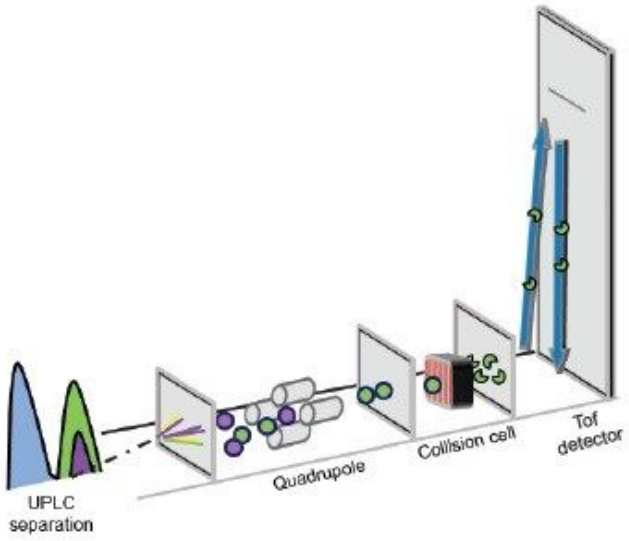

Figure 2. Schematic showing Tof-MRM analysis – in this mode the quadrupole of the QTof is used to isolate a specific precursor mass. Only this mass is permitted to enter the collision cell where it undergoes fragmentation. In this example, the quadrupole is set to isolate the m/z of the green peak. The Tof detector records the masses of all fragment ions – typically a quantifier and qualifier ion is monitored (Table 2).

Results and Discussion

An initial evaluation of Tof-MRM mode, using calibrators, revealed an increased sensitivity over Tof-MS^{E}; increases ranged from 5 to 20-fold. Owing to the aim to use a simple sample preparation protocol (effectively a 10-fold dilution of the sample) the targeted acquisition mode was utilized for the remaining study.

Samples were prepared using a simplified in-well sample protocol (Figure 1) and subsequently analyzed using a 15 minute chromatographic separation (Table 1), combined with optimized dual-transition Tof-MRM monitoring (Figure 3, Table 2). The method was highly sensitive and also permitted differentiation of structural isomers (Figure 4).

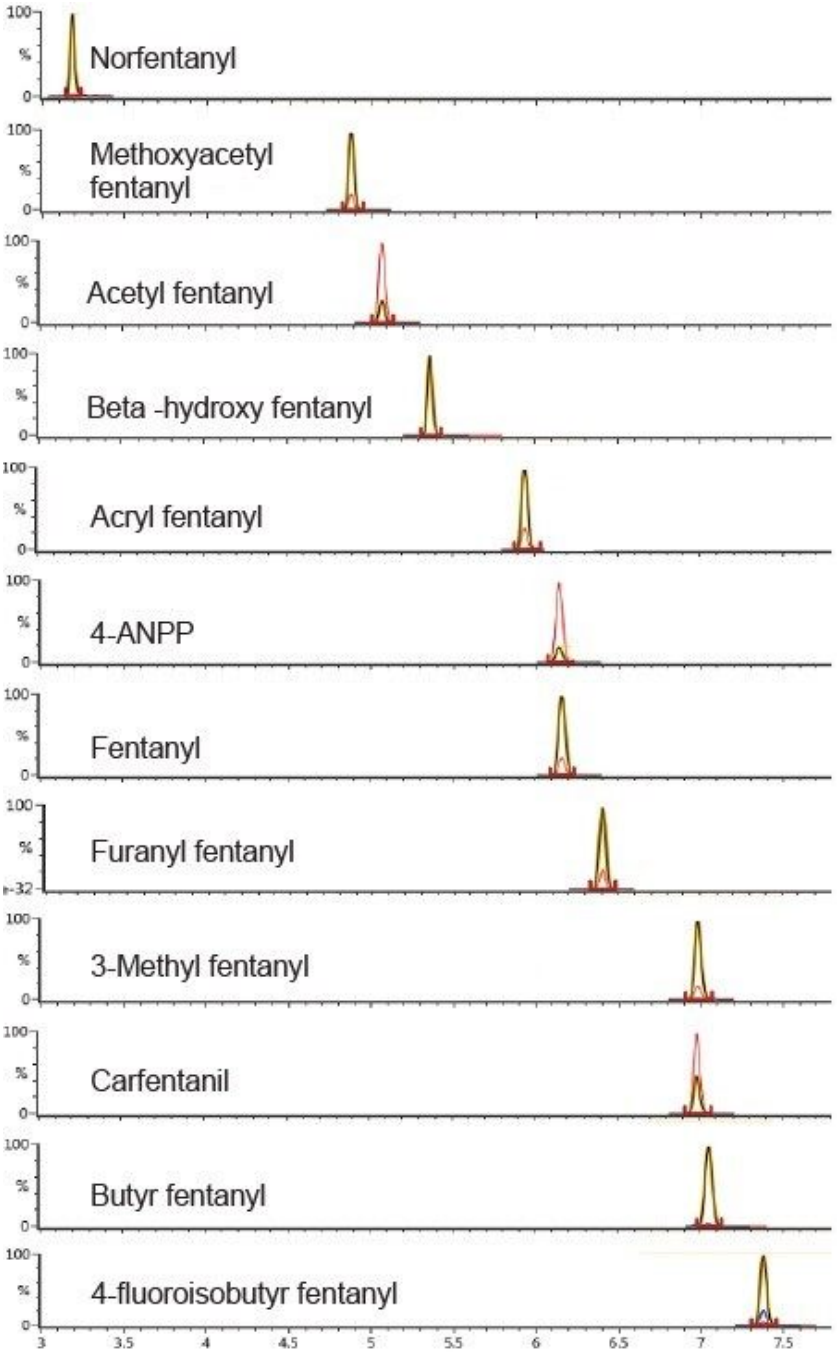

Figure 3. Tof-MRM mass chromatograms for an analyte-free urine sample enriched with the fentanyls at a 10 ng/mL; quantifier (yellow-trace) and qualifier (red-trace) are shown overlaid.

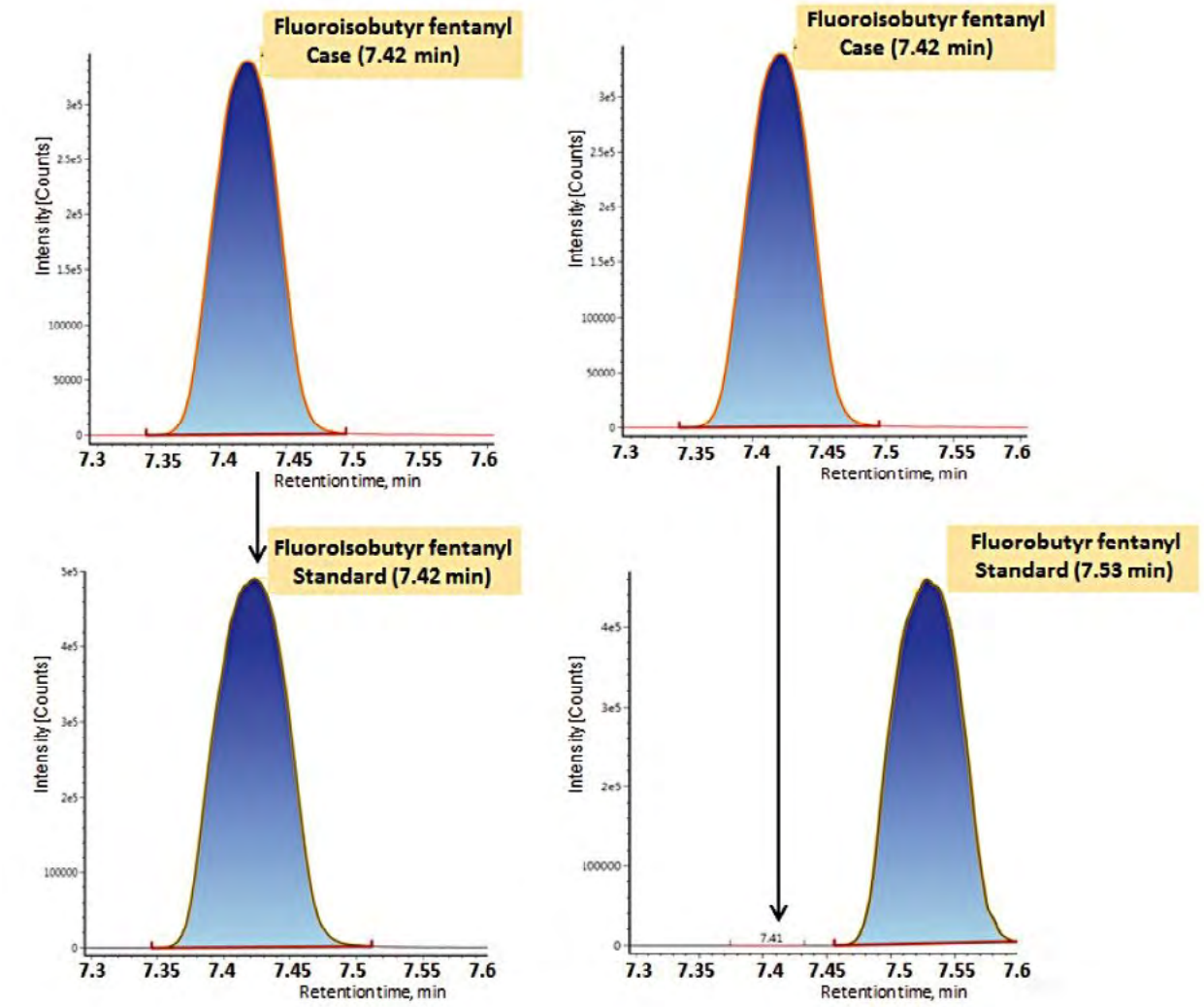

Figure 4. Tof-MRM analysis of 4-fluoroisobutyr fentanyl in a forensic case (upper) compared with reference standard analysis of 4-fluoroisobutyr fentanyl (lower-left) and 4-fluorobutyr fentanyl (lower-right).

Validation studies were conducted over 17 analytical runs and were designed in accordance with New York State Department of Health guidelines. The innovative TAC approach normalized the matrix effect and allowed consistent, threshold-accurate detection of all fentanyls investigated. Acceptable precision and accuracy was demonstrated for analyte concentrations around the cut-off i.e., at 0.8, 1.5, 3.0, and 10.0 ng/mL (Figure 5).

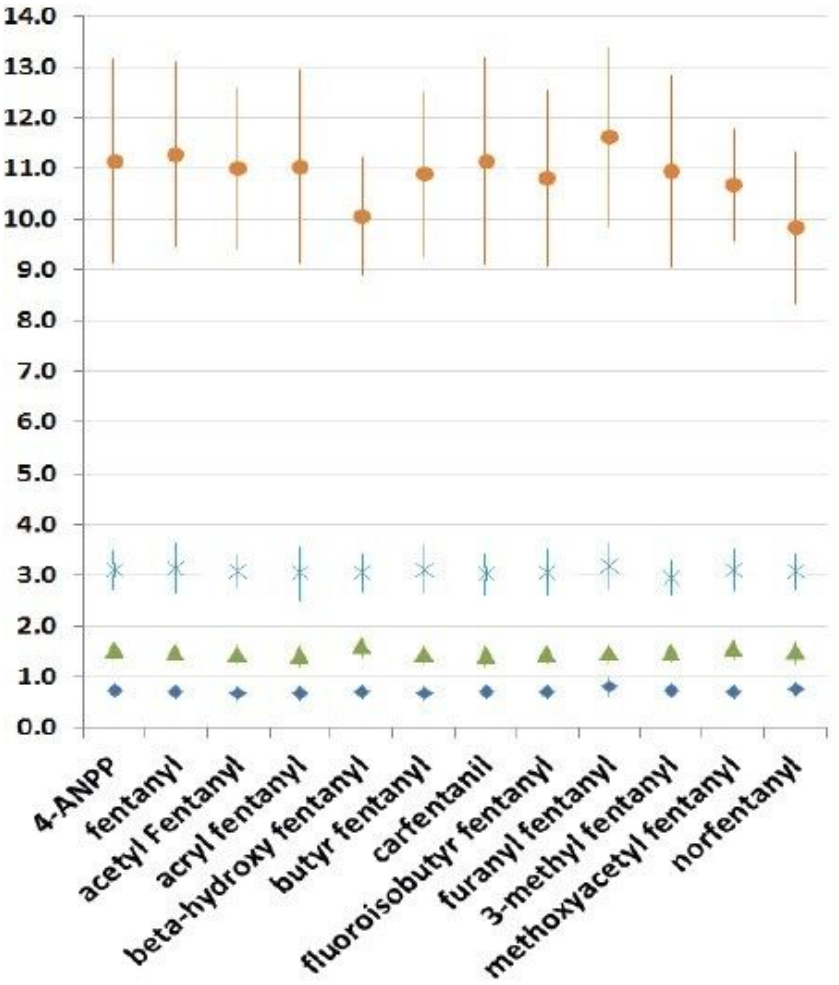

Figure 5. Accuracy and precision data for the QCs, over 17 analytical runs. Mean ± SD is shown for the target concentrations of 0.8, 1.5, 3.0, and 10.0 ng/mL.

The confirmatory method was applied to 25 forensic case samples that had been also analyzed by a separate, fast, UPLC-MS/MS based screening method and the Tof-MRM demonstrated 100% concordance with the independent screen.8 All case findings are summarized in Table 3, with fentanyl, norfentanyl, beta-hydroxy fentanyl, and 4-ANPP among the most commonly found. 4-ANPP is an intermediate, used in the manufacture of fentanyls and as such can be found as an impurity in fentanyl preparations; it is also understood to be a metabolite of fentanyl and some of the analogues including furanyl fentanyl, acetyl fentanyl, and acryl fentanyl.

Case no.	Fentanyls
1	Furanyl fentanyl, 4-ANPP
2	Furanyl fentanyl, fluoroisobutyr fentanyl, 4-ANPP
3	Norfentanyl, 4-ANPP
4	Methoxyacetyl fentanyl, 4-ANPP
5	Fluoroisobutyl fentanyl, fentanyl, norfentanyl
6	Fentanyl, norfentanyl, beta-hydroxyfentanyl
7	Fentanyl, norfentanyl, 4-ANPP
8	Fentanyl, norfentanyl, methoxyacetyl fentanyl
9	Fentanyl, norfentanyl, 4-ANPP
10	Methoxyacetyl fentanyl, 4-ANPP
11	Methoxyacetyl fentanyl, 4-ANPP
12	Fentanyl, norfentanyl, beta-hydroxyfentanyl
13	Fentanyl, norfentanyl, beta-hydroxyfentanyl, acetyl fentanyl, fluoroisobutyr fentanyl, 4-ANPP
14	Fentanyl, norfentanyl, beta-hydroxyfentanyl, acetyl fentanyl
15	Fentanyl, norfentanyl, beta-hydroxyfentanyl, 4-ANPP
16	Fentanyl, fluoroisobutyr fentanyl
17	Fentanyl, norfentanyl, beta-hydroxyfentanyl
18	Fentanyl, norfentanyl, beta-hydroxyfentanyl, methoxyacetyl fentanyl, 4-ANPP
19	Fentanyl, norfentanyl, beta-hydroxyfentanyl
20	Fentanyl, norfentanyl, beta-hydroxyfentanyl, 4-ANPP
21	Fentanyl, norfentanyl, beta-hydroxyfentanyl
22	Fentanyl, norfentanyl, 4-ANPP
23	Fentanyl, norfentanyl, beta-hydroxyfentanyl
24	Fentanyl, norfentanyl, fluoroisobutyr fentanyl
25	Fentanyl, norfentanyl, acetylfentanyl

Table 3. Forensic case findings by UPLC-MS/MS screening and UPLC-QTof MRM confirmation methods.

Conclusion

This application note describes a confirmatory method for use in forensic toxicology.

In line with previous reports, Tof-MRM provided increased sensitivity over the non-targeted Tof-MS^{E} approach.

The method utilizes some innovative approaches to produce a simple, yet accurate and precise qualitative method for the analysis of fentanyl and fentanyl analogues in urine.

The TAC approach, resulted in an accurate qualitative confirmation without the requirement of deuterated internal standards, which may not always be available, particularly for newer drug analogues.

The TAC approach also means that the method is adaptable and can be readily updated as new fentanyl analogues emerge.

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720006515, March 2019

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Application Note

Extraction of Synthetic Fentanyl Compounds and Removal of Phospholipids from Whole Blood Using Oasis PRiME MCX for Forensic Toxicology

Jonathan P. Danaceau, Michelle Wood, Lisa J. Calton

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This Application brief describes to develop an efficient extraction method for the UPLC-MS/MS analysis of synthetic fentanyl analogues from whole blood while removing residual phospholipids for forensic toxicology.

Benefits

Synthetic fentanyl compounds were rapidly and reproducibly isolated from whole blood while minimizing residual phospholipids.

Introduction

The need for the analysis of synthetic fentanyl analogues from blood for forensic toxicology has increased significantly in the last few years as the abuse of these compounds, as well as associated overdoses and fatalities, have increased substantially.[1] One of the main analytical challenges is the low concentrations of these compounds typically seen in blood samples. Because of this, robust and reproducible sample preparation methods can be crucial to achieving the required sensitivity. One of the components of biological samples that can impact sensitivity is the presence of phospholipids. Traditional mixed-mode cation exchange methods tend to have substantial amounts of residual phospholipids compared to other ion-exchange sorbents. This is of particular relevance when working with whole blood, which comprises up to 45% red blood cells, and contains a larger amount of phospholipids than plasma.

This work details the extraction of fentanyl analogs from whole blood using Oasis PRiME MCX µElution plates. Extraction efficiency, matrix effects, and phospholipid removal are all highlighted. In addition, sample pretreatment was optimized to efficiently process whole blood samples prior to solidphase extraction.

Results and Discussion

Synthetic fentanyl compounds were spiked into whole blood. Blood samples (100 µL) were treated with a solution of zinc sulfate and ammonium acetate ($ZnSO_4$/NH_4OAc) to lyse the cells, followed by precipitation with 50:50 ACN:MeOH. After centrifugation, the supernatant was diluted with 4% phosphoric acid (H_3PO_4) and extracted using Oasis PRiME MCX µElution plates. Samples were eluted with 50:50 ACN:MeOH containing 5% ammonia solution. Extracts were analyzed using an ACQUITY™ UPLC I-Class/Xevo TQ-S micro System.

Figure 1 shows the recovery of the synthetic fentanyl analogs from whole blood. Recoveries ranged from 67 to 91%, with an average of 86.4%. The values represent the mean obtained for three different concentrations (250, 1000, and 10,000 pg/mL). Recoveries were consistent, with all %RSDs under 10%. Matrix effects were calculated and are also shown in Figure 1. All matrix effects were less than 10% with RSDs less than 5% demonstrating that there was no ion suppression associated with this method. No concentration dependent bias was seen for recovery or matrix effects.

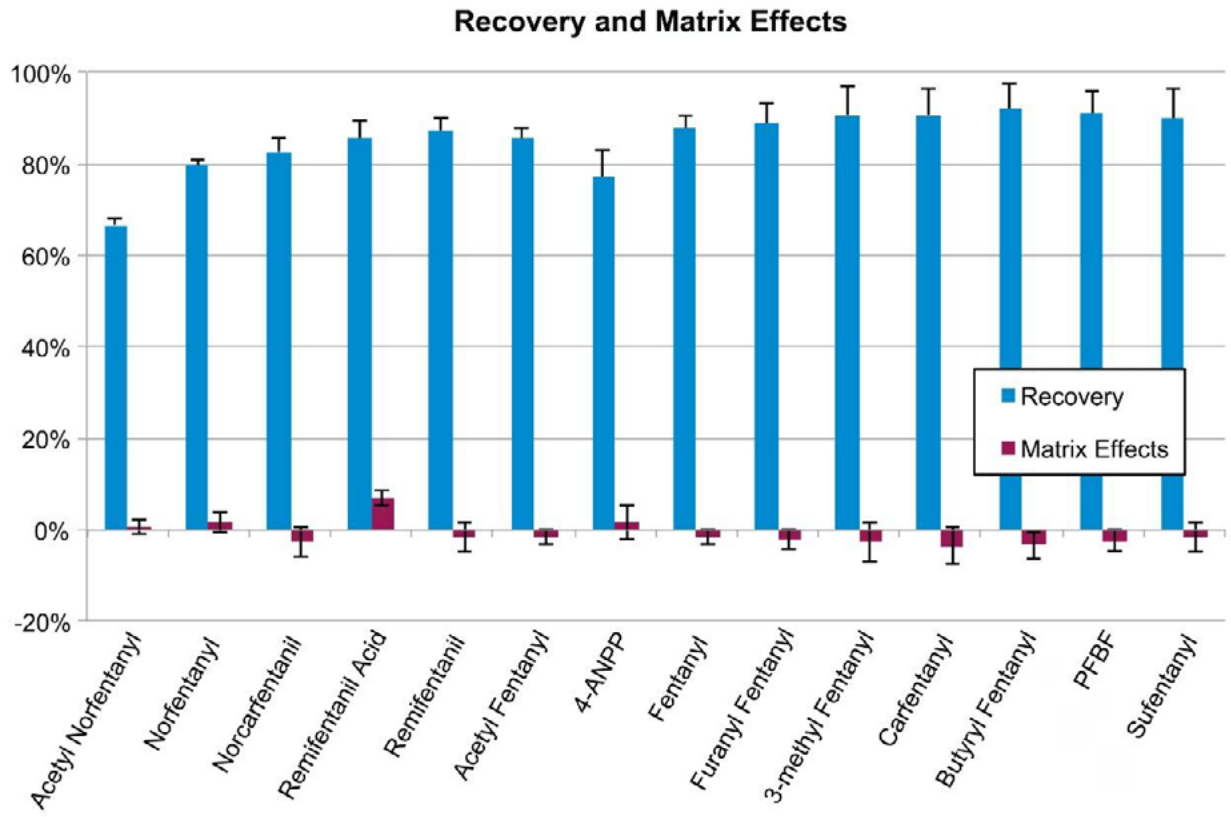

Figure 1. Recovery and matrix effects of synthetic fentanyl analogs from whole blood. Recovery and matrix effects were assessed at 250, 1000, and 10,000 pg/mL (N=4 at each concentration). Bars represent the mean value of all three concentrations and error bars represent standard deviations. No concentration related bias was seen for either recovery or matrix effects.

One of the novel properties of Oasis PRiME MCX is a superior removal of phospholipids. Figure 2 compares the phospholipids remaining from whole blood samples after protein precipitation, extraction with Oasis MCX, and extraction with Oasis PRiME MCX. The chromatogram and associated graph show that Oasis PRiME MCX removes greater than 99% of phospholipids compared to protein precipitation and 97.4% of phospholipids compared to Oasis MCX, a substantial improvement of an already clean extract.

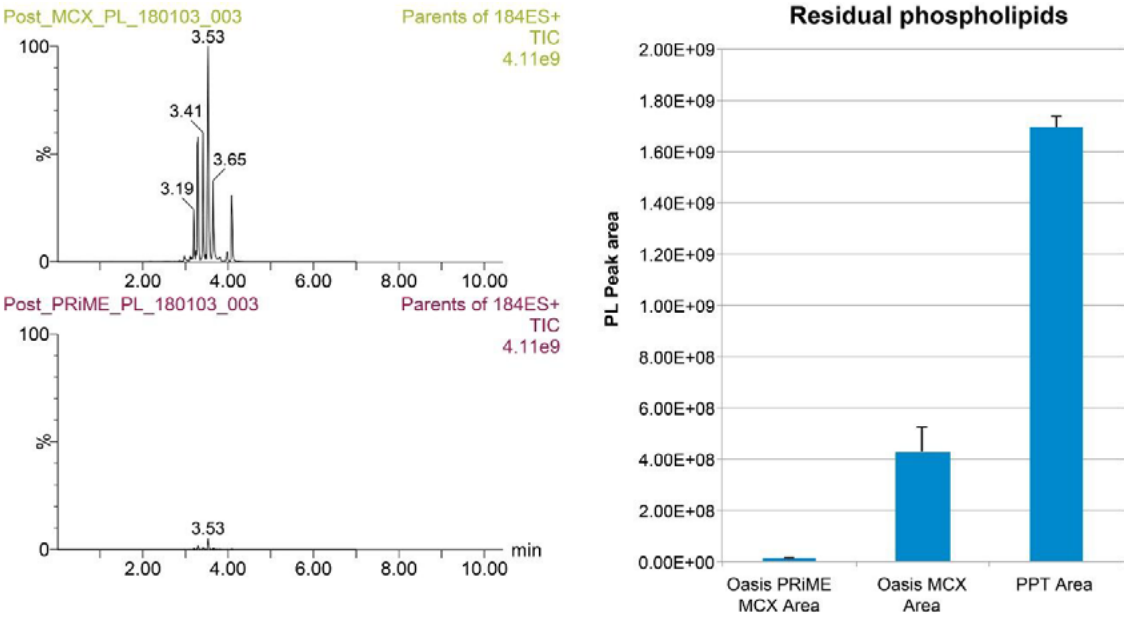

Figure 2. Residual phospholipids in remaining in extracted whole blood samples. The traces on the left show chromatograms of residual phospholipids from whole blood sample prepared with Oasis MCX (top) and Oasis PRiME MCX (bottom), respectively. The bar graph on the right represents the mean area of phospholipid traces from Oasis PRiME MCX, Oasis MCX, and protein precipitation. The total areas of phospholipids were compared for three preparation procedures (N=4 each).

Conclusion

Synthetic fentanyl compounds were extracted from whole blood using Oasis PRiME MCX µElution plates prior to UPLC-MS/MS analysis for forensic toxicology. In addition to providing high and consistent recoveries for all analytes with negligible matrix effects, residual phospholipids were nearly eliminated, with greater than 99% removed compared to protein precipitation and greater than 97% removed compared to Oasis MCX. This translates to cleaner extracts that can improve column lifetime, minimize MS maintenance, and is likely to contribute to the lack of matrix effects seen with this assay. This group of fentanyl analogues spans a wide range of polarities and this method should be applicable to additional fentanyl analogues as necessary.

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720006330, July 2018

Forensic Toxicology Screening

Application Notes

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Application Note

The Utility of MS^E for Toxicological Screening

Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief investigates the utility of the innovative data acquisition mode MS^E for the screening of toxicants in human specimens.

Introduction

Laboratories are frequently required to perform broad screening techniques on complex biological samples to identify drugs of abuse and other toxicants. In recent years there has been an increased interest in the use of Time-of-Flight (Tof) instruments for this purpose owing to the high level of specificity offered by accurate mass data.

While theoretical or exact mass libraries can be automatically generated without reference material *i.e.*, from molecular formulae, the lack of additional information can lead to false positive results in the analysis of

authentic samples. Thus, where possible, additional information ***e.g.***, an associated retention time (RT) and confirmatory fragment ions should be used to increase confidence in drug identification and to improve the ease and speed of data review and reporting.

MS^E is a novel, patented mode of data acquisition that permits the seamless collection of a comprehensive catalogue of information for both precursor and fragment ions in a single analysis.[1–3] This is achieved by rapidly alternating between two functions ***i.e.***, the first, acquired at low collision energy provides an accurate mass measurement of the precursor ion. The second, at elevated energy provides accurate masses of the fragment ions. In addition to providing increased confidence in identification, fragmentation can help to differentiate between isobaric compounds.

This application brief includes examples of MS^E data for toxicological compounds and summarizes some of the key benefits of this acquisition mode in comparison to conventional data-dependent techniques. We describe the flexibility around data processing and summarize the contents of the UNIFI™ Waters™ Forensic Toxicology Library.

Experimental

LC-MS system configuration

ACQUITY™ UPLC™ I-Class PLUS (FTN) System in combination with the Xevo™ G2 XS QTof Mass Spectrometer

LC-MS conditions

Column:	ACQUITY UPLC HSS C_{18}, 1.8 µm, 2.1 x 150 mm (p/n: 186003534)
Run time:	15 min
Ionization mode:	ESI+

Acquisition range:	*m/z* 50–1000
MS^E conditions	Collision energy function 1:6 eV Collision energy function 2:ramp 10–40 eV

Software and Library

UNIFI Software was used in combination with the Waters Forensic Toxicology Scientific Library.

Results and Discussion

Certified reference material (CRM) for toxicologically-relevant compounds were obtained from Merck (Dorset, UK) and were analysed using UPLC-Tof in MS^E mode. Figure 1 shows the MS^E data obtained following analysis of a representative substance, buflomedil. The figure illustrates how a confident identification can be obtained from the ability to measure the mass of the precursor ion to four decimal places (precursor mass is shown in the low energy spectrum). When MS^E is utilized, even greater confidence in identification can be achieved by additional incorporation of the masses of the specific fragment ions which are generated when the collision energy is ramped (high energy spectrum). Furthermore, RT can also be included in the identification criteria.

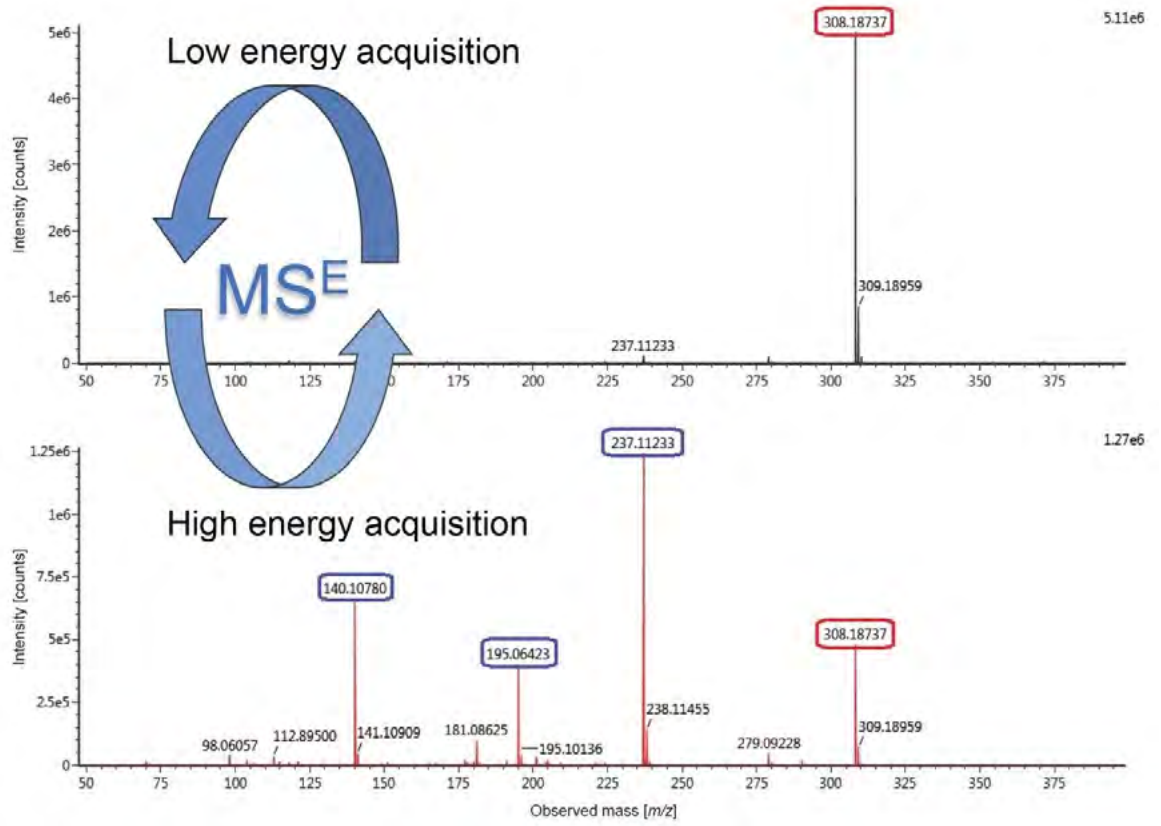

Figure 1. With MS^E the dataset is complete; full accurate mass data for both precursors (shown in red boxes) and fragment ions (blue boxes) is always acquired, even in the case of closely or co-eluting analytes.

MS^E is a data-independent technique meaning that full accurate mass data is being acquired continually under both low and high energy conditions throughout the analysis. This is in contrast to data-dependent (or information-dependent) techniques, where typically the instrument commences collecting full accurate mass data at low energy to provide mass information of the intact molecules until a 'trigger' is received which then instructs the instrument to switch to collecting data in tandem mass spectrometry (MS/MS) mode. The trigger could be any precursor ion exceeding a response threshold, or alternatively the instrument may be set to trigger on detection of specifically targeted precursor ion(s). The disadvantage of data-dependent approaches can be that while the instrument is collecting MS/MS - it is not collecting full scan MS data, thus the data is incomplete. In forensic toxicology a complete and unrestricted dataset is particularly advantageous as it provides the ability to retrospectively examine the data without fear that potentially relevant data has been omitted by use of

targeted acquisition techniques such as data-dependent analysis such as conventional MS/MS. In other words, the user can reprocess existing data without the need to reanalyze or reacquire additional data for the sample.

However, the key benefit of having the complete data means that it opens up the ability to process data using three complementary workflows as summarized in Figure 2.

Figure 2. These complementary workflows are possible owing to the complete nature of the MS^E data.

Targeted Analysis

Targeted analysis is the most straightforward approach, where the acquired data is simply matched against a reference library. The UNIFI Forensic Toxicology Screening Solution includes a comprehensive library, in which each library entry comprises a reference RT together with the exact mass of the precursor ion and verified diagnostic exact mass fragment ions. Figure 3 shows an image of a representative entry from the Waters Forensic Toxicology Library.

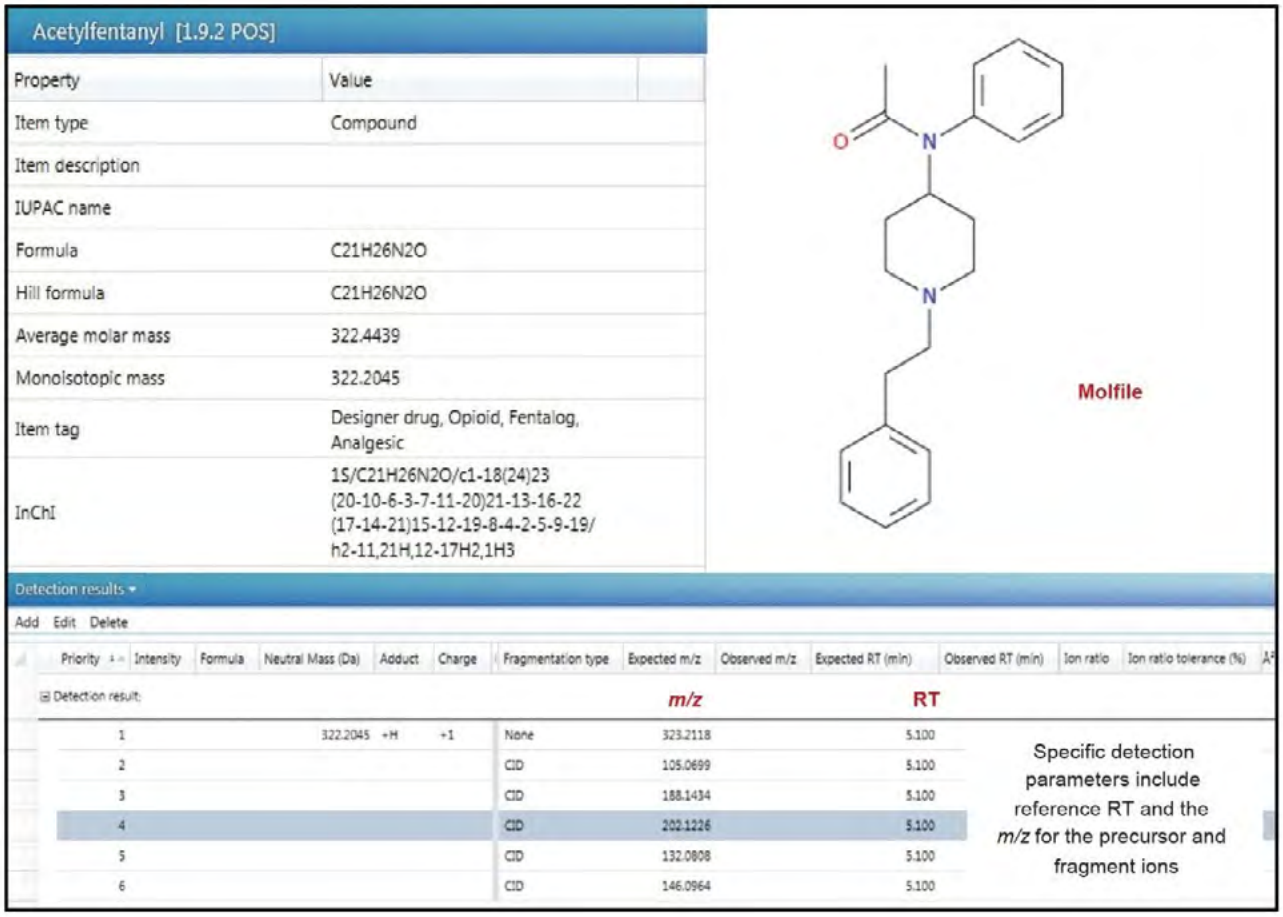

Figure 3. Example library item for a representative compound in the UNIFI Forensic Toxicology Library.

Figure 4 shows the results browser for a typical sample processed in this way and demonstrates the wealth of information that is available for use in the library matching process, and which provides the user with a fast, clear, and confident identification.

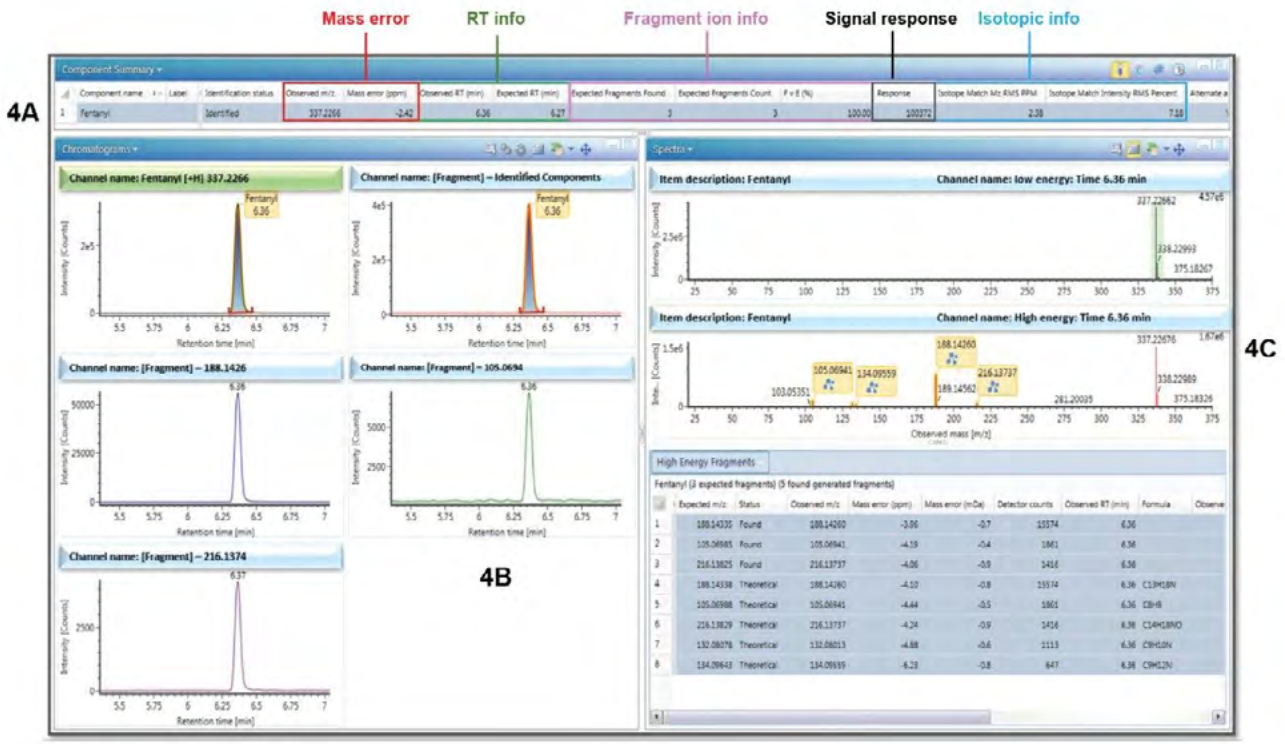

Figure 4. Detection of fentanyl in a sample following targeted analysis. A wealth of information is available and may be viewed if desired. In this figure the upper table (4A) details the results of the comparison of acquired data against the reference information contained in the Toxicology Library. Panel 4B displays the extracted ion chromatograms for the targeted precursor and the 3 diagnostic fragment ions and demonstrates that all ions are time-aligned at 6.36 min. The low and high energy spectra are shown in Panel 4C (upper). Details of the fragment ions are listed in the lower table.

Semi-targeted Analysis

One of the key benefits of high resolution/accurate mass instruments is that even in the absence of a fully characterized library entry, the user still has the ability to screen for drug substances on the basis of their exact mass. This is especially beneficial for forensic toxicology laboratories as it enables the user to screen for novel or emerging drug substances without the requirement of CRM; this is invaluable as access to commercial reference material for new analogues is often delayed. In semi-targeted processing, a Molfile is utilized; this file describes the elemental formula and overall arrangement of the bonds of the compound of interest. During the automated processing, evidence of the *m/z* for the precursor ion (as determined from the Molfile) is sought in the low

energy trace (Figure 5). While this information alone, is useful for a tentative identification, in addition UNIFI performs *in-silico* fragmentation of the Molfile to generate theoretical fragment ions which are then sought in the high energy data. A sample containing evidence of both precursor ***and*** theoretical fragment ions for a particular drug substance, demonstrates a higher confidence in likely identity.

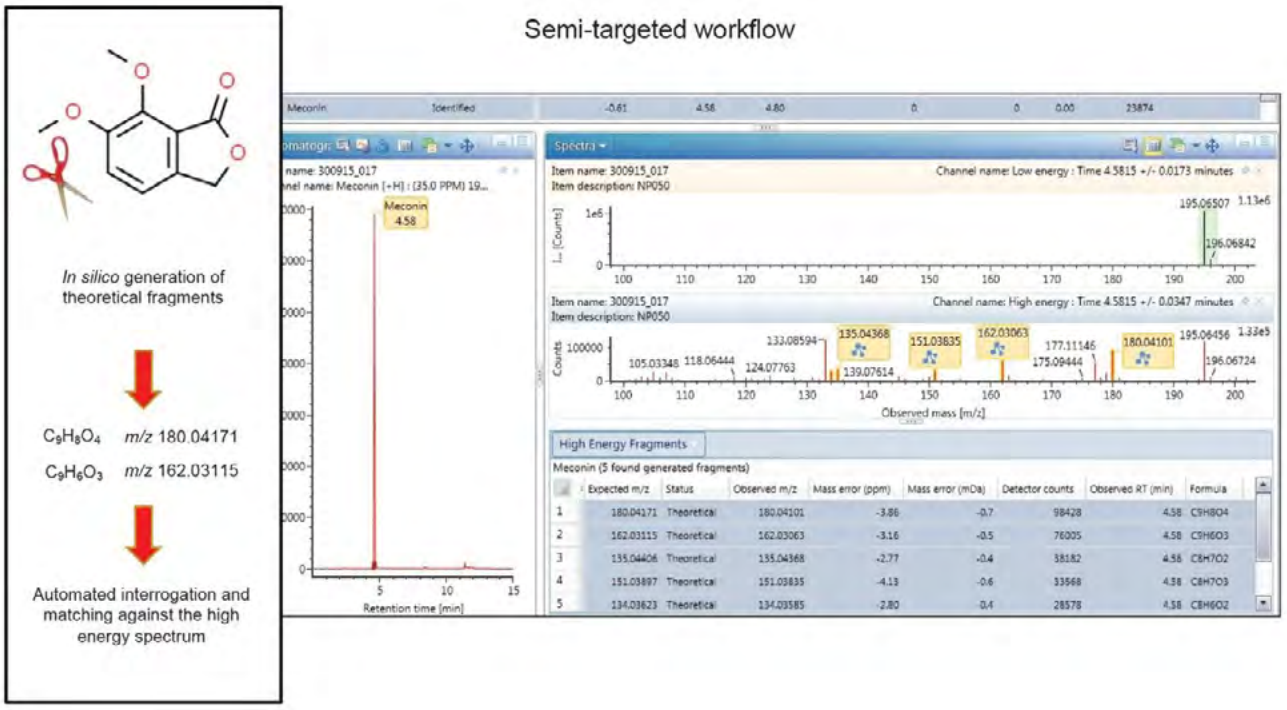

Figure 5. Tentative detection of meconin in a urine sample by screening by use of a Molfile. In this sample processing highlighted an unknown peak with a mass of m/z 195.0651, which was consistent with that of the noscapine metabolite meconin. Meconin has been proposed as a marker for illicit opiate use. In-silico fragmentation is automatically performed for all Molfiles added to the library and yields theoretical fragments which are then sought in the high energy MS^E data. This sample also screened positive for several opiates including noscapine, papaverine and heroin. The tentative detection of meconin was subsequently confirmed following analysis of CRM.

Non-targeted (discovery)

Discovery workflows can be applied where there is an unknown peak in the data that is not identified by either targeted, or semi-targeted, workflows. Under these circumstances, UNIFI offers a full suite of discovery tools that can be used to elucidate the structure of the unknown. The first step in the discovery process is to determine the likely elemental formula(e) of the substance; UNIFI achieves this based on the accurate mass and isotopic information of the precursor mass in the low energy trace of the MS^E data. The second step is to assign any likely substances that correspond to that measured formula. UNIFI achieves this by searching online chemical databases such as those contained within Chemspider and simultaneously accessing the Molfile associated with that substance. In the third step and final step, UNIFI performs the *in-silico* fragmentation process for any proposed substances and compares the theoretical ions with the fragment ions observed in the high energy trace of the unknown substance. The greater the similarity of the acquired to the theoretical, the higher the confidence in the proposed identity. Further confirmation of this type of preliminary tentative identification would require verification of retention time and fragment ions through analysis of CRM. Figure 6 shows an example of a tentative identification of cyclopropylfentanyl using the discovery workflow. A more detailed description of the discovery workflow, with illustrated examples, is available in another Application Note.[4]

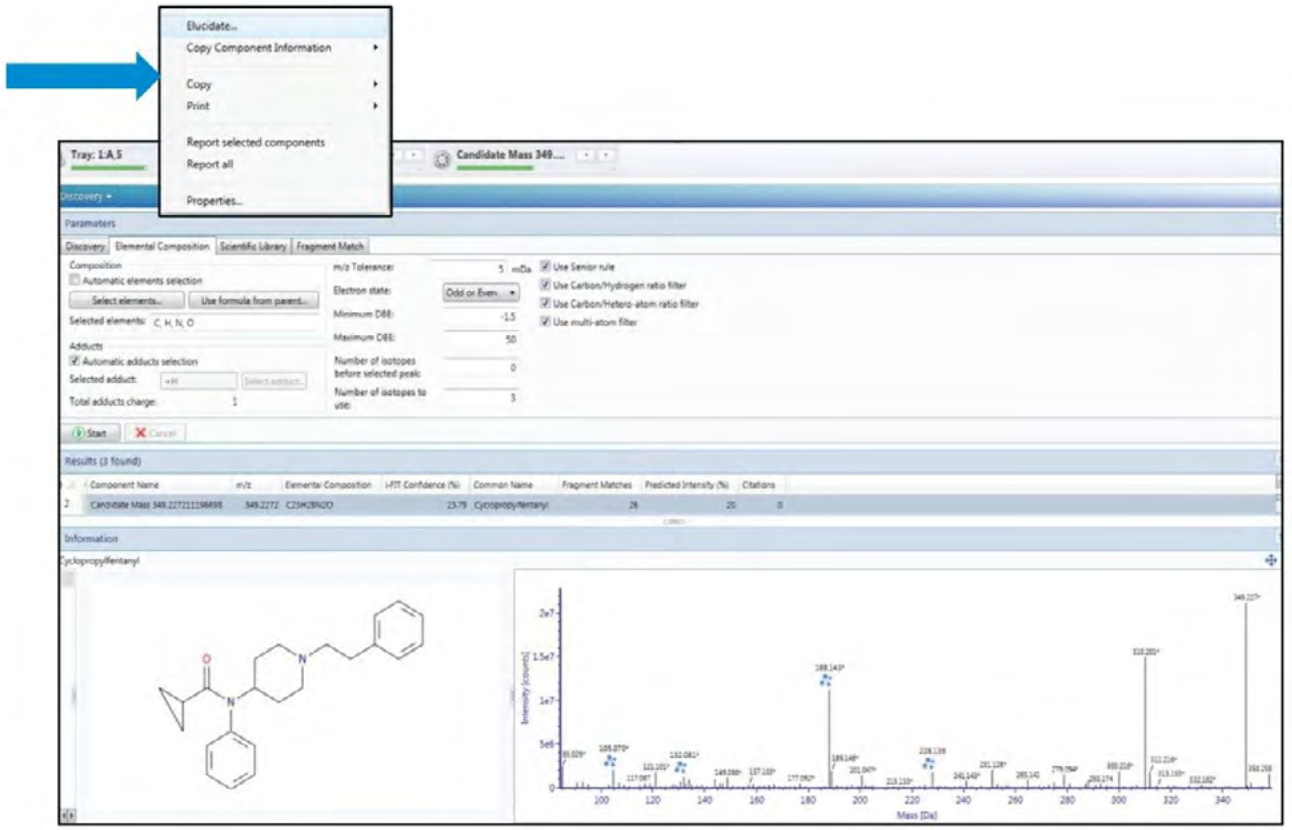

Figure 6. On selection of the component peak of the unknown for the elucidation process, an automated discovery sequence is triggered which includes searching external Scientific libraries e.g., Drugbank,ChEMBL, to provide proposal of likely substances which correspond to the measured accurate mass and matching of theoretical fragments with observed fragment ions. In this example the proposal of cyclopropylfentanyl , based on measured mass m/z 349.2272 together with the isotopic information was supported by 26 matched fragment ions.

Conclusion

MS^E is a powerful acquisition mode that provides a complete catalogue of accurate mass data. Identification by this technique is based on a combination of retention time and an accurate mass fingerprint of the analyte. While the use of Tof instruments provide the capability to assign masses to four decimal places and offers improved

specificity over nominal mass data, the additional information of the diagnostic fragments provided by use of MS^{E} represents further specific identification parameters and minimizes false positives. Ion ratios of the diagnostic can also be incorporated into the user's identification criteria.[5] Furthermore, fragment ions can be useful to differentiate isomers. Together, these capabilities translate into faster, easier data review for the user, and an overall high confidence in identification.

As a complete, non-restricted catalogue of accurate mass information MS^{E} also enables discovery workflows which can be applied to facilitate identification of substances that may be new, emerging psychoactive substances and analogues.

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Featured Products

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Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry <https://www.waters.com/134798222>

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720005198, Revised March 2022

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Application Note

Screening for a Panel of 12 Toxicologically-Relevant Drugs in Urine using Tof-MRM

Jeff Goshawk, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application note describes the application of Tof-MRM using a Xevo G2-XS QTof with UNIFI Scientific Information System for the analysis of forensically-relevant drugs in urine.

Benefits

The use of Tof-MRM to confirm the presence, or absence, of 12 drugs in authentic urine using the Xevo G2-XS QTof and UNIFI.

Introduction

Laboratory testing for illicit drug substances frequently employs a combination of immunoassay-based screening, for common drug classes, followed by confirmatory testing using targeted LC-MS/MS based techniques. Some service providers have successfully replaced this multiple step approach with a single LC-MS/MS procedure targeted for a selection of key analytes.[1] While both of these strategies represent an effective procedure for a limited panel of analytes, the approach does not provide information for a broad range of drug substances. Additionally, these methods are also unlikely to include some of the newer, emerging drug substances.

Previously we have described a time-of-flight (Tof) screening method with the potential to screen for an unlimited number of toxicologically-relevant substances within 15 minutes.[2,3] This technique employs Tof-MS ^{E}, a non-targeted data acquisition mode which yields a complete dataset from which thousands of substances may be screened. The same mass spectrometer may also be used in targeted mode i.e., multiple reaction monitoring mode (Tof-MRM), providing enhanced sensitivity; this mode allows isolation of a precursor mass using the quadrupole followed by Tof detection of specific fragment ions.[4]

Here we present the analysis of 12 common drugs in urine using Tof-MRM. The technique uses the same well-established chromatographic separation as that used for the Forensic Toxicology Screening Application Solution with UNIFI[3] and as such, provides the user with ability to perform screening and confirmation on a single platform.

Experimental

Sample preparation

Authentic drug-free urine was collected from volunteers and pooled. The pooled urine was spiked with a mixture of 12 drug substances to yield a series of samples at the following concentrations: 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50 ng/mL. A blank urine sample was also prepared. Samples were further diluted 5 five-fold with mobile phase prior to analysis.

LC-MS method conditions

Data was acquired using the ACQUITY UPLC I-Class (FTN) together with the Xevo G2-XS QTof in Tof-MRM mode. Two product ions (quantifier and qualifier) were monitored for each of the 12 drugs shown in Table 1. All transitions were acquired with a collision energy ramp from 10 eV to 40 eV, with the exception of norbuprenorphine, for which a constant collision energy of 40 eV was used to monitor of the *m/z* 101.0961 fragment ion.

Tof-MRM conditions

Compound	Precursor ion *m/z*	Product ions *m/z*	Acquisition time (min)
Cotinine	177.1022	**98.0601**, 146.0601	0.57–1.57
Morphine	286.1437	**201.0911**, 165.0699	0.60–1.60
Norcodeine	286.1437	**268.1333**, 225.0911	1.10–2.10
Codeine	300.1593	**215.1067**, 225.0911	1.20–2.20
Dihydrocodeine	302.1750	**199.0754**, 201.0911	1.20–2.20
6-monoacetylmorphine	328.1543	**211.0754**, 165.0699	1.60–2.60
Amphetamine	136.1120	**91.0543**, 119.0856	1.75–2.75
Benzoylecgonine	290.1386	**168.1020**, 105.0335	2.45–3.45
Norbuprenorphine	414.2638	**396.2534**, 101.0961	4.58–5.58
Buprenorphine	468.3108	**414.2639**, 396.2170	6.47–7.47
EDDP	278.1903	**234.1278**, 249.1512	6.79–7.79
Methadone	310.2165	**265.1587**, 105.0335	7.94–8.94

Table 1. Tof-MRM conditions for the included analytes; the quantifier ion is shown in bold.

Results and Discussion

Data were acquired and processed using UNIFI. Processing comprised automatic extraction of the mass chromatogram, for each transition, followed by peak integration. Figure 1 shows representative qualifier chromatograms for three of the substances investigated. The corresponding data for the blank urine sample is included for comparison. Similar results were obtained for the other nine drugs in the study. The standard curve for the quantifier ion of 6-monoacetylmorphine is shown in Figure 2 and demonstrates excellent linearity over the entire concentration range.

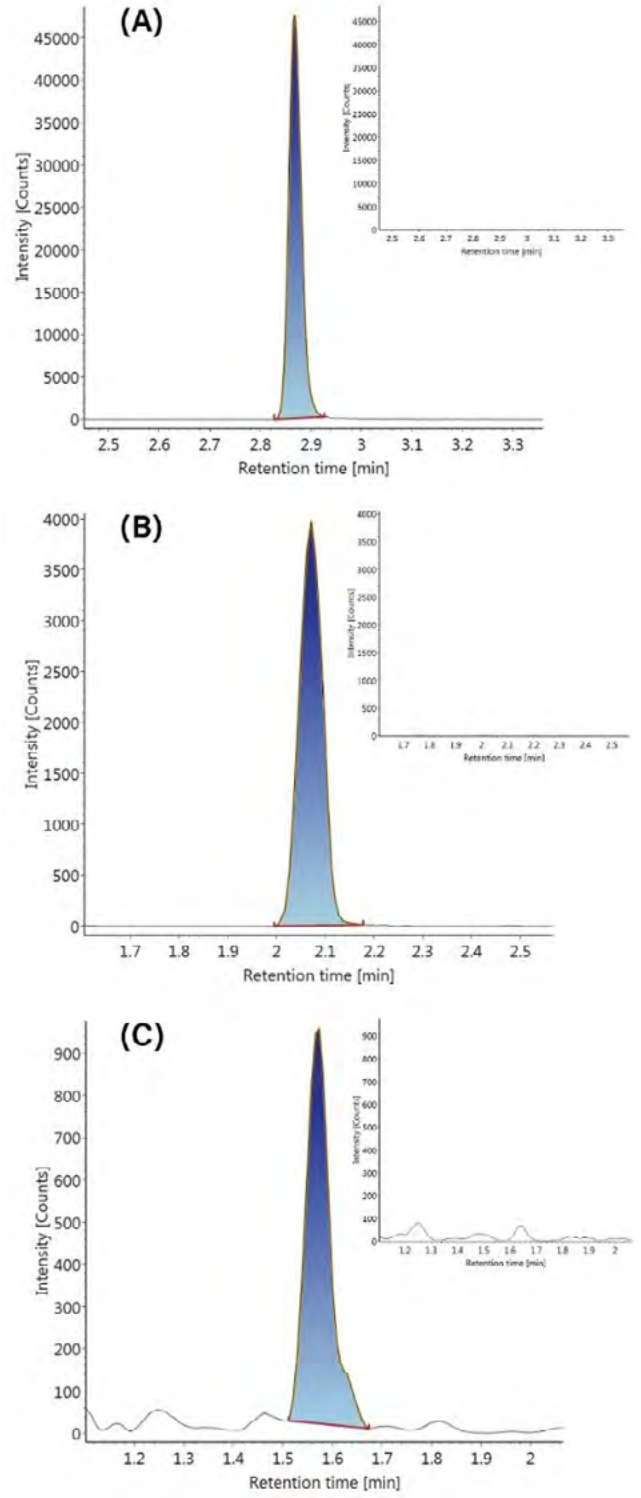

Figure 1. Chromatograms of the quantifier ion for (A) benzoylecgonine (B) 6-monoacetylmorphine and (C) norcodeine. The chromatograms are from the urine sample spiked at a concentration of 10 ng/mL and diluted prior to injection. For comparison, the inset chromatograms provide responses for a blank urine.

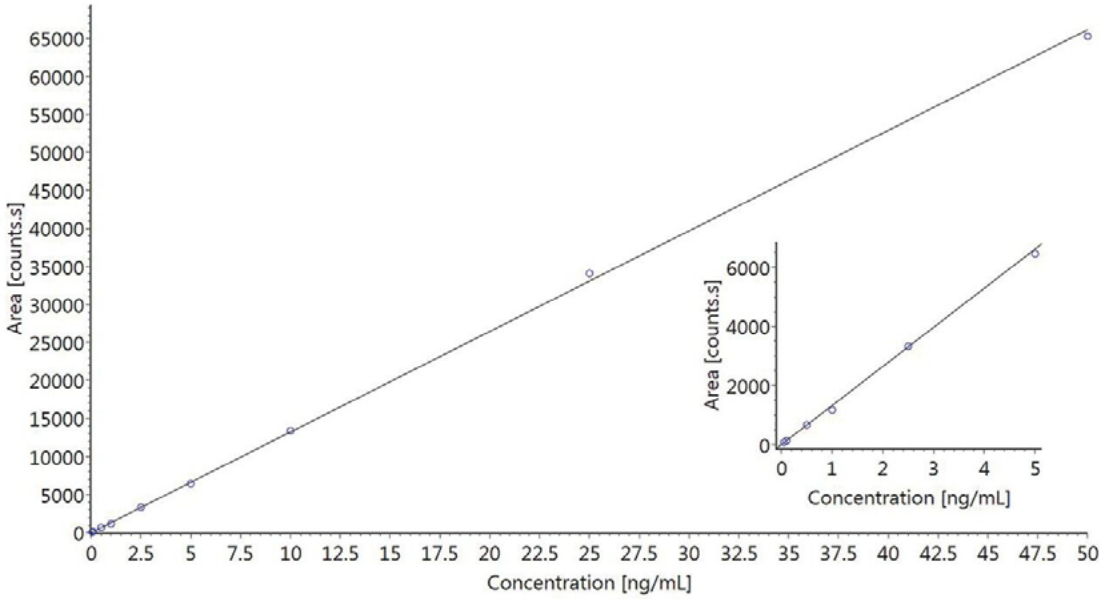

Figure 2. The standard curve for the quantifier ion of 6-monoacetylmorphine. The inset graph shows the detail for the samples at the lower concentrations.

Conclusion

Tof-MRM has been successfully used, on the Xevo G2-XS QTof, to analyze a panel of 12 drug substances. The enhanced selectivity of Tof-MRM permitted detection of the analytes in urine spiked at low ng/mL concentrations and prepared by a simple dilution.

Tof-MRM combined with the ability to perform Tof-MS^E enables comprehensive non-targeted screening and confirmation on a single platform.

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Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry <https://www.waters.com/134798222>

UNIFI Scientific Information System <https://www.waters.com/134801359>

Forensic Toxicology Screening Application Solution with UNIFI <https://www.waters.com/134779723>

720005943, April 2017

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Application Note

No Compromise! Improved Sensitivity for Negatively-Ionizing Substances

Robert Lee, Scott Freeto, Michael Wakefield, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief demonstrates to assess the impact of alternate mobile phases on the sensitivity for a series of negatively ionizing substances.

Benefits

Altered mobile phases provide improved sensitivity when using the Waters Forensic Toxicology Screening Application Solution with UNIFI in negative ionization mode.

Introduction

In 2014, Waters released the first version of the Forensic Toxicology Screening Application Solution with UNIFI,[1] which comprised acquisition of MS^E data using a time-of-flight mass spectrometer operated in electrospray positive ionization mode (ESI+). Data were subsequently compared with a comprehensive library containing more than 1,000 toxicologically-relevant substances.[2,3]

Since this time, on-going efforts have been underway to further improve the forensic solution by continuing to expand the library content, to include novel psychoactive substances and their metabolites, but also to include substances that may preferentially ionize in negative mode (ESI-), such as the barbiturates, cannabinoids, diuretics, and the non-steroidal anti-inflammatory drugs (NSAIDs).[4,5] For convenience, some screening approaches employ the same chromatographic conditions for both positive and negative ionization modes however, the impact of this approach should be evaluated, particularly with regards to the effect on sensitivity.

Consequently, the aim of this study was to compare the sensitivity obtained for a series of negatively-ionizing substances when analyzed using the mobile phases that are usually employed in ES+ mode (Method 1), with some alternative chromatographic conditions, that are based on a previously-reported method for barbiturates (Method 2).[6]

Experimental

Sample preparation

Individual standards were prepared at 1 mg/mL in methanol, then diluted in 10% acetonitrile in water for injection. The final concentrations ranged from 20 ng/mL to 2,500 ng/mL.

LC conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC HSS C_{18}, 2.1 x 150 mm, 1.8 µm
Vials:	Waters Maximum Recovery Vials
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A method 1:	5 mM ammonium formate pH 3.0
Mobile phase A method 2:	Water containing 0.001% formic acid
Mobile phase B method 1:	Acetonitrile containing 0.1% formic acid
Mobile phase B method 2:	Acetonitrile containing 0.001% formic acid
Gradient:	Isocratic at 87% A for 0.5 min then to 5% A at 4.5 min, hold for 1 min before switching to 87% A
Run time:	7.5 min

MS^E conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI-
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin [M-H]- m/z = 554.2620
Acquisition range:	m/z 50-1000
Scan time:	0.1 s
Capillary voltage:	1.5 KV
Cone voltage:	20 V
Collision energy:	Function 1: 6 eV Function 2: Ramped 10 to 40 eV

Results and Discussion

Sixty-two compounds, including barbiturates, cannabinoids, diuretics, NSAIDs, and steroids, were analyzed in triplicate. The retention times for a selection of the compounds, under both sets of mobile phases evaluated, are listed in Table 1, together with the observed increase in 3-dimensional (3D) peak response and signal-to-noise ratio, with the alternative mobile phases.

Sixty of the sixty-two compounds evaluated showed an increase in 3D peak response when using the alternate mobile phases, with 75% of the compounds tested showing a greater than two-fold increase, and only two compounds showing a reduced 3D peak response. The greatest increase in 3D response was for THC, which is illustrated in Figure 1, and showed an increase of more than 50-fold alongside a dramatic increase in signal-to-noise ratio.

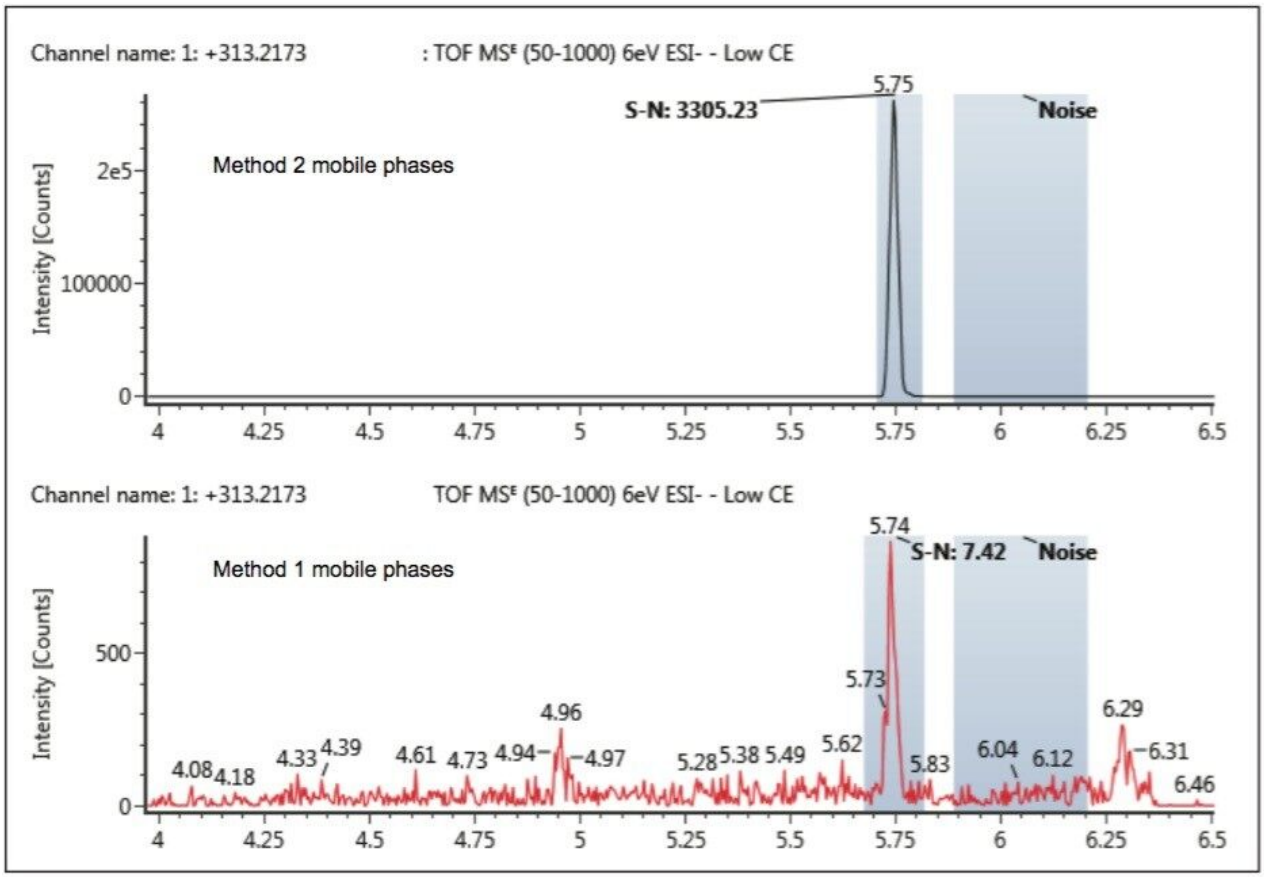

Figure 1. Improvement in both 3D peak response and signal-to-noise ratio for 2500 ng/mL injection of THC standard when using the altered mobile phases (upper chromatogram) in comparison with the original mobile phases (lower chromatogram).

A small number of compounds were only identified, at the concentrations investigated, when using the altered mobile phases. The signal-to-noise ratio comparison between the two methods, for a selection of compounds, is highlighted in Table 1, and complements the increase in 3D response. Only small differences in retention time were observed when switching between the two mobile phases.

Analyte	Drug class	Injection conc.	Degree of improvement (method 2/1)		Retention time (min)	
		ng/mL	3D peak response	Signal-to-noise ratio	Method 1	Method 2
Phenobarbital	barbiturate	100	15	17	2.9	2.9
Secobarbital	barbiturate	100	11	11	3.5	3.5
Carboxy-THC	cannabinoid	20	3	3	5.0	5.0
THC	cannabinoid	2500	67	445	5.7	5.8
Amiloride	diuretic	250	3	3	1.1	1.0
Furosemide	diuretic	250	7	3	3.1	3.1
Naproxen	NSAID	400	23	20	3.8	3.8
Ibuprofen	NSAID	1000	52	73	4.4	4.5
Hydrocortisone	steroid	400	6	5	3.0	3.0
Triamcinolone	steroid	400	5	3	2.6	2.6

Table 1. Improvement in 3D peak response and sensitivity for a selection of analytes, using the two sets of mobile phases along with their retention times.

Conclusion

While it is certainly possible to use the same mobile phases for screening analysis in both positive and negative mode, this simple study clearly demonstrates that these compromises in chromatographic conditions can influence analytical performance and sensitivity, and in some cases, this can be significant. In a toxicological screening this can yield false negative results, particularly for the cannabinoids which are the most-commonly encountered illicit drug substances. For this reason the expanded Waters Forensic Toxicology Screening Application Solution with UNIFI includes a fully-optimized chromatographic method for more efficient, more accurate toxicological screening of negatively-ionizing substances.

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ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Forensic Toxicology Screening Application Solution with UNIFI <https://www.waters.com/134779723>

720005479, August 2015

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Application Note

Analysis of Plant Alkaloids Through Accurate Mass Screening and Discovery

Jeff Goshawk, Michelle Wood

Waters Corporation

For forensic toxicology use only.

Abstract

In this study, the Forensic Toxicology Application Screening Solution with UNIFI was applied to a selection of plant alkaloids. The ease by which the scientific library items can be created and updated has been clearly demonstrated. The UNIFI Scientific Information System v1.8 was used to process the MS^E data and for these plant alkaloids multiple adducts were detected. The fragment match functionality was also able to assign sub-structures to high-energy ions. Additionally, the new discovery tool has been shown to enhance the elucidation of unknown components.

Benefits

Analyze plant alkaloids using the Forensic Toxicology Application Screening Solution with UNIFI[1] to demonstrate the simplicity of library creation and expansion. This application note also showcases the power of the latest suite of discovery tools within the UNIFI Scientific Information System v1.8.

Introduction

Over the last decade there has been a significant increase in the popularity of time-of-flight mass spectrometry (Tof-MS) for multi-residue analysis. Accurate mass imparts high specificity for substance identification and, together with the isotopic data, can provide the user with the opportunity to propose likely elemental compositions. The proposal of elemental formulae is often the starting point for a complex multi-stage process to elucidate chemical structures.

For screening, accurate mass instrumentation presents a significant, and key, advantage over its nominal mass counterpart, i.e., an ability to implement screening methodologies without the requirement of reference material. In this particular workflow the theoretical (expected) exact mass can be determined empirically from the elemental formula. In a toxicological setting this can provide a valuable means with which the analyst may 'prospectively' target novel psychoactive drugs, or new substances and metabolites where reference material may not yet, be available.

An on-going initiative to expand the UNIFI Toxicology Scientific Library led to the analysis of a series of plant alkaloids. These nitrogen-containing compounds are derived from plants and plant material. They are pharmacologically active and have been used for many centuries for both medicinal and recreational

purposes. Consequently, their analysis is of forensic importance. Analysis of these substances provided an opportunity to evaluate the tools within the UNIFI Scientific Information System for both target assignment and structural elucidation.

ACQUITY UPLC conditions

System:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY HSS C_{18}, 2.1 x 150 mm, 1.8 µm
Run time:	15 min
Vials:	Waters Maximum Recovery Vials
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM aqueous ammonium formate, adjusted to pH 3.0
Mobile phase B:	Acetonitrile containing 0.1% formic acid

Gradient:

Time	%A	%B
0.00	87	13
0.50	87	13

Time	%A	%B
10.00	50	50
10.75	5	95
12.25	5	95
12.50	87	13
15.00	87	13

MS conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI+
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M+H]^+$ = *m/z* 556.2766
Acquisition range:	m/z 50–1000
Scan time:	0.1 s
Capillary voltage:	0.8 kV
Cone voltage:	25 V
Collision energy:	Function 1: 6 eV Ramped 10 to 40 eV

Data management

Forensic Toxicology Screening Application Solution with UNIFI v1.8

Experimental

Materials

The following plant alkaloids were obtained from Sigma-Aldrich (Poole, UK) as solid material: amygdalin, berberine chloride, bufalin, coumarin, digitoxin, gitoxin, lanatocide C, neriifolin, and α-solanine.

Sample preparation

Individual stock solutions of the plant alkaloids were initially prepared, by dilution with methanol, to a concentration of 10 µg/mL; these solutions were stored at -20 °C until further use. Prior to Tof-MS analysis, the stock solutions were further diluted with mobile phase A to yield samples for injection at a concentration of 1 µg/mL.

Results and Discussion

Prior to analysis, a new UNIFI Scientific Library was created specifically for plant alkaloids, by simply entering the names of the nine alkaloids. A MOL file describing the structure of each substance was added to each entry in the library (Figure 1). Individual solutions of the plant alkaloids were injected and data were acquired using the standard screening conditions supplied with the Forensic Toxicology Screening Application Solution with UNIFI.[1] These data were subsequently processed using the UNIFI Scientific Information System and screened against the new plant alkaloid library.

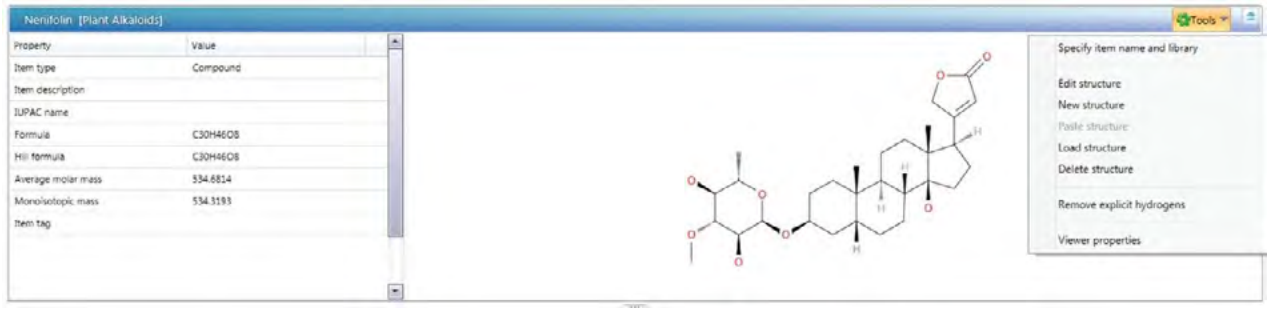

Figure 1. Creating a library entry for neriifolin. Existing MOL file structures can be appended (Load structure) or created by standard chemical drawing packages and subsequently appended (New structure).

Identification through the application of in-silico fragmentation techniques

The presence of each plant alkaloid was confirmed through the mass accuracy of the protonated precursor ion in combination with theoretical fragment ions that were automatically generated from the structure of each substance and matched to ions in the high-energy spectrum.

Figure 2 shows the identification of α-solanine as presented in UNIFI. The Component Summary table presents the information related to the identification of this alkaloid and includes; the observed *m/z* value together with the deviation from the expected *m/z* value, the difference between measured and theoretical isotope patterns in terms of both *m/z* and intensity distributions, the observed retention time, the number of theoretical fragment ions found, and the detector counts, which represents the abundance of all the low-energy ions associated with the detected compound.

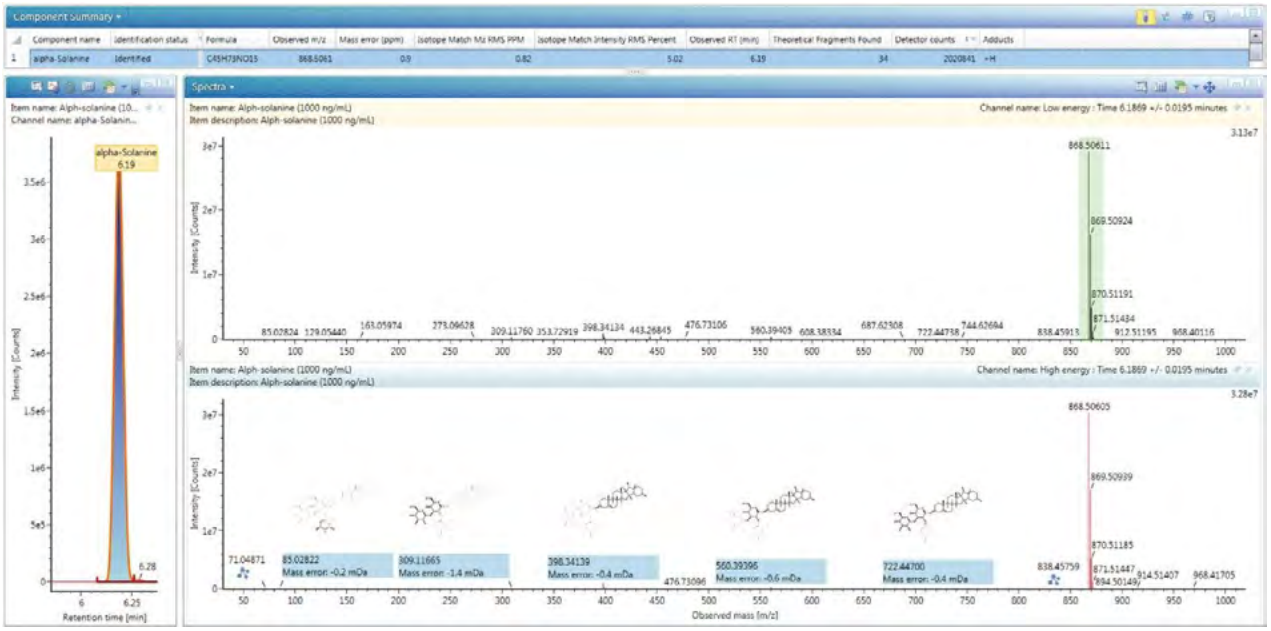

Figure 2. Identification of a-solanine in the UNIFI Scientific Information System.

Updating library entries

All of the alkaloids were identified on the basis of the mass accuracy of the precursor ion and theoretical fragment ions generated during processing. Upon identification, a retention time was associated with each substance. With UNIFI, the library entries can be updated directly from the analysis such that they contain the expected retention time and the expected *m/z* value for each assigned adduct and fragment ion. Following the update, a typical library entry has information similar to that shown for neriifolin in Figure 3. This additional information can be used to target the substance in subsequent analyses.

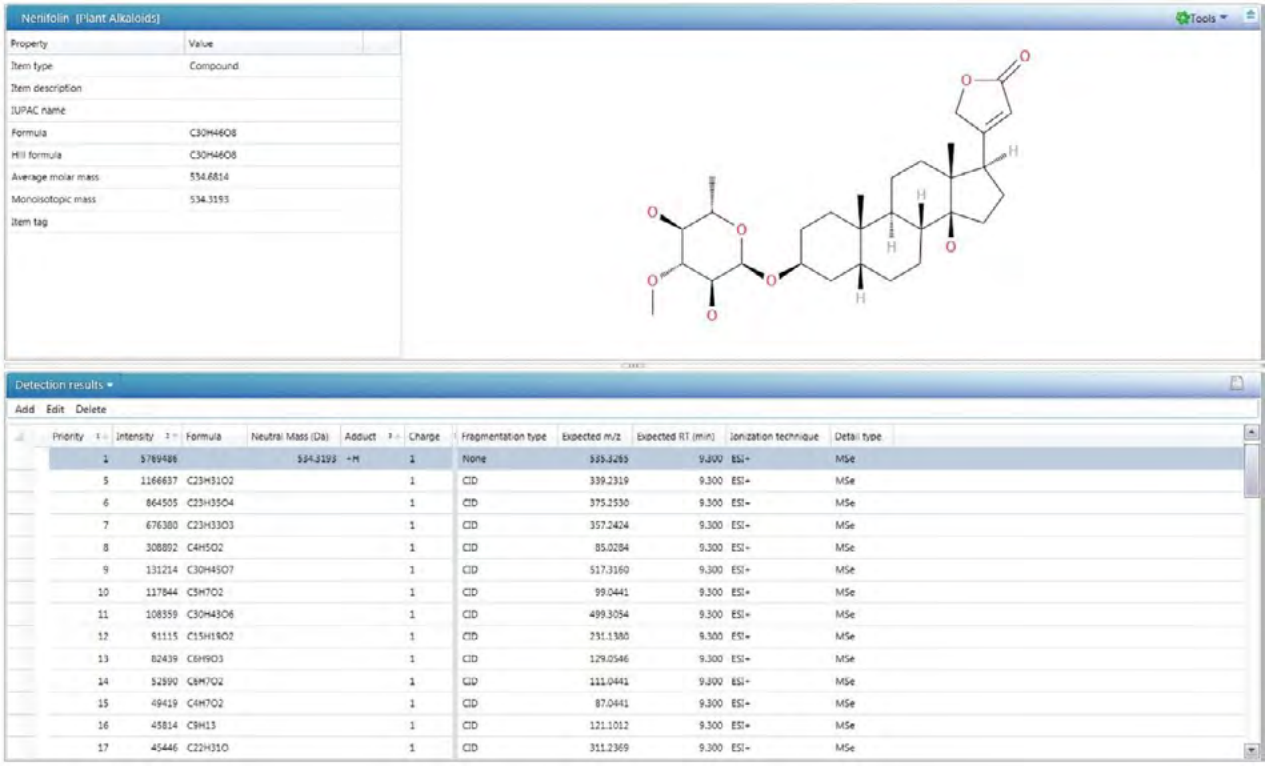

Figure 3. Library entry for neriifolin. The lower section of the composite is now populated with expected retention time and the expected m/z values of precursor and fragment ions.

Multiple adducts

Data for gitoxin, one of the other alkaloids investigated in this study, is shown in Figure 4. The low-energy ions assigned to this substance are highlighted in green within the spectrum and correspond to the protonated isotope cluster. The detector counts determined for the protonated isotope cluster of gitoxin is 568. The high-energy spectrum is annotated with sub-structures of gitoxin, as determined automatically by UNIFI and associated to the high-energy spectral peaks as fragment ions.

Figure 4. Identification of gitoxin in the UNIFI Scientific Information System.

Further examination of the low-energy spectrum for this substance revealed that some of the ions may correspond to other adducts of gitoxin. Consequently the data was reprocessed to target the $[NH_4]^+$, $[Na]^+$, and $[K]^+$ adducts in addition to the protonated species. Figure 5 details the isotope clusters in the low-energy data assigned to each adduct following reprocessing. The assignment of the additional adducts to gitoxin has been reflected in the detector counts which has increased from 568, determined from the isotope cluster of the protonated adduct, to 118680. Similar results were obtained for the other substances in this analysis.

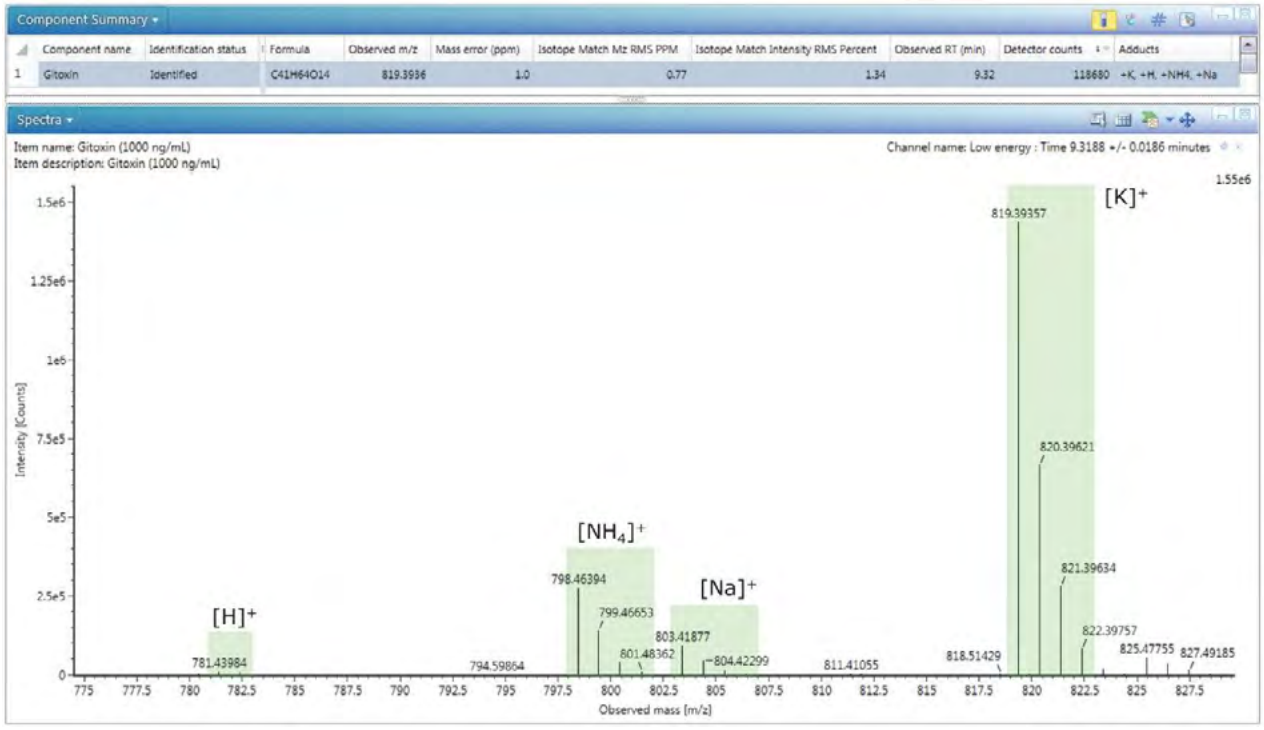

Figure 5. Multiple adduct assignment for gitoxin

The discovery tool

Another new feature in the UNIFI Scientific Information System v1.8 is the discovery tool, which chains together elemental composition, library searching and fragment match functionality into a single step process making it easier to obtain the identity of unexpected substances within a sample. The parameters used to run the discovery tool are detailed in Figure 6A–D.

The first set of parameters, displayed in Figure 6A, control the maximum number of elemental compositions returned for each component, and the number of library hits returned for each elemental composition. For each component selected, the measured m/z value is submitted to the elemental composition application, the parameters of which are displayed in Figure 6B. Each scientific formula returned by the elemental composition application is then automatically submitted to the list of selected libraries. The libraries can either belong to the UNIFI Scientific Library or, if connected to the internet, ChemSpider. The dialog showing the selection of ChemSpider libraries is presented in Figure 6C.

Every hit for each scientific formula that is returned from the library search is then automatically submitted to the fragment match application, provided the library hit has an associated structure in the form of a MOL file.

The fragment match application performs a systematic bond disconnection of each structure, applying the parameters selected through the dialog displayed in Figure 6D, and matches the *m/z* values of theoretical sub-structures to measured high-energy fragment ions. The number of fragment ions matched and the percentage of the intensity of the high-energy spectrum accounted for by those matches are both determined.

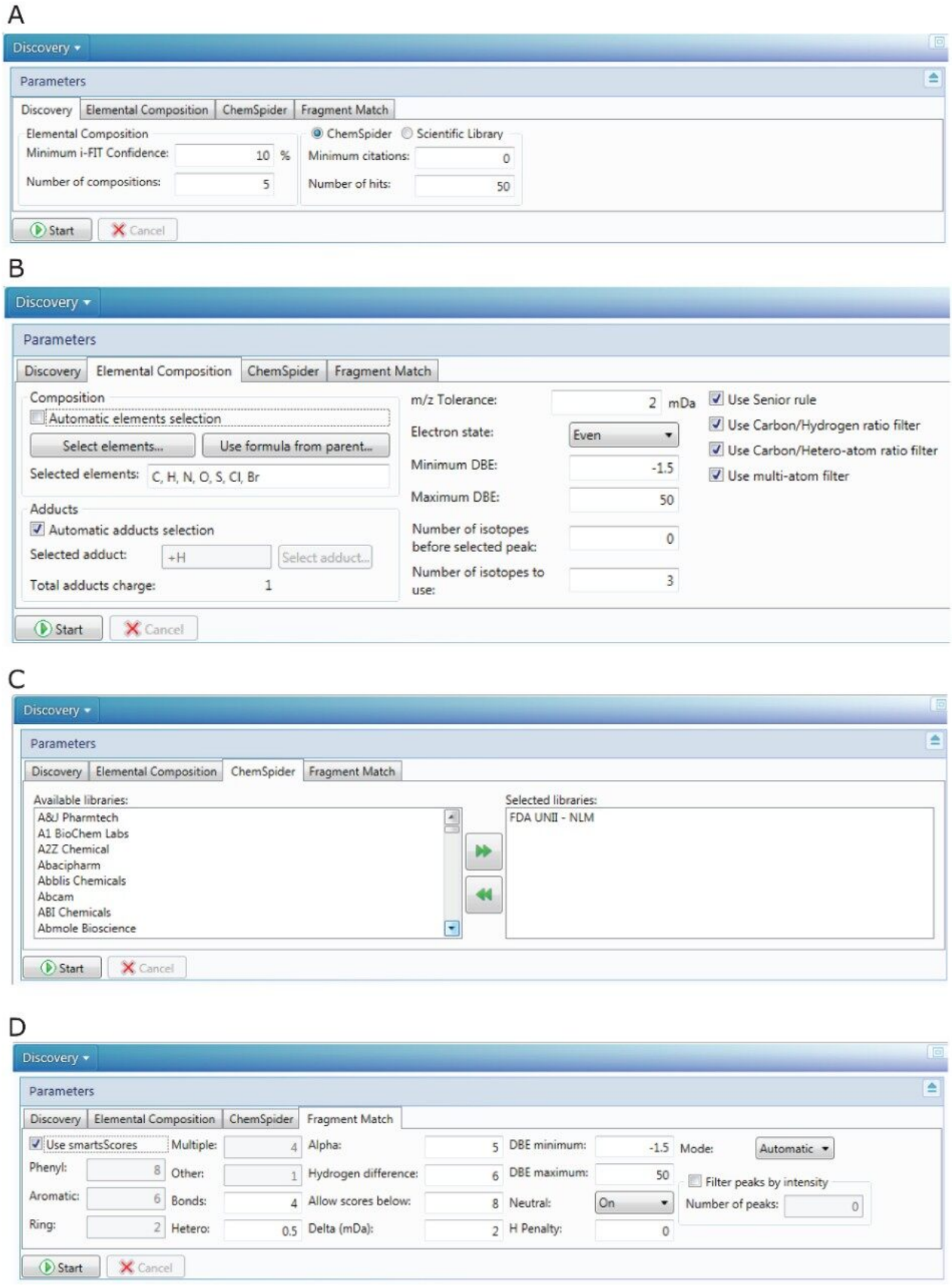

Figure 6. Discovery tool in UNIFI. A) General discovery tool parameters. B) Elemental composition parameters. C) ChemSpider parameters. D) Fragment match parameters.

For the purposes of illustration, the candidate component identified as amygdalin in the targeted analysis was submitted to the discovery tool. The results, upon running the application with respect to the parameters shown in Figure 6A–D, are presented in Figure 7.

The component submitted to the discovery tool was Candidate Mass *m/z* 458.1649. The results show that one elemental composition, $C_{20}H_{27}NO_{11}$, with an i-FIT confidence of 89% was determined for *m/z* 458.1649. This elemental composition, was automatically submitted to the FDA UNII – NLM library within ChemSpider and a hit for amygdalin was returned with a list of synonyms, a structure and the number of citations. The structure was used automatically in conjunction with fragment match and appropriate sub-structures were assigned to the high-energy spectrum associated with Candidate Mass *m/z* 458.1649, as shown in Figure 7. The number of high energy fragments matched by sub-structures and the percentage of the intensity of the high energy spectrum accounted for by those fragment matches are displayed for the library hit.

Access to this information for a range of components, elemental compositions, and library hits enables the analyst to make an informed decision with respect to the identity of unexpected substances in their samples.

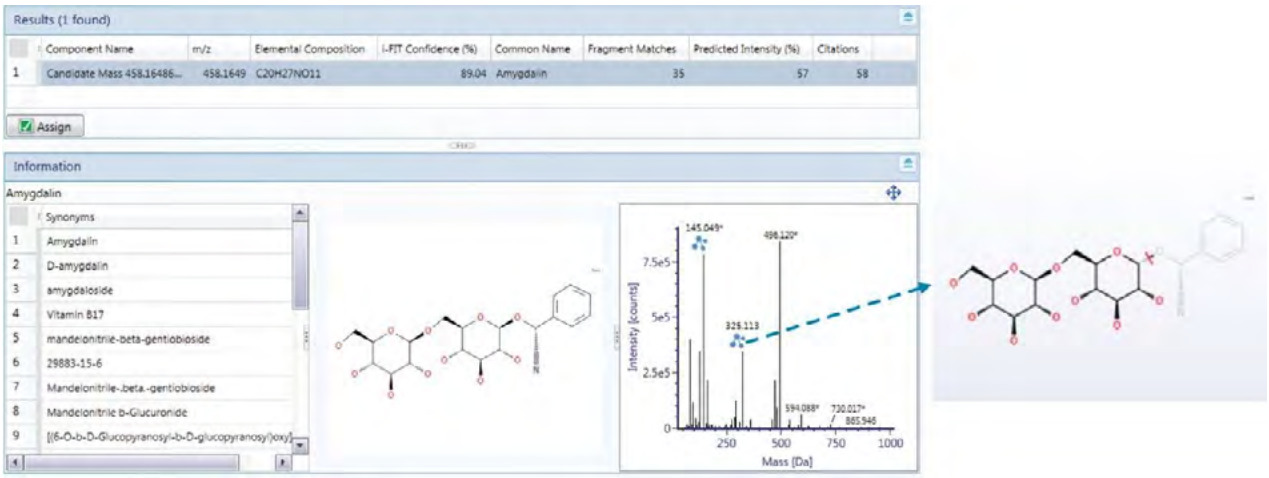

Figure 7. A typical result in the discovery tool.

Conclusion

In this study, the Forensic Toxicology Application Screening Solution with UNIFI[1] was applied to a selection of plant alkaloids. The ease by which the scientific library items can be created and updated has been clearly demonstrated. The UNIFI Scientific Information System v1.8 was used to process the MS^E data and for these plant alkaloids multiple adducts were detected. The fragment match functionality was also able to assign sub-structures to high-energy ions. Additionally, the new discovery tool has been shown to enhance the elucidation of unknown components.

References

1. Forensic Toxicology Screening Application Solution. Waters Brochure (P/N 720004830EN).

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Forensic Toxicology Screening Application Solution with UNIFI <https://www.waters.com/134779723>

Available for purchase online

ACQUITY UPLC HSS C18 Column, 100Å, 1.8 µm, 2.1 mm X 150 mm, 1/pkg <
https://www.waters.com/waters/partDetail.htm?partNumber=186003534>

720005461, July 2015

Waters™

Application Note

Screening for Cannabinoids Using the Waters Forensic Toxicology Application Solution with UNIFI

Robert Lee, Michelle Wood

Waters Corporation

For forensic toxicology use only.

Abstract

This application note demonstrates the sensitivity and selectivity of the expanded Forensic Toxicology Application Solution with UNIFI using negative ionization in providing a consistent comprehensive determination of cannabinoids. It can be applied as both a screen for selected cannabinoids and a method suitable for quantifying these analytes, at levels below the current EWDTS urine screening cut-off (50 ng/mL for cannabis metabolites), using a simple five-fold dilution. The excellent linear dynamic range of this system is demonstrated by simple automatically generated calibration plots.

Benefits

Expanded Forensic Toxicology Application Solution with UNIFI enabling the detection and quantitation of negative ionizing cannabinoids in urine.

Introduction

Cannabis is the most widely used illicit substance in the world and long-term use can lead to dependency. Cannabinoids are one of the most commonly detected classes of illegal drugs; consequently their analysis is of key importance in both forensic and workplace testing. Δ-9 tetrahydrocannabinol (THC) and cannabinol (CBN) are psychoactive elements present in the plant *Cannabis sativa*.[1] THC produces a number of metabolites such as 11-nor-9-hydroxy-Δ9 tetrahydrocannabinol (THC-OH), but the most significant metabolite for urine drug testing is 11-nor-9-carboxy-Δ9 tetrahydrocannabinol (cTHC), which is the major metabolite eliminated in urine, as the free acid or the ester-linked β-glucuronide.[2] Cannabidiol (CBD) is a major constituent of cannabis resin but is believed to have limited psychoactive properties.

The Waters Forensic Toxicology Application Solution with UNIFI currently comprises acquisition of accurate mass MS^E data on an orthogonal acceleration time-of-flight mass spectrometer operating in electrospray positive ionisation mode (ESI+), followed by comparison of the data with a comprehensive library containing more than 1000 toxicologically-relevant substances.[3,4,5] A number of compounds, such as the cannabinoids, also ionise in negative electrospray mode (ESI-) and the aim of the recent work was to further extend the Forensic Toxicology Application Solution to include those compounds. The new method was used to

determine the presence of cannabinoids in urine, particularly at concentrations, below the current screening cut-off,[6] and to compare the values obtained using this method with a recently published fully validated UPLC-MS/MS assay.[7]

Experimental

ACQUITY UPLC conditions

UPLC system:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC HSS, 100Å, 1.8 µm, C_{18}, 2.1 mm x 150 mm (p/n 186003534)
Vials:	Maximum Recovery Vials (p/n 186000327C)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	Water containing 0.001% formic acid
Mobile phase B:	Acetonitrile containing 0.001% formic acid
Gradient:	Isocratic at 87% A for 0.5 min, then to 5% A at 4.5 min, hold for 1 min before switching to 87% A
Run time:	7.5 min

MS conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESISource
temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin [M-H]- *m/z* = 554.2620 Acquisition range *m/z* 50–1000
Scan time:	0.1 s
Capillary voltage:	1.5 KV
Cone voltage:	20 V
Collision energy:	Function 1: 6 eV Function 2: ramped 10 to 40 Ev

Materials

Reference standards THC, CBD, CBN, THC-OH, and cTHC (1 mg/mL) were purchased from LGC Standards (Teddington, UK); cTHC-glucuronide and the deuterated (d-3) analogue of cTHC (for use as internal standard; ISTD), were obtained from the same supplier at 0.1 mg/mL.

Prior to use the individual standards were diluted to 5000 ng/mL in acetonitrile and the internal standard was diluted to 100 ng/mL in 0.001% formic acid.

Bio-Rad normal control urine and Bio-Rad Liquichek Urine Toxicology Controls Level C2 and Level S10 reference urines were obtained from Bio-Rad Laboratories (Hemel Hempstead, UK).

All other chemicals used were of the highest grade available and stored according to the supplier's instructions.

Standards preparation

Standards (0.1 mL) were added to 0.9 mL 0.001% formic acid in a Maximum Recovery Vial and vortex-mixed to give a concentration of 500 ng/mL.

Sample preparation

Acetonitrile (0.1 mL) was added to 0.2 mL urine and ISTD (0.7 mL). The sample was vortex-mixed, for 5 min at 1200 rpm, and then centrifuged at 8000 *g* for 10 min. Supernatant was transferred to a Maximum Recovery Vial.

Results and Discussion

The cannabinoids investigated in this analysis are listed in Table 1, along with their exact neutral mass, high energy fragment ions, and UPLC retention times.

Analyte	Neutral monoisotopic mass	Fragment ions (*m/z*)		Retention time (min)
cTHC-glucuronide	520.2308	343.1915 245.1547	299.2017 175.0248	4.7
cTHC	344.1988	299.2017 191.1078	245.1547 179.1078	5.0
THC-OH	330.2190	311.2017 268.1469	281.1547 267.1391	5.1
Cannabidiol	314.2246	245.1547 179.1078	229.1234 135.1179	5.4
Cannabinol	310.1933	279.1391 222.0686	252.1156 159.0815	5.6
THC	314.2246	245.1547 191.1078	229.1234 149.0972	5.8
cTHC-d3	347.2176	302.2191	248.1739	5.0

Table 1. Analyte neutral mass, high energy fragment ions, and retention times.

The acceptance criteria for a positive identification of each analyte was as follows: three dimensional (3D) low energy ion count intensity greater than 250, retention time to be within 0.35 min of reference, and the observed precursor mass to be within 5 ppm of expected. For additional confirmation, a minimum of one diagnostic fragment ion had to be found in the high energy function. Chromatographic separation of

cannabinoid standards at 100 ng/mL (50 ng/mL for cTHC-glucuronide and 500 ng/mL for cannabinol) is shown in Figure 1.

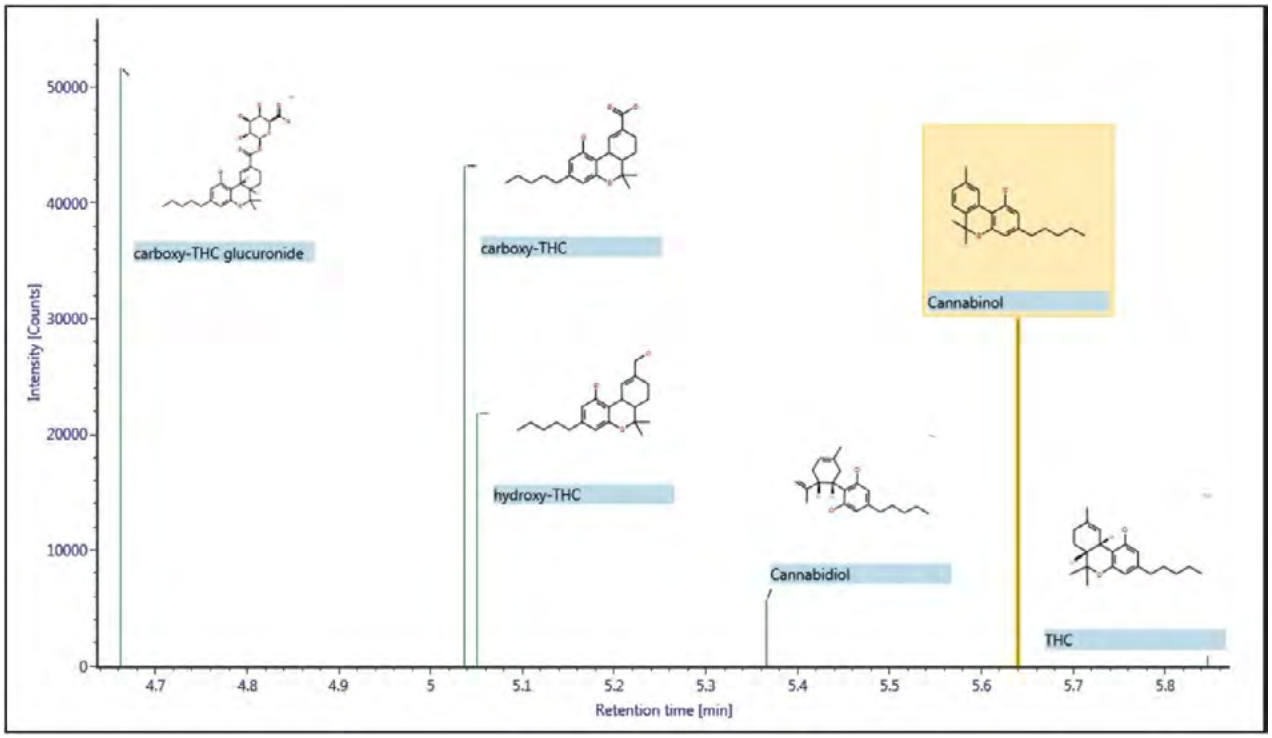

Figure 1. Component plot showing positively identified cannabinoids in a mixture of standards (ISTD not shown). The plot shows chromatographic separation for the isomers cannabidiol and THC.

To investigate linearity, Bio-Rad control urine was spiked with cTHC and cTHC glucuronide over a range from 0 to 500 ng/mL (250 ng/mL for cTHC-glucuronide) and prepared in duplicate, as described above. The 3D peak response for each positively identified analyte trace was generated automatically during processing and referenced to the ISTD response; calibration curves were plotted using a 1/x weighting and a linear fit was applied to both analytes. The correlation coefficient (r^2 value) was ≥0.995 for both analytes; a calibration curve for cTHC is shown in Figure 2. The lowest calibrator positively identified, using the criteria detailed above, was 6.25 ng/mL for cTHC-glucuronide and 12.5 ng/mL for cTHC.

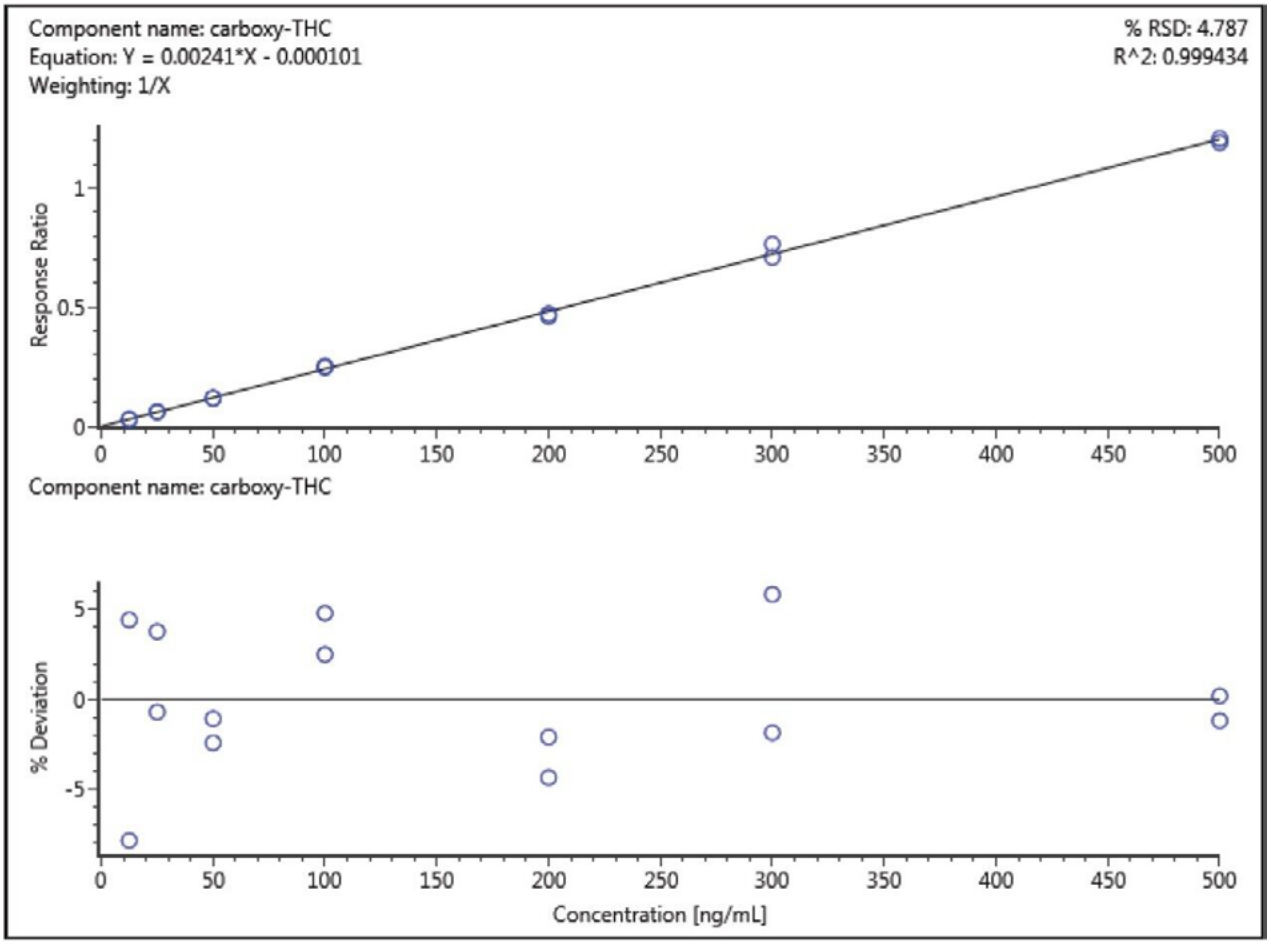

Figure 2. A spiked urine calibration curve for cTHC over the range 0 to 500 ng/mL using a linear fit with 1/x weighting applied.

Analysis of authentic urine samples Twenty-six authentic urine samples and two commercial reference urines (Bio-Rad Liquichek Level C2 and S10) were analyzed following the sample preparation method described here. The authentic samples comprised anonymized samples that had previously been quantified using a fully validated UPLC-MS/MS assay.[7] The UPLC-MS/MS results for the 26 authentic samples are shown in Table 2, along with the results from the UPLC-QTof-MS^E assay, showing whether cTHC or cTHC-glucuronide were positively identified based on the criteria stated previously.

Sample	cTHC		cTHC-glucuronide	
	UPLC-MS/MS (ng/mL)	UPLC-QTof-MS^E (positive ID)	UPLC-MS/MS (ng/mL)	UPLC-QTof-MS^E (positive ID)
001	20	YES	40	YES
002	0	NO	33	YES
003	68	YES	285	YES
004	0	NO	0	NO
005	0	NO	0	NO
006	0	NO	0	NO
007	0	NO	0	NO
008	12	YES	70	YES
009	3	NO	16	YES
010	6	NO	33	YES
011	0	NO	0	NO
012	18	YES	49	YES
013	7	NO	21	YES
014	14	YES	206	YES
015	11	YES	10	YES
016	0	NO	12	YES
017	54	YES	14	YES
018	15	YES	76	YES
019	10	YES	177	YES
020	0	NO	0	NO
021	0	NO	0	NO
022	67	YES	120	YES
023	0	NO	0	NO
024	83	YES	233	YES
025	0	NO	0	NO
026	0	NO	7	NO

Table 2. Comparison of individual sample results for either the UPLC-MS/MS or UPLC-QTof-MS^E methods. Positive identification criteria for the UPLC-QTof-MS^E assay were 3D low energy ion count intensity greater than 250, retention time to be within 0.35 min of reference, observed precursor mass to be within 5 ppm of expected and a minimum of one high energy fragment ion detected.

The UPLC-QTof-MS^E method positively identified cTHC in 11 samples and cTHC-glucuronide in 16 samples, and overall demonstrated excellent concordance with the UPLC-MS/MS data (Table 3). This demonstrated

that this method can consistently detect cTHC and cTHC-glucuronide in urine, using a simple fivefold dilution, at concentrations below the current European Workplace Drug Testing Society (EWDTS) screening cut-off of 50 ng/mL for cTHC.[6]

cTHC			cTHC-glucuronide		
UPLC-MS/MS		UPLC-QTof-MS^E	UPLC-MS/MS		UPLC-QTof-MS^E
Blank	12	15 NEG	Blank	9	10 NEG
Positive <10 ng/mL	3		Positive <10 ng/mL	1	
Positive ≥10 ng/mL	11	11 POS	Positive ≥10 ng/mL	16	16 POS

Table 3. Summary of results for 26 authentic urine samples obtained using the quantitative UPLC-MS/MS methodology[7] and the described UPLC-QTof-MS^E assay.

Furthermore the method detected and correctly assigned cTHC in both commercial reference urines. The semi-quantitative results obtained using this method for the analysis for the Bio-Rad Liquichek Level C2 and S10 reference urines were in accordance with the manufacturer's stated reference values, and are shown in Table 4. The additional confirmation provided by the presence of 4 high energy fragments for cTHC in the Bio-Rad Liquichek level S10 reference urine is shown in Figure 3.

Reference urine	GC/MS (ng/mL)	UPLC-QTof-MS^E (ng/mL)
Bio-Rad Liquichek Level C2	11.5	11.1
Bio-Rad Liquichek Level S10	35.3	40.5

Table 4. Comparison between the values obtained using the UPLC-QTof-MS^E method for the analysis of the Bio-Rad Liquichek reference urines and the values stated by the manufacturer.

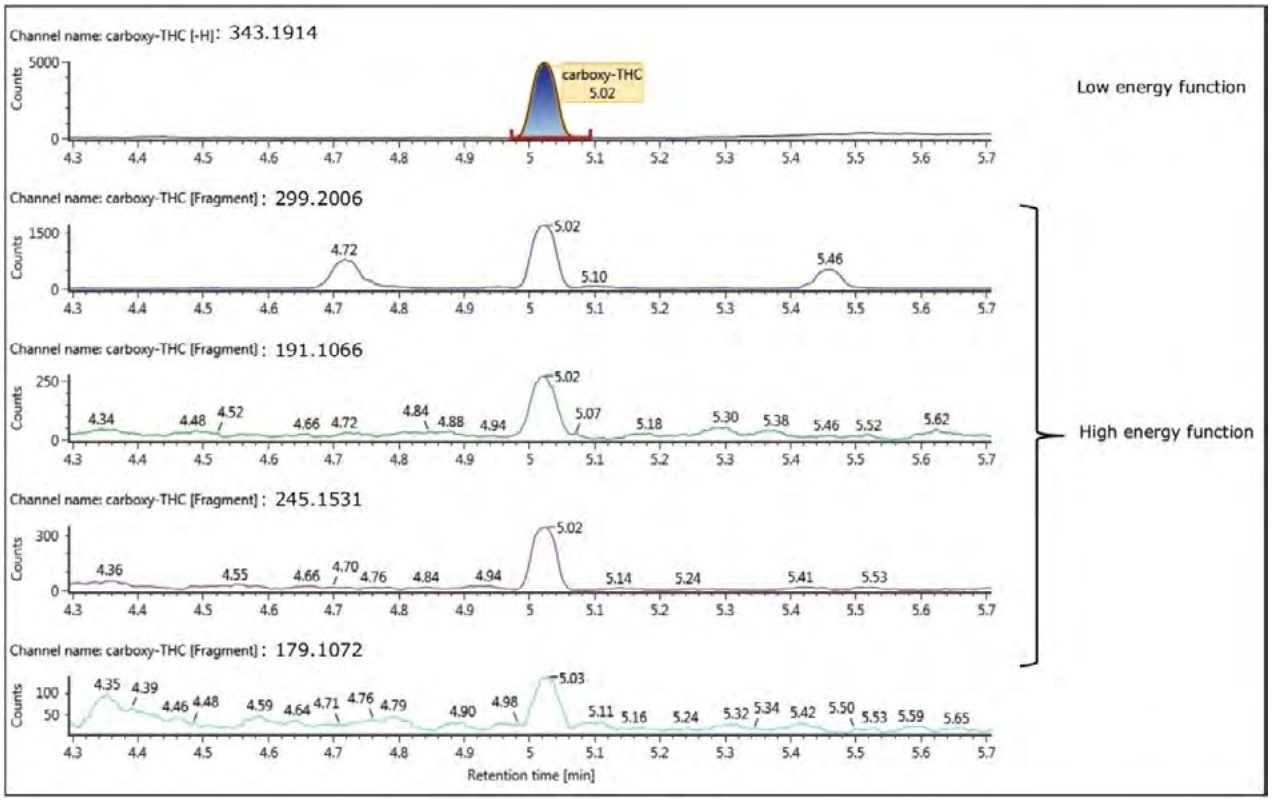

Figure 3. Data for cTHC in the Bio-Rad Liquichek Level S10 reference urine; additional confirmation is achieved by the presence of 4 fragment ions in the high energy function.

Conclusion

This application note demonstrates the sensitivity and selectivity of the expanded Forensic Toxicology Application Solution with UNIFI using negative ionisation in providing a consistent comprehensive determination of cannabinoids. It can be applied as both a screen for selected cannabinoids and a method suitable for quantifying these analytes, at levels below the current EWDTS urine screening cut-off (50 ng/mL for cannabis metabolites), using a simple five-fold dilution. The excellent linear dynamic range of this system is demonstrated by simple automatically generated calibration plots.

Acknowledgements

CEDAM Italia, Bresso, Italy and Bianalisi Analisi Mediche, Carate Brianza, Italy for supplying the anonymized authentic urine samples.

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1. Dewey WL. Cannabinoid Pharmacology. *Pharmacol Rev.* 1986 Jun;38(2):151–78.
2. Pallente S, et al. Synthesis and Characterization of Glucuronides of 5′-Hydroxy-delta-9-THC and 11-Hydroxydelta- THC. *Drug Metab Dispos*. 1978 Jul Aug;6(4):389–95.
3. Wood M. The Utility of MS^E for Toxicological Screening. Waters technology brief. 2010. p/n 720005198EN.
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7. Lee R and Wood M. Using UPLC-MS/MS for Workplace Drug Testing. Waters application note. 2014. p/n 720005032EN.

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Forensic Toxicology Screening Application Solution with UNIFI <https://www.waters.com/134779723>

Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry <https://www.waters.com/134798222>

Available for Purchase Online

ACQUITY UPLC HSS C18 Column, 100Å, 1.8 µm, 2.1 mm X 150 mm, 1/pkg <
https://www.waters.com/waters/partDetail.htm?partNumber=186003534>

LCGC Certified Clear Glass 12 x 32mm Screw Neck Max Recovery Vial, with Cap and Preslit PTFE/Silicone Septa, 2 mL Volume, 100/pkg <
https://www.waters.com/waters/partDetail.htm?partNumber=186000327C>

A full validation by the user would be necessary prior to adoption in a laboratory.

720005413, May 2015

Waters™

Application Note

Using the Waters Forensic Toxicology Screening Application Solution With UNIFI to Determine Diuretics in Urine

Robert Lee, Michelle Wood

Waters Corporation

For forensic toxicology use only.

Abstract

The rise of sports doping testing has highlighted the need for a quick, accurate, reliable, and robust method to initially screen large numbers of samples. Expanding the Forensic Toxicology Screening Application Solution with UNIFI to determine negative ionising compounds enables the determination of diuretics in diluted urine at levels which will allow this method to be applied to anti-doping labs that comply with the WADA guidelines.

Benefits

Expanded Forensic Toxicology Screening Application Solution with UNIFI, enabling the detection and quantitation of negative ionising diuretics in urine.

Introduction

Diuretics are a class of pharmaceutical compounds whose primary aim is to promote urine production. As such they can be used to treat a number of medical conditions including congestive heart failure and hypertension. In sports, diuretics can be abused by athletes to generate rapid weight loss or to maintain low body weight. They can also be used to mask the presence of banned or illegal substances by facilitating dilution of the urine and aiding excretion. Consequently the use of diuretics is prohibited for athletes by the World Anti-Doping Agency (WADA). The list of banned compounds can be found in the WADA technical document[1] and includes at least 25 compounds with diuretic properties. To ensure consistency of measurement amongst doping control laboratories, WADA defines the minimum required performance level (MRPL), which is the concentration of a prohibited substance that laboratories are expected to detect; currently this is set at 200ng/mL.[2]

The Forensic Toxicology Screening Application Solution with UNIFI currently comprises acquisition of accurate mass data on an orthogonal acceleration time-of-flight mass spectrometer, operating in MS^E mode using electrospray positive ionisation mode (ESI+), followed by comparison of the data with a comprehensive library containing more than 1000 toxicologically-relevant substances.[3-5] However, as a number of the diuretics only ionise in negative electrospray mode (ESI-), the aim of the recent work was to further extend the Forensic Toxicology Screening Application Solution with UNIFI to include compounds that ionise in negative mode and to use the method to determine the presence of diuretics in urine, particularly at concentrations below the WADA MRPL.

Experimental

ACQUITY UPLC conditions

UPLC System:	ACQUITY UPLC I-Class (FTN)
Column:	ACQUITY UPLC HSS C_{18}, 100A, 1.8 µm, 2.1 mm x 150 mm, (p/n 186003534)
Vials:	Maximum Recovery Vials, 12 x 32mm, screw neck (p/n 186000327c)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	Water containing 0.001% formic acid
Mobile phase B:	Acetonitrile containing 0.001% formic acid
Gradient:	Isocratic at 87% A for 0.5 min then to 5% A at 4.5 min, hold for 1 min before switching to 87% A
Run time:	7.5 min

MS^E conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M-H]^-$ *m/z* = 554.2620
Acquisition range:	*m/z* 50-1000
Scan time:	0.1 s
Capillary voltage:	1.5 KV
Cone voltage:	20 V
Collision energy:	Function 1: 6 eV Function 2: ramped 10 to 40 eV

Materials

Ioxinyl, for use as internal standard (ISTD), was purchased from Sigma-Aldrich (Poole, UK). A stock solution was prepared at 1 mg/mL in methanol and stored at -20 °C. Prior to use, the stock was diluted to 100 ng/mL in 0.001% formic acid.

All other chemicals used were of the highest grade available and stored according to the supplier's instructions.

Bio-Rad normal control urine was obtained from Bio-Rad Laboratories (Hemel Hempstead, UK).

Sample preparation

Acetonitrile (0.1 mL) was added to 0.2 mL urine and ISTD (0.7 mL).The sample was vortex-mixed, for 5 min at 1200 rpm, and then centrifuged at 8000 *g* for 10 min. Supernatant was transferred to a Maximum Recovery Vial (p/n 186000327c).

Results and Discussion

Sixteen diuretics that ionise in ESI- were included in this analysis and are listed in Table 1, along with their exact neutral mass and UPLC retention times. The list includes five compounds that solely ionise in negative mode (bendroflumethiazide, benzthiazide, furosemide, hydrochlorothiazide, and hydroflumethiazide).

Analyte	Neutral monoisotopic mass	Retention time (min)
Acetazolamide	221.9881	1.7
Chlorothiazide	294.9488	1.8
Hydrochlorothiazide	296.9645	1.9
Hydroflumethiazide	330.9908	2.4
Chlorthalidone	338.0128	2.6
Furosemide	330.0077	3.1
Metolazone	365.0601	3.2
Benzthiazide	430.9835	3.4
Indapamide	365.0601	3.4
Cyclothiazide	389.0271	3.5
Bendroflumethiazide	421.0378	3.5
Canrenoic acid	358.2144	3.4
Xipamide	354.0441	3.7
Bumetanide	364.1093	3.8
Probenecid	285.1035	3.9
Tolvaptan	448.1554	4.1

Table 1. Analyte retention times and neutral mass.

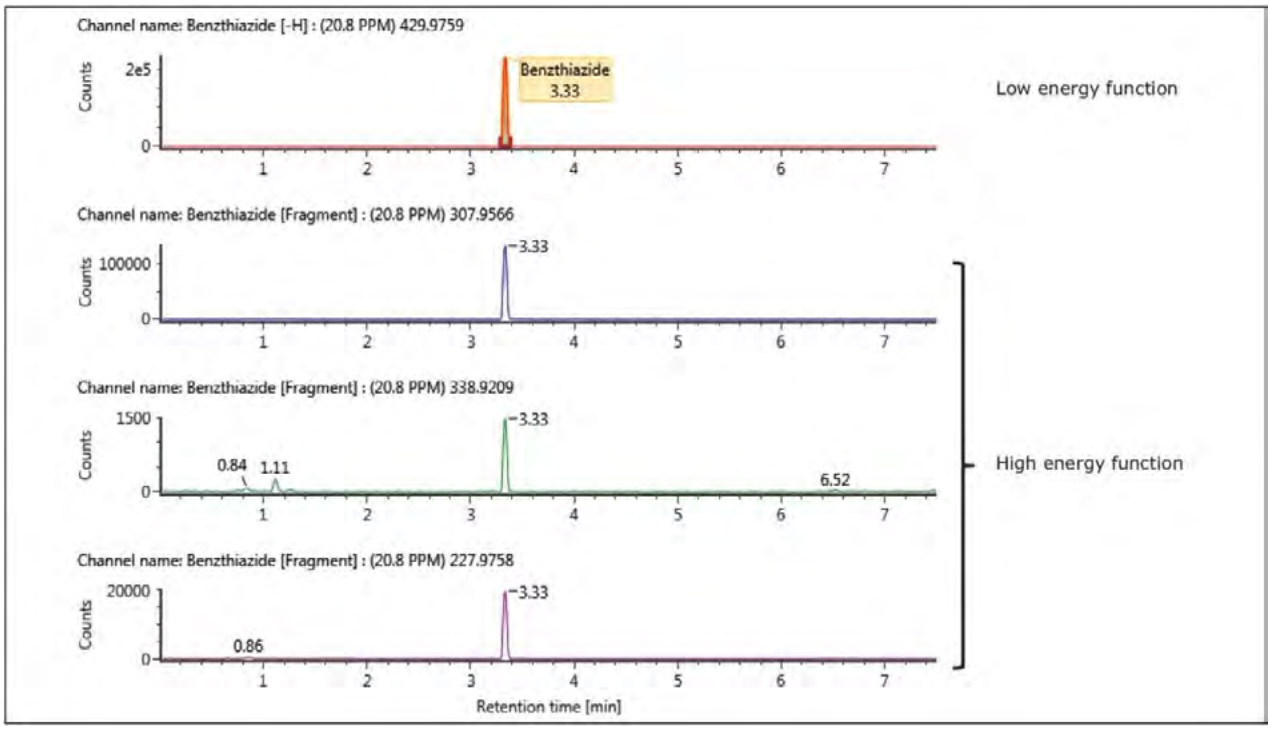

Figure 1. Data for benzthiazide in a blank urine sample that has been spiked at the WADA MRPL (200 ng/mL); additional confirmation is achieved by the presence of three fragment ions in the high energy function.

The acceptance criteria for a positive identification of each analyte was as follows: retention time to be within 0.35 min of reference and the observed precursor mass to be within 5 ppm of expected. For additional confirmation, a minimum of one diagnostic fragment ion had to be found in the high energy function.

The utility of the MS^E approach and associated fragment ion data generated under the higher energy condition is further demonstrated in Figure 2. The figure displays the high energy data for metolazone and indapamide which have identical elemental composition (C16H16ClN3O3S) and, under the chromatographic conditions employed here, are also closely-eluting i.e., within 0.2 min. Under these conditions it could be challenging to differentiate between the two diuretics; however the figure shows clear differentiatiation when the diagnostic fragment ions are taken into account.

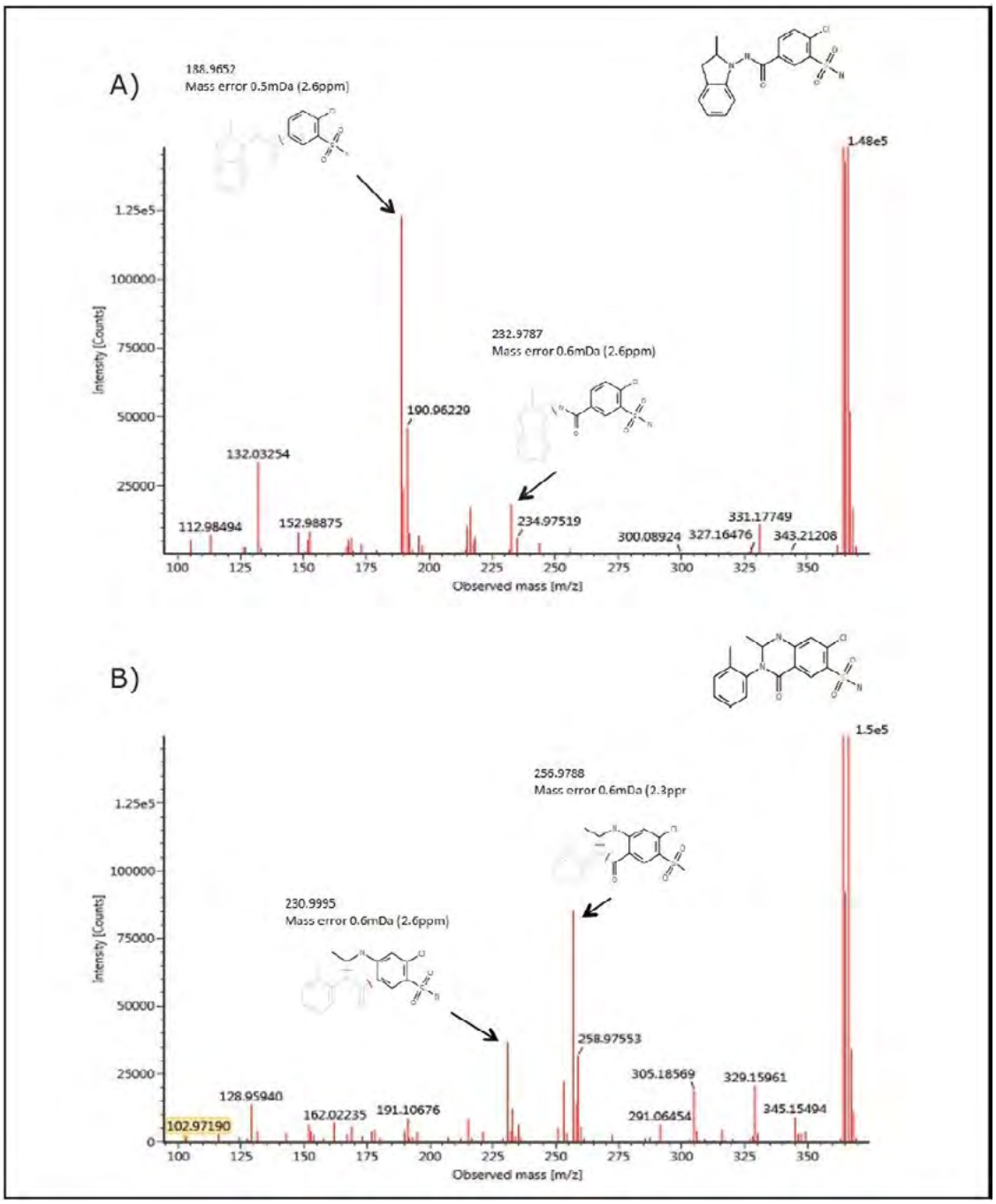

Figure 2. Spectra showing the high energy data for indapamide (Figure 2A) and metolazone (Figure 2B) highlighting the differences in the high energy fragments detected.

To investigate linearity, control urine was spiked with the diuretics over a range from 0 to 2000 ng/mL and prepared, in duplicate, as described above. The response for each analyte trace was generated automatically during processing and referenced to the ISTD response. Semi-quantitative calibration curves were plotted

using a 1/x weighting and a quadratic fit was applied to all the analytes. The correlation coefficient of determination was >0.99 for each analyte. A calibration curve from 0 to 2000 ng/mL for furosemide is shown in Figure 3. Urine spiked at the WADA MRPL was quantified against the relevant calibration curves; all analytes were positively identified at this level.

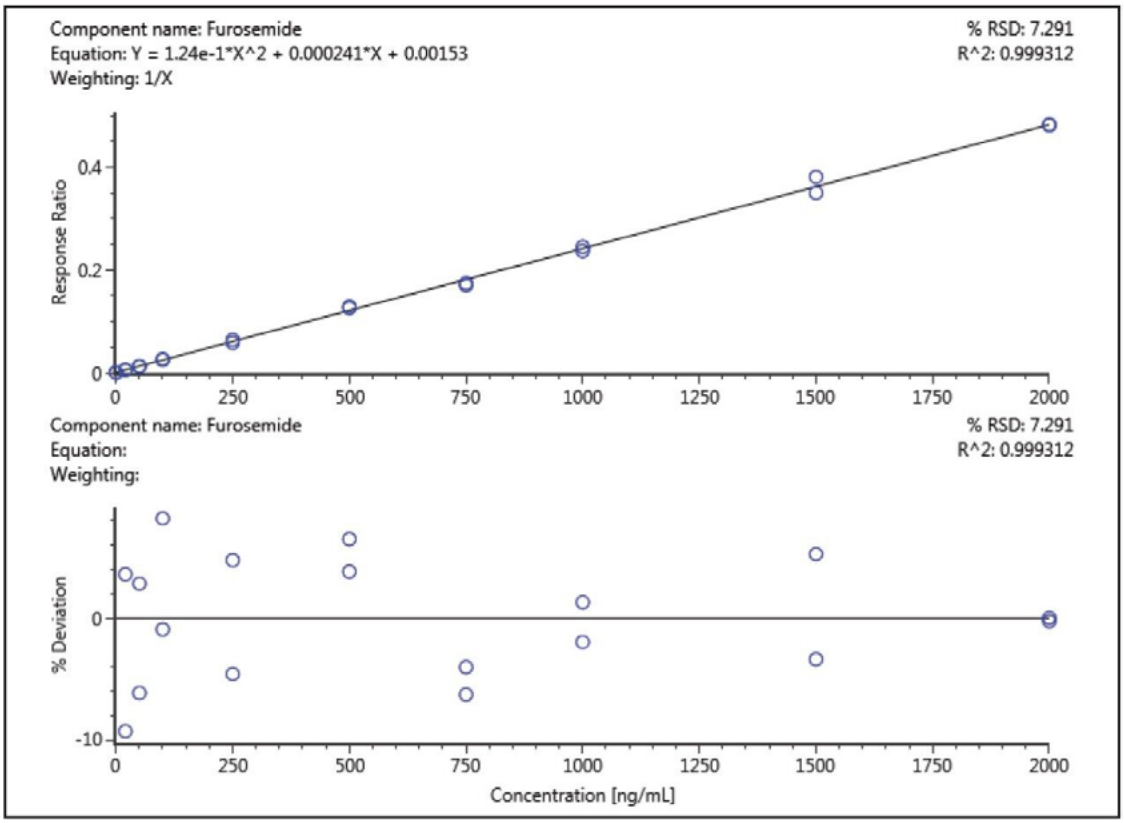

Figure 3. A spiked urine calibration curve for furosemide over the range 0 to 2000 ng/mL using a quadratic fit with 1/x weighting applied.

A comparison between the high energy fragments identified in an analytical standard and those identified in a blank urine sample spiked with furosemide is shown in Figure 4.

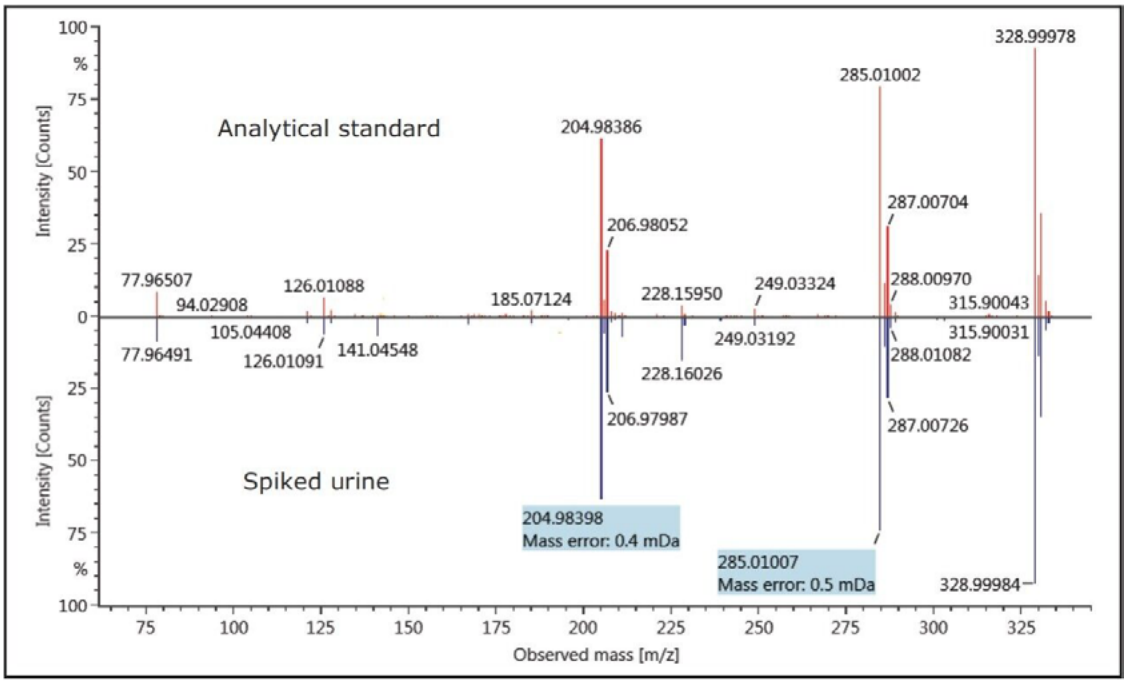

Figure 4. Results of the binary comparison analysis for furosemide (the plot is scaled to the relative percentages of the identified fragments). The data have been generated using the binary compare tool in UNIFI and highlight the mass error between the standard (upper-trace) and the sample data (lower-trace).

Analysis of Authentic Urine Sample

An authentic urine sample was analysed following the sample preparation method described and was shown to contain the diuretic furosemide. Further investigation of the data using UNIFI's metabolite identification (Met ID) tools indicated the presence of both phase 1 and phase 2 metabolites. The retention times for the parent molecule and the most prevalent metabolite, a glucuronide conjugate, (2.69 min) are shown in Figure 5. The software highlights metabolic transformation with observed retention time along with observed *m/z* and the mass error in ppm.

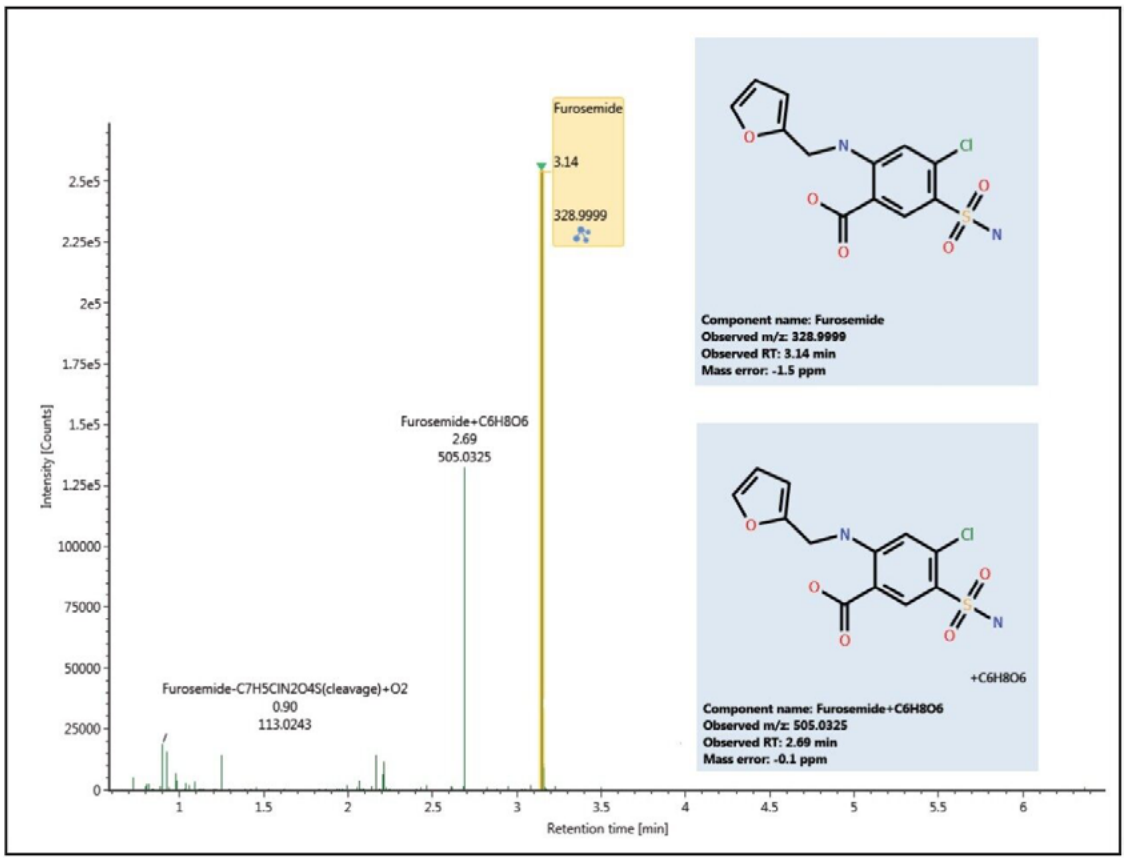

Figure 5. A selection of UNIFI Met ID proposed metabolites (showing observed m/z, mass error in ppm, retention time, and transformation) detected in an authentic urine sample.

Conclusion

The rise of sports doping testing has highlighted the need for a quick, accurate, reliable, and robust method to initially screen large numbers of samples. Expanding the Forensic Toxicology Screening Application Solution with UNIFI to determine negative ionising compounds enables the determination of diuretics in diluted urine at levels which will allow this method to be applied to anti-doping labs that comply with the WADA guidelines.

The use of the binary compare and metabolite identification tools within UNIFI can increase the confidence in the data by highlighting high energy fragment matches and facilitates discovery of metabolites in the sample which are not present in the database. These metabolites can subsequently be added to the library.

References

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4. Forensic Toxicology Screening Application Solution. Waters Brochure. p/n 720004830EN.
5. Roberts M and Wood M. Analysis of Beta-Blockers using UPLC with Accurate Mass Screening. Waters application note. 2014. p/n 720005188EN.

A full validation by the user would be necessary prior to adoption in a laboratory.

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Forensic Toxicology Screening Application Solution with UNIFI <https://www.waters.com/134779723>

Available for Purchase Online

ACQUITY UPLC HSS C18 Column, 100Å, 1.8 µm, 2.1 mm X 150 mm, 1/pkg < https://www.waters.com/waters/partDetail.htm?partNumber=186003534>

LCGC Certified Clear Glass 12 x 32mm Screw Neck Max Recovery Vial, with Cap and Preslit PTFE/Silicone Septa, 2 mL Volume, 100/pkg < https://www.waters.com/waters/partDetail.htm?partNumber=186000327C>

720005391, April 2015

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Application Note

Analysis of Beta-Blockers Using UPLC with Accurate-Mass Screening

Mark Roberts, Michelle Wood

Waters Corporation

For forensic toxicology use only.

Abstract

This application note demonstrates the sensitivity and selectivity of the Forensic Toxicology Application Solution with UNIFI in providing comprehensive screening of beta-blockers at low levels of concentration in human urine and achieving the MRPL with minimal sample preparation.

Benefits

- Enables analysts to confidently screen and identify beta-blocker drugs in urine
- Data is processed automatically and presented to the user with fully customizable workflows and reports.

Introduction

Propranolol was the first, clinically successful beta-blocker. Synthesized by JW Black in the early 1960s,[1] it revolutionized the management of angina pectoris and spawned the development of additional beta-blockers. Beta-blockers competitively block the action of beta-adrenergic agonists at the beta-receptors in the cells of heart muscle and other tissues of the sympathetic nervous system. They are legally prescribed and used primarily for the management of hypertension, angina, and cardiac arrhythmias. These substances however, can be abused by athletes who want to decrease their heart rate, lower their blood pressure, or improve their fine motor skills. Consequently, the World Anti-Doping Agency (WADA) includes beta-blockers in its 2014 Prohibited List[2] (Category P2), limiting the prohibition to sports like archery, golf, and shooting.

Recent advances in liquid chromatography and mass spectrometry can help determine the presence of beta-blockers in urine.

Experimental

Sample preparation

A mixed, methanolic standard containing the following beta-blockers was prepared at a concentration of 50 µg/mL: acebutolol, alprenolol, atenolol, bunolol, bisoprolol, carazolol, celiprolol, levobunolol, metipranolol,

metoprolol, nadolol, nebivolol, oxprenolol, pindolol, sotalol, and timolol. Blank human urine was spiked with the mixed standard, resulting in final concentrations of 50, 100*, 250, and 500 ng/mL. A simple five-fold dilution with mobile phase A was used to prepare each spiked urine sample for injection.

* Minimum required performance level (MRPL) for a WADA-accredited laboratory.

Method conditions

LC conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Run time:	15 min
Column:	ACQUITY UPLC HSS C_{18} 2.1 x 150 mm, 1.8 µm
Vials:	Waters Maximum Recovery Vials
Column temp.:	50 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM aqueous ammonium formate, adjusted to pH 3.0
Mobile phase B:	Acetonitrile with 0.1% formic acid
Gradient:	87% A to 50% A over 10 min, reduce to 5% A and hold for 1.5 min before returning to 87% A

MS^E conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI+
Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M+H]^+$ = 556.2766
Acquisition range:	*m/z* 50–1000
Scan time:	0.1 s
Capillary voltage:	0.8 kV
Cone voltage:	25 V
Collision energy:	Function 1: 6 eV Function 2: ramped 10 to 40 eV

Results and Discussion

The diluted spiked urine samples were injected and data was acquired using the standard MS^E -based toxicology screen.[3,4] Data were subsequently processed using the UNIFI Forensic Toxicology Library comprising more than 1,000 toxicologically-relevant substances. Qualitative identification was achieved through a combination of mass accuracy, retention time (RT) and the presence/absence of expected fragment ions. In the same processing step, UNIFI Scientific Information System also generates and displays any quantitative data.

UNIFI uses a simple workflow approach to guide the user through the sample results; data is automatically filtered and presented to the user according to the degree of confidence in the identification, thereby decreasing the requirement for analyst's review. Workflows are fully customizable - an example of the criteria that may be used is shown in Figure 1.

All of the beta-blockers were successfully identified at the lowest concentration investigated in this study (50 ng/mL) and met the user-defined criteria for a "Positive" drug finding. Figures 1 through 4 provide an illustrative example of some of the data that is automatically-displayed or available to the user on a "single-click" from the Review pane.

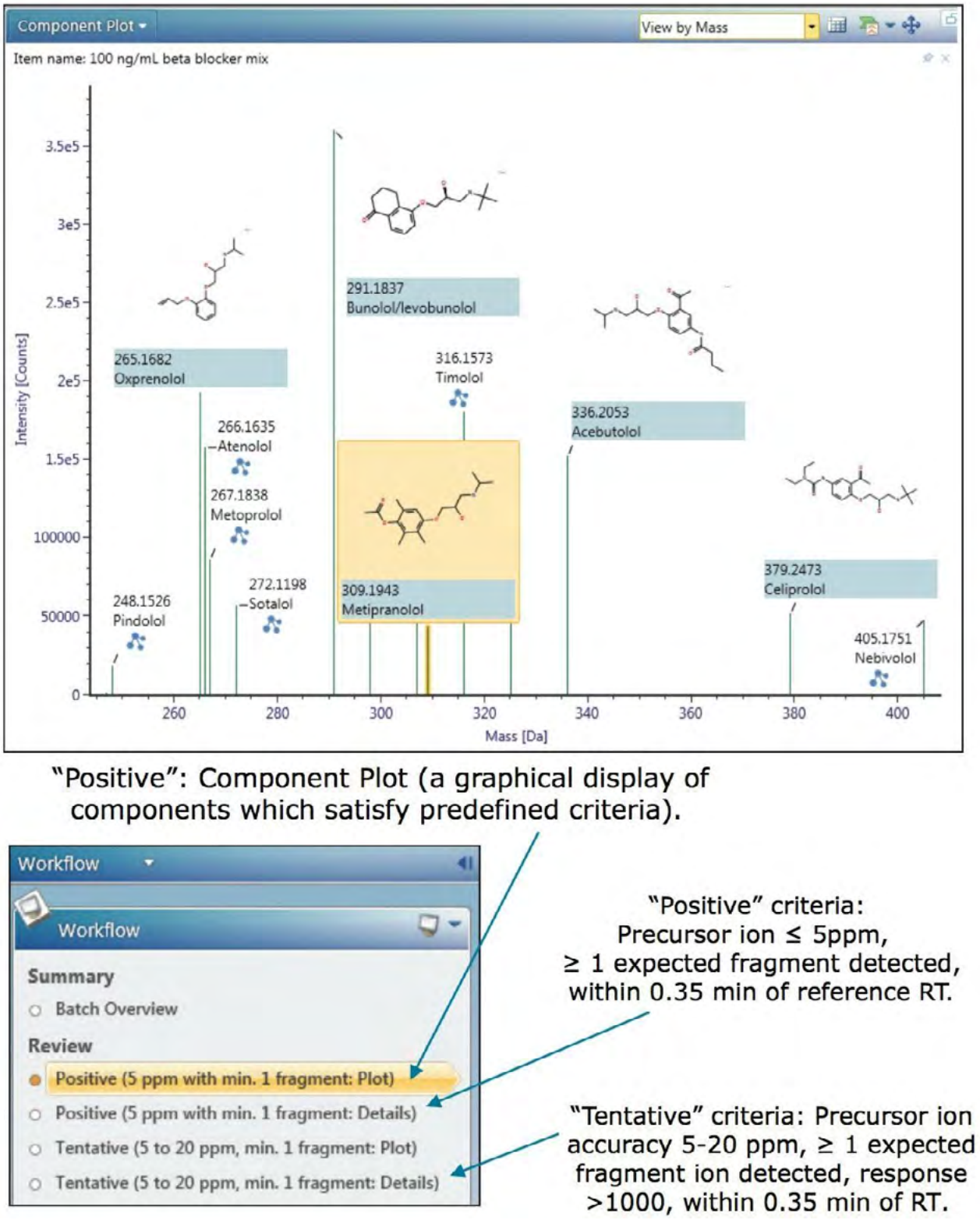


Figure 1. Example of a workflow based on two differing categories of identification ("Positive" and "Tentative") together with a summary of the criteria used for each.

In contrast to the plot view, which provides a very simple graphical display of detected components, full details of each identification can be viewed by selection of the Component Summary (Figure 2). This is a user-friendly table that summarizes key characteristics of identified peaks including mass accuracy, confirmatory fragment ions and isotope information (in this example, only those components that matched the “Positive” criteria are shown).

The extracted mass chromatograms for the precursor ion and all of the high collision energy fragment ions for a particular component can also be displayed if required, as shown in the Chromatograms window (lower left of Figure 2).

Further information is available by viewing the low and high energy spectra for a component as shown in the Spectra window. This view highlights the precursor ion in the top trace and the found fragment ions in the bottom trace. UNIFI provides improved three-dimensional (3D) chromatographic peak detection with its integrated ApexTrack algorithm, which facilitates the generation of cleaner mass spectra, enabling better library matching of fragment ions.

In addition to viewing the spectra, it is often useful to display a summary of the confirmatory fragment ion data. Figure 2 also shows the Fragments table which contains details for the expected fragments for acebutolol, the mass error associated with each detected fragment, and the detected fragment intensity.

Component Summary: User-friendly table that summarizes key characteristics of identified peaks including information related to: mass accuracy; RT; presence of confirmatory fragment ions and isotope information.

Figure 2. Details for a 50 ng/mL spiked urine sample. The Component Summary shows details for the first five analytes present in alphabetical order. The F v E (%) column displays the number of found vs number of expected fragments, expressed as a percentage. The Isotope Match Intensity RMS Percent and Isotope Match Mz RMS PPM indicate the degree of matching between the theoretical isotopic pattern and the observed pattern for the precursor ion cluster. The chromatogram's window contains the extracted mass chromatograms for the selected precursor i.e., acebutolol (m/z 337.212) and fragment ions (m/z 116, 319, 98, and 260). The Spectra window displays the low collision energy (upper spectrum) and high collision energy (lower spectrum) for acebutolol and the Fragments table shows each of the expected fragments for acebutolol.

The isotopic pattern obtained for each component can also be an aid in identification. Figure 3 shows the mass spectrum of the low collision energy trace for sotalol, a sulphur-containing compound. The two most abundant stable isotopes of sulphur are ^{32}S and ^{34}S which are present at a ratio of 95:4. An algorithm within UNIFI can be used to indicate the degree of matching between the theoretical and observed isotopic patterns for a component, with a low score indicating a good match. This "Isotope Match Intensity RMS Percent" column can be added to the Component Summary table as an extra point of confirmation. A further UNIFI algorithm is used to evaluate the level of agreement between the expected *m/z* and found *m/z* of each isotopic peak and these results are shown in the Isotope Match Mz RMS PPM, again with a low score indicating a good match as shown in the rightmost columns of the Component Summary window of Figure 2.

Isotopic information:
UNIFI includes both a graphical display as well as calculated comparisons of measured isotopic data against the theoretical (see Figure 2).

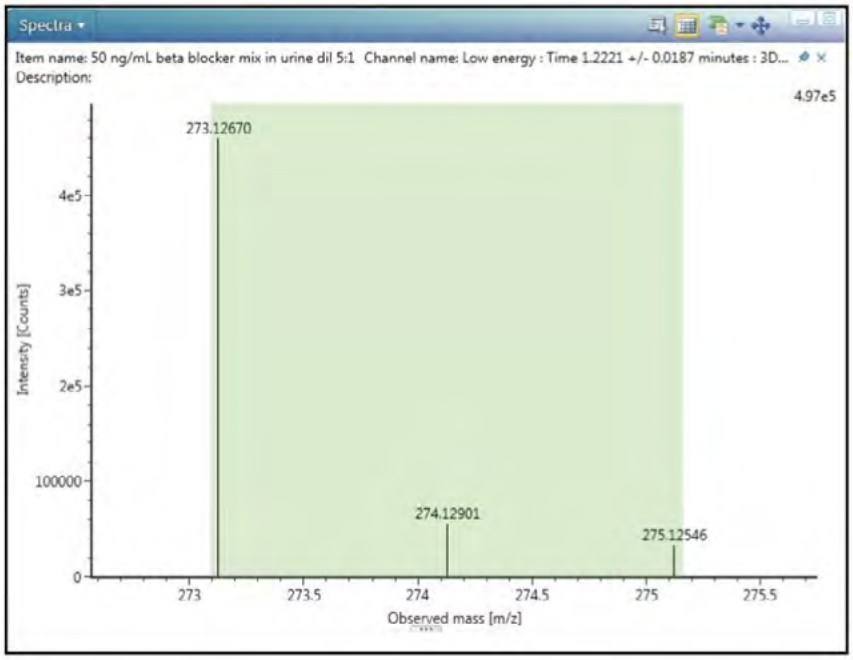


Figure 3. The low collision energy spectrum for sotalol showing the m/z 273 and 275 ions corresponding to the sulphur isotopes 32S and 34S. UNIFI includes algorithms to automatically compare the isotopic data of the measured component with the theoretical for the proposed substance; this data is included in the last two columns of the Component Summary table.

In particular, Figure 4 shows, for each beta-blocker, a semi-quantitative calibration plot that draws data from three replicate injections made at each concentration (50, 100, 250, and 500 ng/mL). The calibrations are calculated from the response value for each analyte, a value that originates with the 3D integration of the monoisotopic precursor-ion peak. Because no internal standards were used in this study, this semi-quantitative data demonstrates only the typical dynamic range of the instrument.

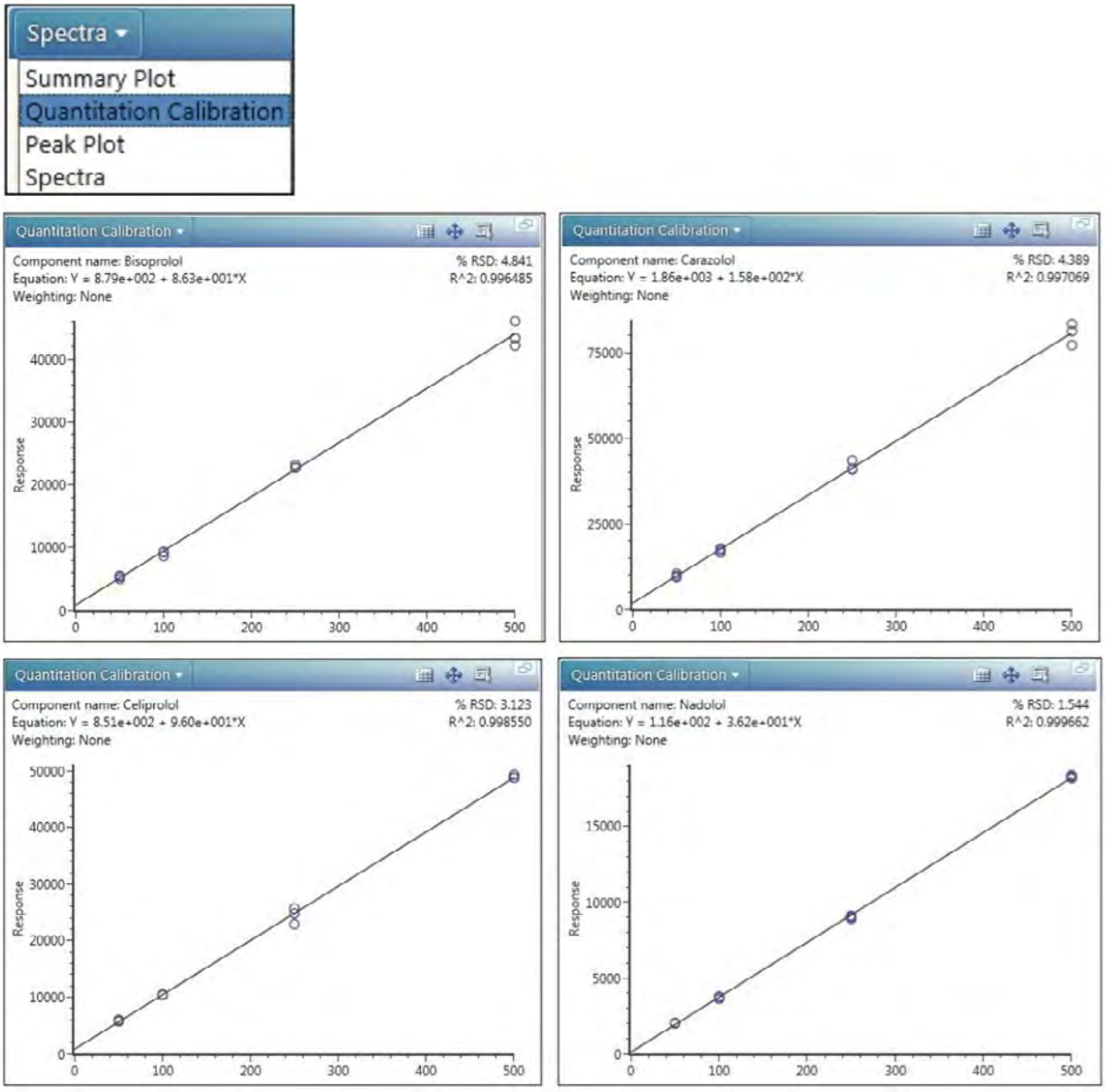


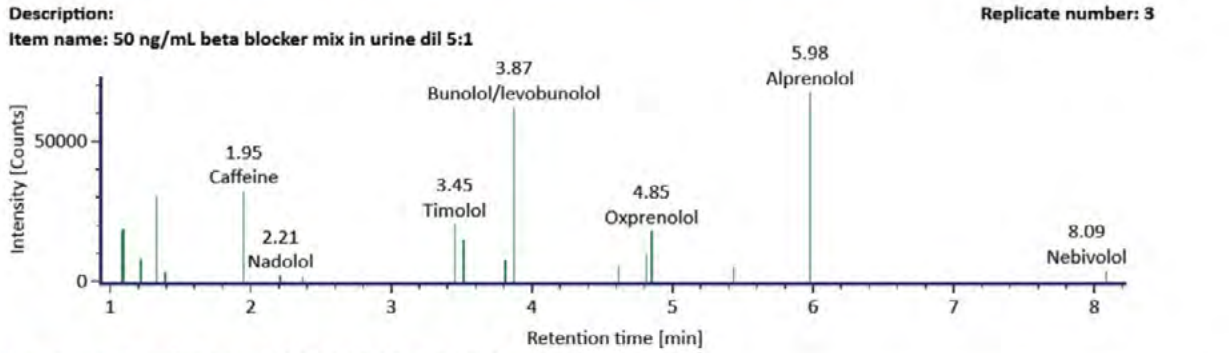
Figure 4. Calibration plots for four beta-blocker analytes spiked into urine (triplicate injections) at 50, 100, 250, and 500 ng/mL using a linear fit with no weighting applied.

Figure 5 illustrates a fully customizable report generated by the UNIFI Software from the results that provided the key details of the identifications made for this sample. A section from this report is shown in Figure 5 and provides a Component Plot as well as a Component Summary for each identification category.

Sample Information

Item name:	50 ng/mL beta blocker mix in urine dil 5:1	Sample type:	
Description:		Replicate number:	3
		Sample position:	1:B,6

Positive Identifications (Mass error <5ppm, at least one diagnostic fragment ion)



Item name: 50 ng/mL beta blocker mix in urine dil 5:1

Component name	m/z	Mass error (ppm)	Expected RT (min)	Observed RT (min)	Identified High Energy Fragments	Expected Fragments Count	F v E (%)	Response	Isotope Match Mz RMS PPM	Isotope Match Intensity RMS Percent
Acebutolol	337.2121	-0.27	3.7	3.5	4	4	100.00	14684	0.48	1.84
Alprenolol	250.1804	1.07	6.2	6.0	2	3	66.00	67513	0.99	1.50

Figure 5. A fully customizable report showing the Component Plot and the first two lines of the Component Summary for this injection.

Conclusion

This application note demonstrates the sensitivity and selectivity of the Forensic Toxicology Application Solution with UNIFI in providing comprehensive screening of beta-blockers at low levels of concentration in human urine and achieving the MRPL with minimal sample preparation. Despite the complex nature of accurate mass MS^E data, the UNIFI Software enables user-friendly, comprehensive data analysis, interpretation, and reporting. The excellent linear dynamic range of this system is demonstrated in four, simple, automatically generated calibration plots.

References

1. Black JW, Crowther AF, Shanks RG, Smith LH, and Dornhurst AC. A new adrenergic beta-receptor antagonist. *Lancet*. 1964 May 16;1(7342):1080–1.
2. The World Anti-Doping Code: The 2014 Prohibited List, International Standard. http://list.wada-ama.org/wp-content/uploads/2013/11/2014-Prohibited-List-ENGLISH-FINAL.pdf (accessed 20 September 2014).
3. Rosano TG, Wood M, Ihenetu K, and Swift TA. Drug screening in medical examiner casework by high resolution mass spectrometry (UPLC-MS^{E}-TOF). *J Anal Toxicol*. 2013 Oct;37(8):580–93. doi: 10.1093/jat/bkt071.
4. Forensic Toxicology Screening. Waters Brochure 720004830EN.

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Forensic Toxicology Screening Application Solution with UNIFI <https://www.waters.com/134779723>

UNIFI Scientific Information System <https://www.waters.com/134801648>

White Papers

Evaluation of Ion Ratios as an Additional Level of Confirmation in Accurate Mass Toxicology Screening

Quality Over Quantity – It's Not Always a Numbers Game

Benefits of High-Resolution Mass Spectrometry in a Forensic Toxicology Laboratory

Componentization Following 3D-Peak Detection in UNIFI Scientific Information System

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Application Note

Extension of the Systematic Toxicological Screening Library for use with the Waters Nominal Mass Screening Solutions

Robert Lee, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This Application brief describes creating an additional library containing toxicologically relevant analytes that can be used for Systematic Toxicological Analysis (STA) with Waters nominal mass full scan screening solutions.

Benefits

An extension to the comprehensive systematic toxicological analysis library for use with either the Xevo TQD or Xevo TQ-S micro Mass Spectrometers has been created to bring the total number of analytes in the solution to 1200.

Introduction

Forensic toxicology laboratories require reliable screening techniques that can detect a wide variety of toxicants in highly complex biological matrices, such as ante and postmortem specimens. The original Waters systematic toxicological screening method used the Waters Alliance 2695 Separations Module in conjunction with the ZQ Single Quadrupole Mass Spectrometer.1 In 2009, this approach was migrated to the ACQUITY TQD System, to deliver the same comprehensive toxicological screening capabilities in half the time.[2]

The solution was further developed over subsequent years, to provide a full scan screening method and associated toxicology libraries, capable of screening for >950 drug substances and metabolites in 15 minutes. This method has been successfully and routinely used in toxicology laboratories worldwide.[3,4] Owing to the popularity of this methodology, in 2013, this solution was transferred to the ACQUITY UPLC I-Class/Xevo TQD System, and the release of the Xevo TQ-S micro in 2016 allowed for further evolution of this successful solution.[5,6]

Forensic toxicology laboratories are under great pressure to keep pace with the ever changing trends of the drug landscape with new psychoactive substances (NPS) or designer drugs being detected and characterized on a weekly basis. This highlights the importance of being able to increase the number of analytes in toxicology libraries to enable laboratories to detect more compounds.

Experimental

Test substances

Single analytes or mixtures of analytes were prepared in 5 mM ammonium formate pH3 at 2500 ng/mL.

Data acquisition

Data was acquired in full scan mode on the ACQUITY UPLC I-Class/Xevo TQD System using the STA method.[5]

Library creation

The acquired data was processed using the ChromaLynx Application Manager and a library was created in the NIST format using the built in ChromaLynx library creation tools.

Results and Discussion

A new library containing 244 analytes was created using the STA method on the ACQUITY UPLC I-Class/Xevo TQD System. This library was then converted for use with the ACQUITY UPLC I-Class/Xevo TQ-S micro System. This brings the total number of analytes in the Waters nominal mass full scan screening solution to 1200, increasing coverage for NPS and newer drug analogs whilst also expanding the applicability to sports doping analysis.

A library containing an additional 244 analytes was created. For each analyte the library contains the analyte name, molecular formula and retention time along with the mass spectra at multiple cone voltages in positive electrospray ionisation mode. The fragmentation pattern obtained for the psychedelic tryptamine 5-MeO-DMT ($C_{13}H_{18}N_2O$, retention time 2.6 minutes) is shown in Figure 1.

The library was converted for use with the ACQUITY UPLC I-Class/Xevo TQ-S micro System, which requires a different version of the acquisition method.[6]

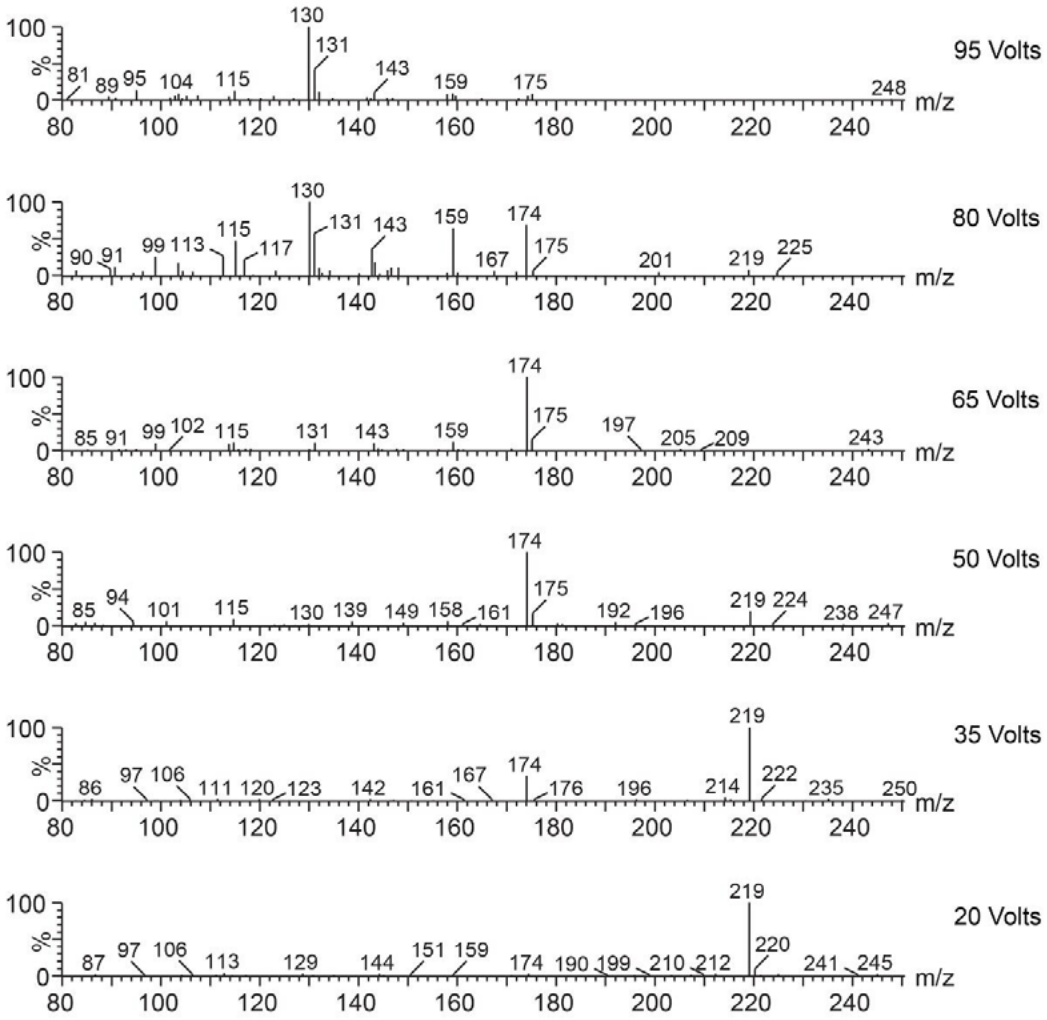

Figure 1. Fragmentation pattern for the psychedelic tryptamine 5-MeO-DMT across the six cone voltages used on the Xevo TQD.

Conclusion

A new library containing 244 analytes was created for use with the Waters nominal mass STA screening solutions to increase the total number of analytes in the forensic toxicology nominal mass screening solution to 1200. This library can be used on both the ACQUITY and Xevo TQD Mass Spectrometers in conjunction with the ACQUITY UPLC I-Class and the converted version can be used with the ACQUITY UPLC I-Class/Xevo TQ-S micro System.

The additional analytes in the library increases the number of "designer" drugs and NPS such as designer benzodiazepines, psychedelic tryptamines, and fentanyl analogs that can now be detected using this screening method. The number of beta blockers, diuretics, steroids and receptor modulator drugs, which are of specific interest to sports doping laboratories, has also increased with this new release.

The easy to use ChromaLynx library creation tool allows customers to prepare their own libraries from either standard material or authentic samples. The additional library is formatted for immediate use and can be simply added to the customer's existing library search method used by ChromaLynx to increase the number of analytes that can be detected using this screening method.

References

1. Humbert L, Lhermitte M, and Grisel F. General Unknown Screening for Drugs in Biological Samples by LC/MS. Waters Application Note. 720001552EN. 2007.
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4. Rosano T, Wood M, and Swift T. Postmortem Drug Screening by Non-Targeted and Targeted Ultra-Performance Liquid Chromatography-Mass Spectrometry Technology. *Journal of Analytical Toxicology*. 35: 411–423. 2011.
5. Roberts M and Wood M. Forensic Toxicology Screening Using the ACQUITY UPLC I-Class System with the Xevo TQD. Waters Application Note. 720004602EN. 2013.
6. Lee R and Wood M. Systematic Toxicological Screening Using the ACQUITY I- Class/Xevo TQ-S micro. Waters Application Note. 720005661EN. 2016.

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Xevo TQD Triple Quadrupole Mass Spectrometry <https://www.waters.com/134608730>

Xevo TQ-S micro Triple Quadrupole Mass Spectrometry <https://www.waters.com/134798856>

ChromaLynx <https://www.waters.com/513759>

720006502, February 2019

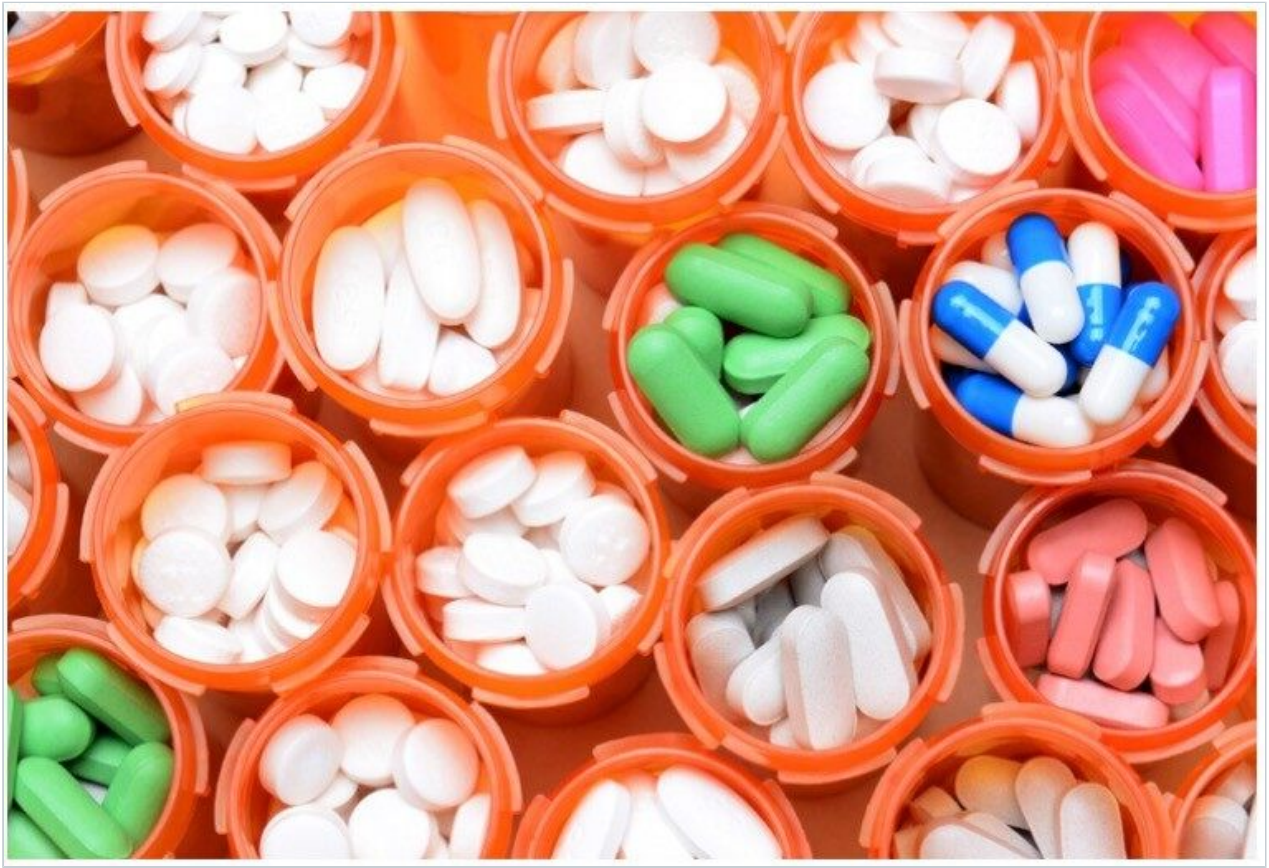
Waters™

Application Note

Evaluation of the Potential of the ACQUITY QDa Mass Detector for Use in Forensic Chemistry and Drug Control Laboratories

Jeff Goshawk, Robert Lee, Michelle Wood

Waters Corporation



For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief assess the transferability of an existing toxicology library to the Acquity QDa mass detector.

Benefits

Application of an existing toxicology library to the qualitative screening of medicines using the ACQUITY QDa — a promising tool for drug control.

Introduction

In recent years, a comprehensive spectral library for use in analytical toxicology has been developed. The library was originally generated using the Waters ACQUITY TQD Mass Spectrometer and was prepared by acquiring full scan mass spectra over multiple cone voltages, to yield compound-specific fragmentation patterns by the process of in-source collision-induced dissociation.[1] Since the first application of this methodology, over a decade ago, the approach has been applied to newer generation instruments[2] and the library has been expanded; it now contains data for over 950 toxicologically-relevant substances. The purpose of the current work was to evaluate the feasibility of using the existing library in combination with the ACQUITY QDa[3] to provide a simple, low-cost, qualitative screening and identification system for use in forensic chemistry and drug control laboratories. For this study, a selection of over-the-counter and prescribed medicines were analyzed as representative agents.

The ACQUITY UPLC I-Class System and ACQUITY QDa Mass Detector.

Experimental

Materials

Preliminary spectral testing was performed for a range of licit and illicit drug substances, using mixtures prepared from certified reference material (Sigma-Aldrich). Eight mixtures, each containing ten compounds, were analyzed.

Authentic samples for subsequent testing were prepared using a selection of over-the-counter and prescribed medicines in tablet, capsule, or liquid form.

Sample preparation

Individual tablets/capsules or 250 µL of medicines supplied in liquid form, were added to 25 mL of a methanol and water mixture (70:30) and sonicated at room temperature for 30 minutes. One millilitre of the resulting solution was transferred to a 2 mL microcentrifuge tube and centrifuged at 13000 rpm for 5 minutes. Fifty microlitres of the supernatant was diluted with 950 µL of water in a maximum recovery vial and vortex-mixed. LC-MS analysis was performed using 10 µL of the resulting solution.

LC-MS method conditions

Chromatographic separation was achieved within 15 minutes using an ACQUITY UPLC I-Class (FTN) and an established toxicology screening gradient.[1,2,4] The ACQUITY QDa was operated in ESI+, and full scan data were acquired over a *m/z* range of 80–650 at the following five cone voltages: 10 V, 20 V, 35 V, 45 V, and 55 V.

Results and Discussion

Data for a selection of medicines were acquired using the ACQUITY UPLC I-Class System combined with the ACQUITY QDa according to a well-established technique in which samples are screened against a library comprising reference retention time and multiple spectra.[1,2,4]

A preliminary assessment of voltages was performed using mixtures of drug standards at a concentration of 200 ng/mL. Data were acquired using the ACQUITY QDa at identical voltages to those used in the preparation of the original ACQUITY TQD library, i.e. from 20 V to 95 V in increments of 15 V. This initial data indicated that for the same cone voltages, the ACQUITY QDa exhibited increased fragmentation; consequently library voltages were adjusted to achieve parity with the ACQUITY QDa. The modified library was subsequently applied to the analysis of eight pharmaceuticals.

The multi cone voltage data acquired for each of the eight samples were processed using MassLynx Software with ChromaLynx Application Manager which detects the components within each sample and provides an identification through library matching. The confidence with which a substance is identified is presented as an average library match factor which has a maximum value of 1000. The average match factor is determined by comparing the measured and library spectra acquired over the five cone voltages.

The active ingredients detected in each of the eight samples are listed in Table 1 together with the average match factors as determined by ChromaLynx. For one of the medicines, Imuran, there was no match with the library, however, a large response was observed at the same retention time for each of the five cone voltages. The package insert for the product indicated that the active ingredient in Imuran is azathioprine, and this was consistent with the spectral data shown in Figure 1. The acquired data was subsequently used to generate library entries for azathioprine.

Medicine	Active ingredients detected (match factor)
LEMSIP® Max Cold and Flu Remedy	Phenylephrine 6.1 mg (707), paracetamol 500 mg (804), caffeine 25 mg (897)
Galpharm™ Hayfever and Allergy Relief	Cetirizine 10 mg (832)
Entrolax®	Bisacodyl 5 mg (889)
Galpharm Extra Power Pain Relief	Paracetamol 200 mg (816), caffeine 45 mg (880)
Buscopan®	Scopolamine butylbromide 10 mg (902)
Prozac®	Fluoxetine 20 mg (883)
Benylin®	Guaifenesin 100 mg/5 mL (701)
Imuran®	Azathioprine 50 mg*

Table 1. The active ingredients in each of the medicines analyzed, together with the disclosed amount and the average match factors to the library entries.

**Azathioprine was not present in the library and the measured spectra were used to create a library entry.*

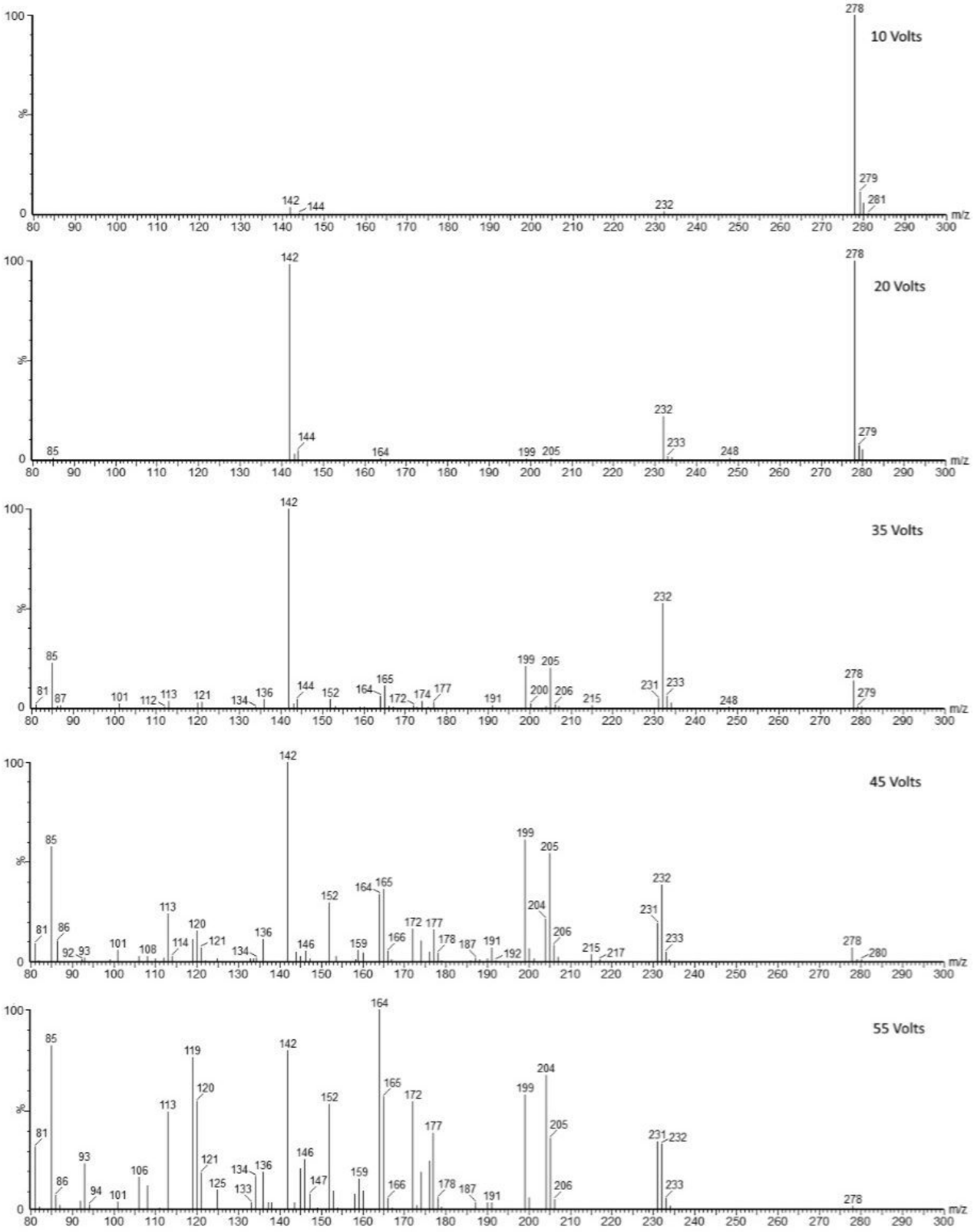


Figure 1. ESI+ spectra for azathioprine (precursor mass m/z 278) on the ACQUITY QDa at the following cone voltages: 10V, 20V, 35V, 45V, and 55V.

An example of the information available in the browser of the ChromaLynx Application Manager is shown in Figure 2 for the analysis of the LEMSIP MAX Cold and Flu Remedy.

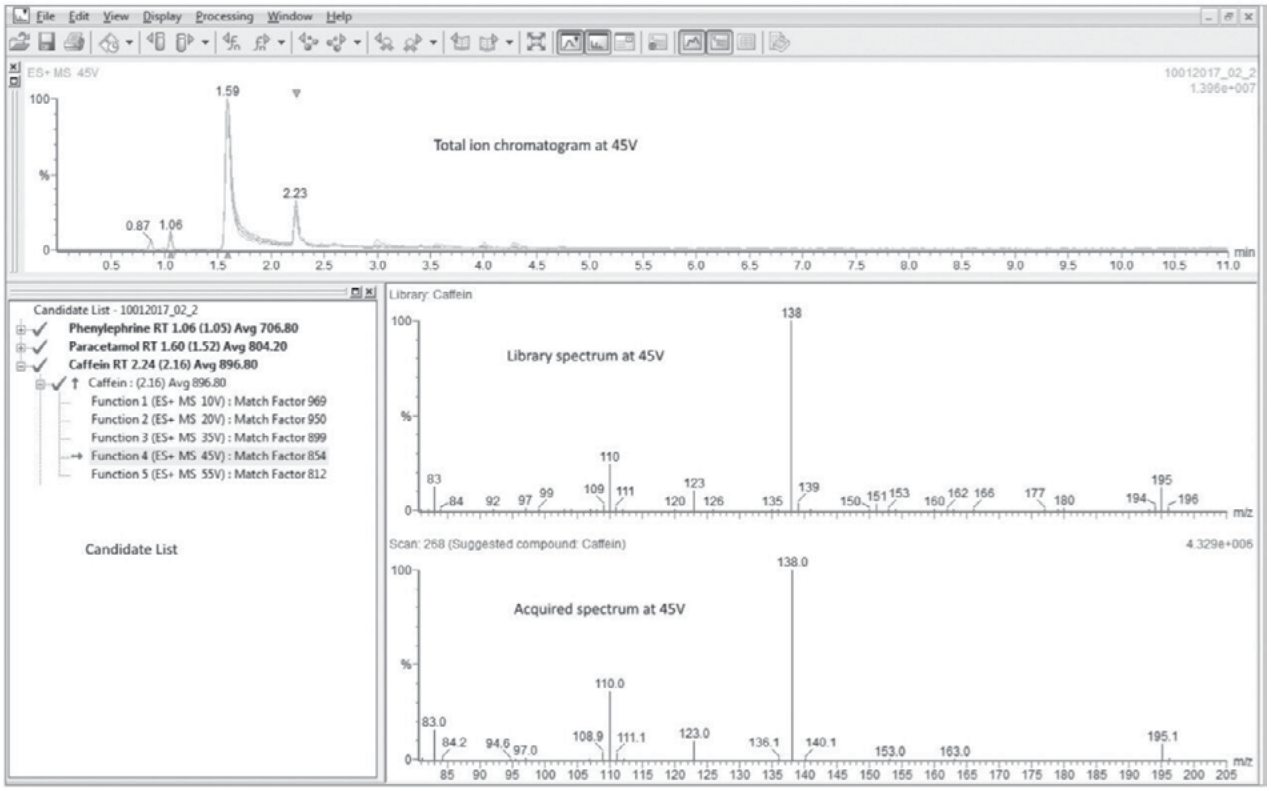


Figure 2. The ChromaLynx browser displaying the results of the analysis of the LEMSIP Max Cold and Flu Remedy, highlighting the identification of caffeine with a precursor ion at m/z 195.

Conclusion

In this study, a series of representative medicines were used to assess the feasibility of applying an existing toxicology library to the ACQUITY QDa Mass Detector. Application of an established chromatographic method together with the qualitative screen demonstrated very good agreement between library spectra and acquired data, leading to the identification of the active ingredients in the medicines. Therefore, the modified toxicology library, in combination with the ACQUITY QDa, appears promising as a low-cost solution for forensic chemistry and drug control testing.

References

1. Screening of Xenobiotics by Ultra-Performance Liquid Chromatography-Mass Spectrometry Using In-Source Fragmentation at Increasing Cone Voltages: Library Constitution and an Evaluation of Spectral Stability, L. Humbert, F. Grisel, C. Richeval and M. Lhermitte, *Journal of Analytical Toxicology* 2010; 34: 571–580.
2. Systematic Toxicological Screening Using the ACQUITY UPLC I-Class/Xevo TQ-S micro, R. Lee and M. Wood, 720005661EN, 2016.
3. ACQUITY QDa Detector Brochure. 720004632EN.
4. Postmortem Drug Screening by Non-Targeted and Targeted Ultra-Performance Liquid Chromatography-Mass Spectrometry Technology, T. Rosano, M. Wood, and T. Swift, *Journal of Analytical Toxicology* 2011; 35: 411–423.

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

ACQUITY QDa Mass Detector <https://www.waters.com/134761404>

720006004, May 2017

Waters™

Application Note

Systematic Toxicological Screening Using the ACQUITY UPLC I-Class/Xevo TQ-S micro

Robert Lee, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

To evaluate the performance of previously published methodology using the Xevo TQ-S micro.

Benefits

A simple, sensitive UPLC-MS method for forensic toxicology screening of compounds in various biological matrices.

Introduction

Forensic toxicology laboratories require reliable screening techniques that can detect a wide variety of toxicants in highly complex biological matrices, such as ante and postmortem specimens. The original Waters systematic toxicological screening method used the Waters Alliance 2695 Separations Module in conjunction with the Waters/Micromass ZQ Single Quadrupole Mass Spectrometer.[1] In 2009, this approach was migrated to the ACQUITY TQD System to deliver the same comprehensive toxicological screening capabilities in half the time.[2] The solution was further developed over subsequent years to provide a full scan screening method and associated toxicology libraries, capable of screening for >950 drug substances and metabolites in 15 minutes. This method has been successfully and routinely used in toxicology laboratories worldwide.[3,4] Owing to the popularity of this methodology, in 2013, this solution was transferred to the ACQUITY UPLC I-Class System and Xevo TQD.[5] The release of the Xevo TQ-S micro allows for further evolution of this successful solution.[6]

Experimental

Test substance

Bio-Rad S10 Liquichek Urine Toxicology Quality Control human urine was obtained from Bio-Rad, Hemel Hempstead, UK.

Sample preparation

The reference urine (250 µL) was extracted using a simple liquid–liquid extraction protocol. Following removal of the upper organic layer and evaporation of the organic solvent, samples were reconstituted in 50 µL of mobile phase A and transferred to a Waters Total Recovery vial.

LC conditions

System:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC HSS C_{18}, 100Å, 1.8 µm, 2.1 mm x 150 mm
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	10 µL
Wash solvent:	Acetonitrile/water (95:5 v/v)
Purge solvent:	5 mM ammonium formate pH 3.0
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM ammonium formate pH 3.0
Mobile phase B:	Acetonitrile containing 0.1% formic acid

MS conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI+
Capillary voltage:	3.0 KV

Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/Hr
Cone gas:	20 L/Hr
Cone voltages:	50 V to 125 V in 15 V increments (preconfigured in provided MS method)
Acquisition range:	*m/z* 80–650

Results and Discussion

Combining the ACQUITY UPLC I-Class System with the Xevo TQ-S micro allows this established UPLC-MS screening methodology to be used on the latest generation of Waters mass spectrometers.

Figure 1. ACQUITY UPLC I-Class System and Xevo TQ-S micro.

The technique uses in-source collision induced fragmentation at various cone voltages followed by library matching using the ChromaLynx Application Manager. Previous analysis of mixtures of drug substances using the Xevo TQ-S micro indicated that the cone voltages required to produce comparable fragmentation patterns were higher than those used with the previous generation mass spectrometers (e.g. Xevo TQD), therefore modified libraries were prepared and evaluated. Figure 2 shows a comparison of spectra obtained on the two platforms, highlighting the additional 30 V applied to the cone for each function on the Xevo TQ-S micro.

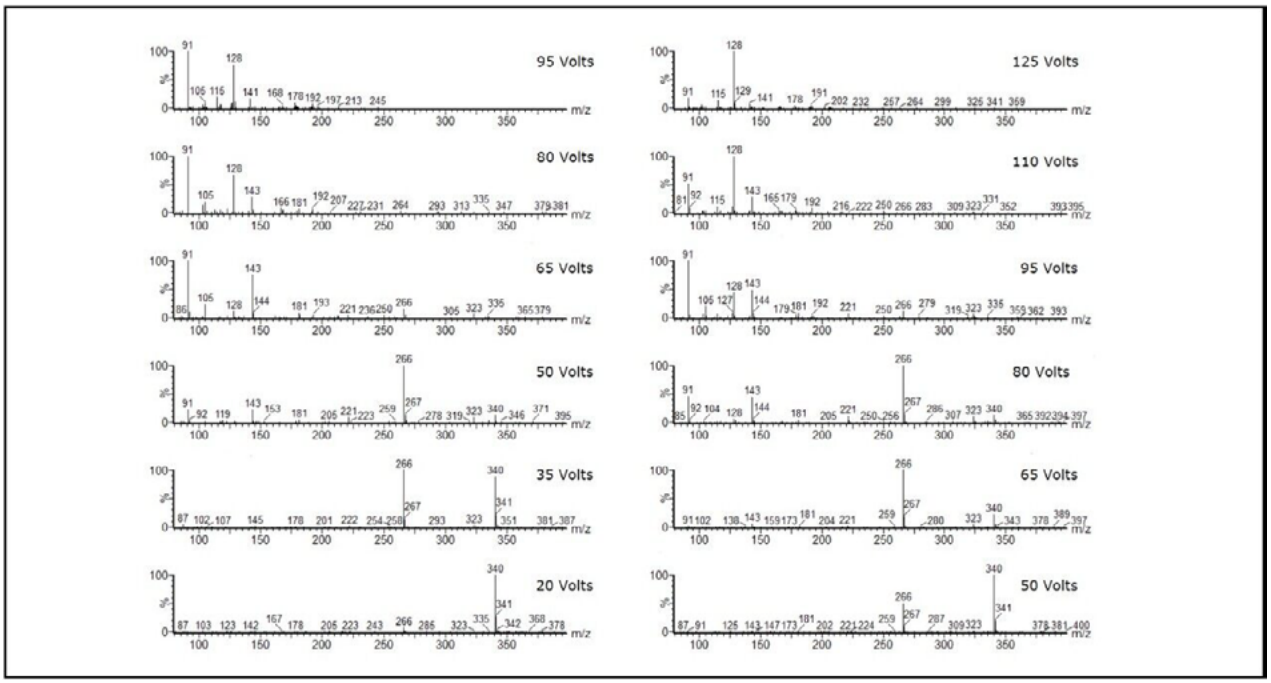


Figure 2. Comparison between the fragmentation patterns obtained using the Xevo TQD (left panel) and the Xevo TQ-S micro (right panel) for propoxyphene in the Bio-Rad S10 Liquichek Urine Toxicology Quality Control reference urine. the Xevo TQD (left panel) and the Xevo TQ-S micro (right panel) for propoxyphene in the Bio-Rad S10 Liquichek Urine Toxicology Quality Control reference urine.

A selection of the information available in the ChromaLynx results browser for the extracted urine sample is shown in Figure 3.

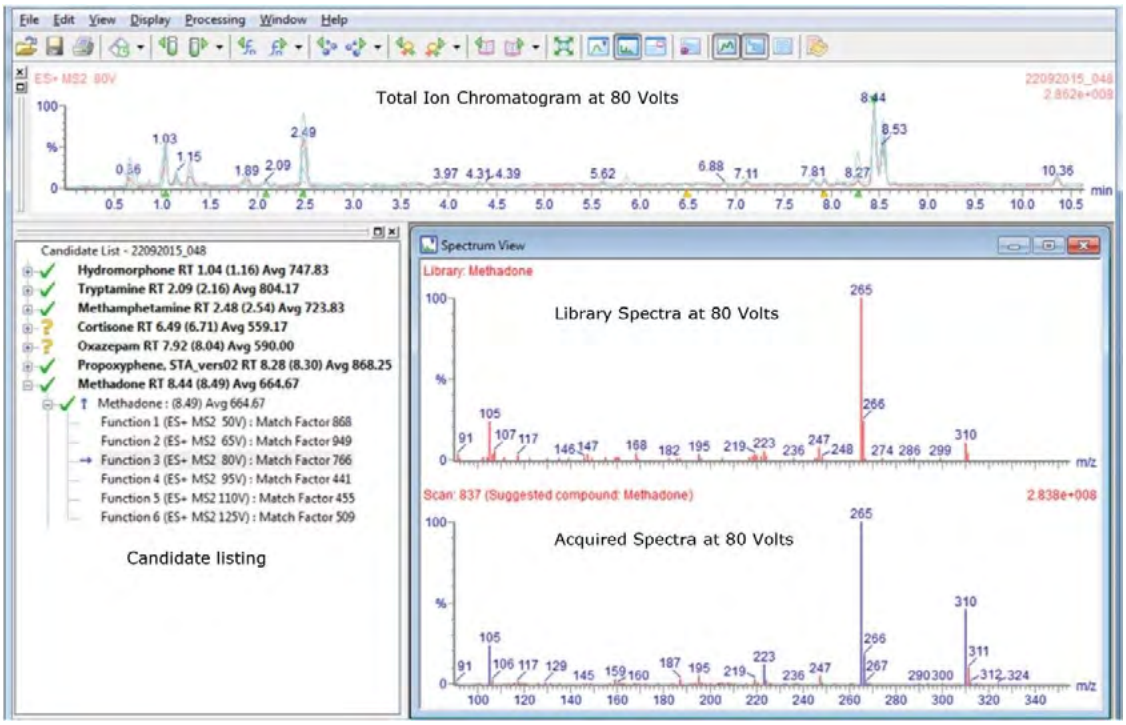


Figure 3. ChromaLynx browser displaying selected information for the analysis of the Bio-Rad S10 Liquichek Urine Toxicology Quality Control urine, highlighting the identification of methadone.

Conclusion

The Xevo TQ-S micro is a tandem mass spectrometer designed to provide rapid, reliable, and reproducible data to deliver consistent low levels of quantitatio over a wide dynamic range. We have shown that the highly successful systematic toxicological screening method can be transferred to the Xevo TQ-S micro by altering the acquisition method to take into account the different energy applied in the source. In conjunction with amended libraries, the Xevo TQ-S micro platform performs to the same high level as previous Waters MS platforms. The Xevo TQ-S micro is a highly versatile instrument for use in toxicology, providing the user with both broad qualitative full scan MS and targeted MRM-based screening capabilities as well as high sensitivity quantitative detection on the same instrument platform.

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6. Xevo TQ-S micro Product Brochure. 2014. Waters Marketing Brochure, 720005046EN.

This is a proof of principle demonstration of an analytical method, which may include examples of typical results that can be achieved with the stated configuration. This method represents a basic starting point from which users should perform their own in-house validation.

Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Xevo TQ-S micro Triple Quadrupole Mass Spectrometry <https://www.waters.com/134798856>

Xevo TQD Triple Quadrupole Mass Spectrometry <https://www.waters.com/134608730>

ChromaLynx <https://www.waters.com/513759>

720005661, March 2016

Waters™

Application Note

Targeted MRM Screening Using the ACQUITY UPLC I-Class/ Xevo TQ-S micro

Robert Lee, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This technical brief has highlighted the increased response of the Xevo TQ-S micro when using a preconfigured targeted MRM qualitative screening method; when compared to data collected from the same samples on the Xevo TQD an improvement in the number of true positives was observed. The ACQUITY UPLC I-Class/Xevo TQ-S micro is a highly-versatile instrument for use in toxicology, providing the user with both broad qualitative screening capabilities as well as high sensitivity quantitative detection on the same instrument platform.

Benefits

A simple, sensitive UPLC-MS/MS method for targeted forensic toxicology screening of compounds in various biological matrices.

Introduction

Forensic toxicology laboratories require reliable screening techniques that can detect a wide variety of toxicants in highly complex biological matrices, such as ante and postmortem specimens. The Waters targeted toxicology screening application using the ACQUITY TQD System was released in 2009.[1] This approach has been used by Rosano ***et al*** to compare screening methodologies for postmortem blood samples.[2] Following its success, this solution was transferred in 2013 to the ACQUITY UPLC I-Class and Xevo TQD system.[3] The release of the Xevo TQ-S micro allows for further evolution of this solution.[4]

Figure 1. ACQUITY UPLC I-Class and Xevo TQ-S micro configuration.

Combining the ACQUITY UPLC I-Class with the Xevo TQ-S micro allows this established UPLC-MS/MS screening methodology to be used on the latest generation of Waters mass spectrometers.

Experimental

Test substances

The following commercial human urine reference controls were obtained: Basis-line U from Medidrug (40201); Blankcheck urine (UR015) and DCT -25% (UR22020A) both from ACQ Science; Urine Toxicology Control DAU HC2 (50701) from UTAK; and the following Liquichek Urine Toxicology Quality Controls from Bio-Rad: Negative Control (460), C2 (442), and S10 (673).

Sample preparation

The commercial reference urines were diluted 5-fold with mobile phase A and vortex-mixed. Following centrifugation the supernatant was transferred to a Waters Maximum Recovery vial and triplicate injections were analyzed.

LC Conditions

System:	ACQUITY UPLC I-Class with FTN
Column:	ACQUITY UPLC HSS C_{18}, 100A, 1.8 µm, 2.1 mm x 150 mm (P/N 186003534)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Wash solvent:	Acetonitrile/water (95:5 v/v)
Purge solvent:	5 mM ammonium formate pH3.0
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM ammonium formate pH3.0
Mobile phase B:	Acetonitrile containing 0.1% formic acid

MS conditions

System:	Xevo TQ-S micro
Ionization mode:	ESI+
Capillary voltage:	3.0 KV

Source temp.:	150 °C
Desolvation temp.:	400 °C
Desolvation gas:	800 L/Hr
Cone gas:	20 L/Hr
Cone voltages:	Preconfigured in provided MRM method
Collision energies:	Preconfigured in provided MRM method

Results and Discussion

The data was collected using the supplied MRM method which contains two transitions (qualifier and quantifier) per compound, with associated preconfigured parameters for cone voltage and collision energies for 178 compounds. The three negative control reference urines (Basis-line U, Blankcheck, and Negative Control) and four positive control reference urines (C2, S10, DAU HC2, and DCT -25%) containing certified levels of analytes, were assayed using the method described above. The data was automatically processed using the TargetLynx Application Manager, following a slight increase in the area threshold reject parameter, as a result of the increased response of the TQ-S micro. Screening results were compared for equivalence to the data obtained from the Xevo TQD platform.

A number of compounds were detected in the negative control reference urines on both platforms, i.e. caffeine and other substances associated with over-the-counter medications, which are routinely detected in urine screens.

For the certified positive control reference urines, both platforms detected the same number of expected compounds in the S10 reference urine. The Xevo TQ-S micro also found the same analytes as the Xevo TQD in the C2 and DAU HC2 urine samples, but in addition was able to detect α-hydroxyalprazolam in the C2 urine and lorazepam in the DAU HC2 urine.

Additional sensitivity for the benzodiazepines was also confirmed through analysis of the ACQ Science DCT -

Additional sensitivity for the benzodiazepines was also confirmed through analysis of the ACQ Science DCT -25% sample. Figure 2 details five additional benzodiazepines which were detected using the Xevo TQ-S micro. This commercial reference urine has certified levels of analytes at a concentration equivalent to 25% lower than the maximum cut-off concentration currently recommended by the European Workplace Drug Testing Society (EWDTS) for confirmation tests in urine;[5] the benzodiazepines detected here are present in this urine at 75 ng/mL.

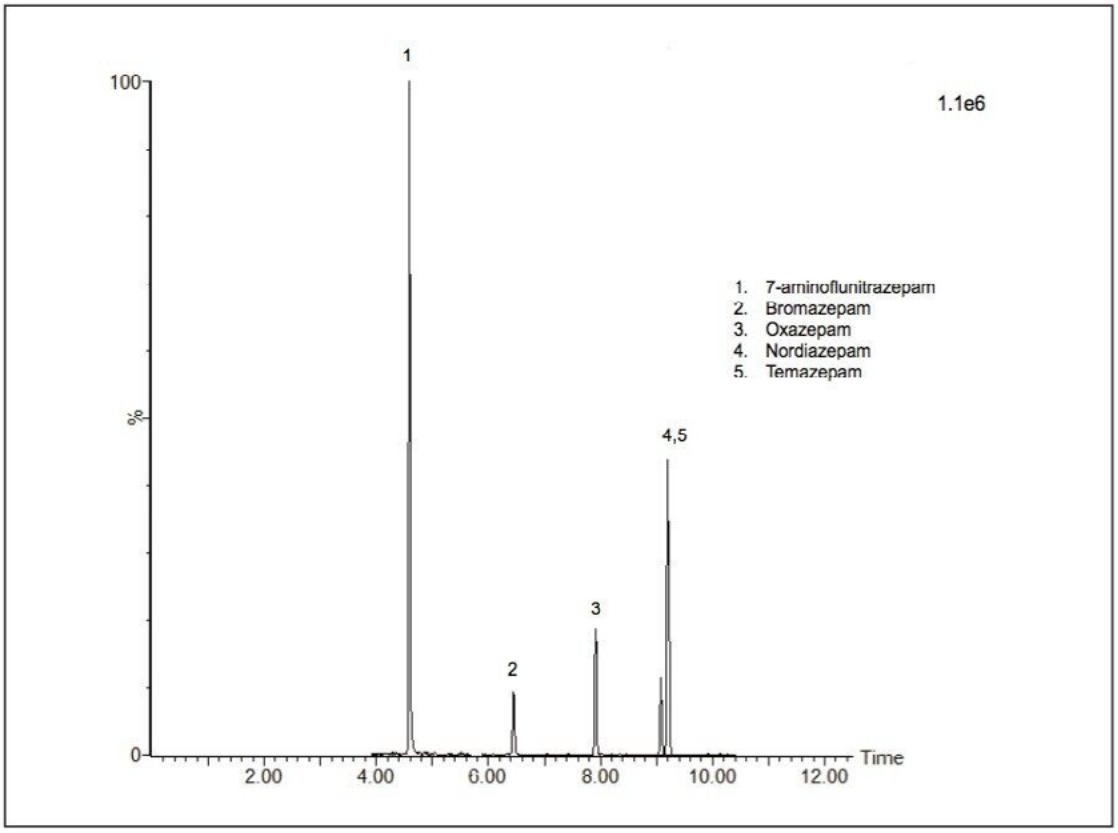

Figure 2. Chromatogram showing benzodiazepines in the ACQ DCT -25% commercial reference urine detected by the Xevo TQ-S micro using the supplied targeted MRM method but not the Xevo TQD. The quantifier ion transition is displayed.

The average number of scans per function has increased as the method has evolved from the ACQUITY TQD System through the ACQUITY UPLC I-Class/Xevo TQD and now to the ACQUITY UPLC I-Class/Xevo TQ-S micro; because the dwell time (10 msec) in the supplied MRM method has not changed this increase can be attributed to the improvements in electronic design that have accompanied each new MS platform. This increased number of scans per function improves precision, reproducibility, and sensitivity.

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4. Xevo TQ-S micro Product Brochure. 2014 Waters Marketing Brochure, 720005046EN.
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Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

Xevo TQ-S micro Triple Quadrupole Mass Spectrometry <https://www.waters.com/134798856>

Quadrupole MS Solutions for Forensic Laboratories <https://www.waters.com/10011568>

TargetLynx <https://www.waters.com/513791>

ACQUITY UPLC Columns <https://www.waters.com/513206>

Available for Purchase Online

ACQUITY UPLC HSS C18 Column, 100Å, 1.8 µm, 2.1 mm X 150 mm, 1/pkg <
https://www.waters.com/waters/partDetail.htm?partNumber=186003534>

720005606, February 2016

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Application Note

Systematic Toxicological Analysis (STA) Using the ACQUITY QDa Mass Detector - Part 1: An introduction to using mass detection in forensic toxicology screening

Robert Lee, Nayan S. Mistry, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief demonstrates to evaluate the Waters STA methodology on the ACQUITY QDa Mass Detector to facilitate screening of toxicologically relevant compounds in various biological matrices.

Benefits

In the first of a two-part series on STA using the ACQUITY QDa Mass Detector, we review the strengths and weaknesses of traditional screening methods and describe a simplified screening technique for the detection of toxicologically-relevant analytes in biological matrices.

Introduction

General unknown screening or systematic toxicological analysis (STA) is an essential element of the workflow applied within forensic toxicology laboratories. The main purpose of this initial screening technique is to identify putative positive samples, i.e., those containing relevant drug substances, while simultaneously eliminating negative specimens from any subsequent analytical interrogation. Typically, urine and plasma/serum are the specimens of choice.

Traditional techniques have included immunoassay (IA), gas chromatography in conjunction with mass spectrometry (GC-MS), and liquid chromatography (LC) with ultraviolet (UV) or photodiode array (PDA). Although commonly used, each of these techniques have some significant limitations, which are summarized in Table 1.

Technique		Strengths	Weaknesses
Immunoassay		Quick and simple as no chromatographic separation required	Not a fully comprehensive screen; multiple immunoassays required for each specimen - can be costly Class-specific test only (e.g., reports 'Benzodiazepine Positive' but does not indicate which specific Benzodiazepine drug is present) Not available for all drugs/drug classes (e.g., NPS) Cross-reactivity for NPS with common immunoassays is unclear or variable Some immunoassays are subject to false positive or false negative results
Gas Chromatography	Mass Spectrometry	Chromatographic separation – good for biological samples and provides reference retention time (RT) for use in identification process Availability and reproducibility of mass spectral libraries based on electron impact (EI) ionization is beneficial. Identification based on mass to charge (*m/z*) of the precursor and fragment ions Retrospective data interrogation is possible	Generic chromatographic conditions need to be applied for a comprehensive screen – may not be optimal conditions for all analytes To achieve appropriate sensitivity chemical derivatization is commonly required. Different derivatization protocols may have to be applied to each specimen Thermolabile analytes such as benzodiazepines are not amenable to the high temperatures applied in GC Poor sensitivity for polar drugs, metabolites and non-volatile substances Degradation products and remaining derivatization compounds can complicate interpretation of spectral data
Liquid Chromatography	Mass Spectrometry	Direct analysis – chemical derivatization is not required LC amenable to widest range of analytes Chromatographic separation – good for complex biological samples and provides a reference RT for use in identification Availability of mass spectral libraries is beneficial and provides high specificity Electrospray is a 'soft' ionization technique and yields an abundance of structural information – identification is based on matching *m/z* of precursor and fragment ions	Generic chromatographic conditions need to be applied for a comprehensive screen – may not provide optimal conditions for all analytes
Liquid Chromatography	UV or PDA detection	Direct analysis – chemical derivatization is not required LC amenable to widest range of analytes Chromatographic separation – good for complex biological samples and provides a reference RT for use in identification Availability of UV or PDA spectral libraries is beneficial	Single spectra used for identification – poor sensitivity and specificity especially in complex mixture and matrices

Table 1. A comparison of the strengths and weaknesses of various screening techniques.

In brief, immunoassays commonly generate class-specific screening results, thus multiple kits are required to provide information for the main drug classes. In addition, assays are not available for all drugs and drug classes, therefore this approach provides a very restricted screen. False positive and false negative identifications are also a significant, and well-documented, disadvantage of immunoassays.[1]

For many years, gas chromatography coupled with mass spectrometry was considered the "gold-standard" comprehensive screen and the mainstay of many toxicology laboratories. The availability of large commercial libraries based on electron impact (EI) ionization containing data for xenobiotics is certainly beneficial to the user; EI is considered to be a hard ionization technique and some analytes may be poorly detected owing to excessive fragmentation. In order to improve sensitivity, chemical derivatization is often used, which is both time-consuming and can bring additional problems. For these reasons, over the last two decades, liquid chromatographic techniques have been steadily replacing GC.

In contrast to GC, LC permits direct analysis of the drug substances without the need to chemically alter the molecule and is suitable for a wider range of drugs. Techniques based on the combination of LC and MS offer a simpler, yet more sensitive approach. Mass spectrometry detection also offers superior selectivity over PDA or UV detection and provides analyte specific detection rather than the class-specific approach of immunoassay. These advantages combine to make LC-MS the ideal tool for screening in the forensic toxicology market.

Waters first introduced the combination of LC with MS detection for STA in 2007.[2] Over the intervening years, this methodology evolved to reflect the advancements in technology available from Waters and users can now search against a spectral library containing 1200 compounds.[3-5]

Waters introduced the ACQUITY QDa Mass Detector in 2013. This instrument has already been shown to be applicable to both pharmaceutical and illicit drug identification.[6]

In this technology brief, we describe the application of the same approach to biological samples for the purposes of comprehensive toxicological screening.

Results and Discussion

Screening Method

LC-MS using electrospray ionization (ESI) is ideally suited to polar, non-volatile, and thermally unstable compounds, and therefore provides a powerful means of identifying many toxicologically-relevant

compounds rapidly without the need for sample derivatization, unlike GC-MS. Electrospray is a soft ionization technique that mainly leads to protonated molecules in positive mode and to deprotonated molecules in negative mode. In order to get more specific structural information, it is possible to induce fragmentation of these molecular ions in the source region of the QDa. This can be achieved by increasing the voltage applied to the sampling cone. Molecular ions then collide with neutral molecules in the source region and fragment into characteristic ions. This is referred to as in-source collision-induced dissociation (CID). Using in-source CID it is possible to generate mass spectra of different fragmentation patterns according to the value of cone voltage applied in the source (see Figure 1). Through this process, reproducible LC-MS mass spectra can be used to produce a library of mass spectra.

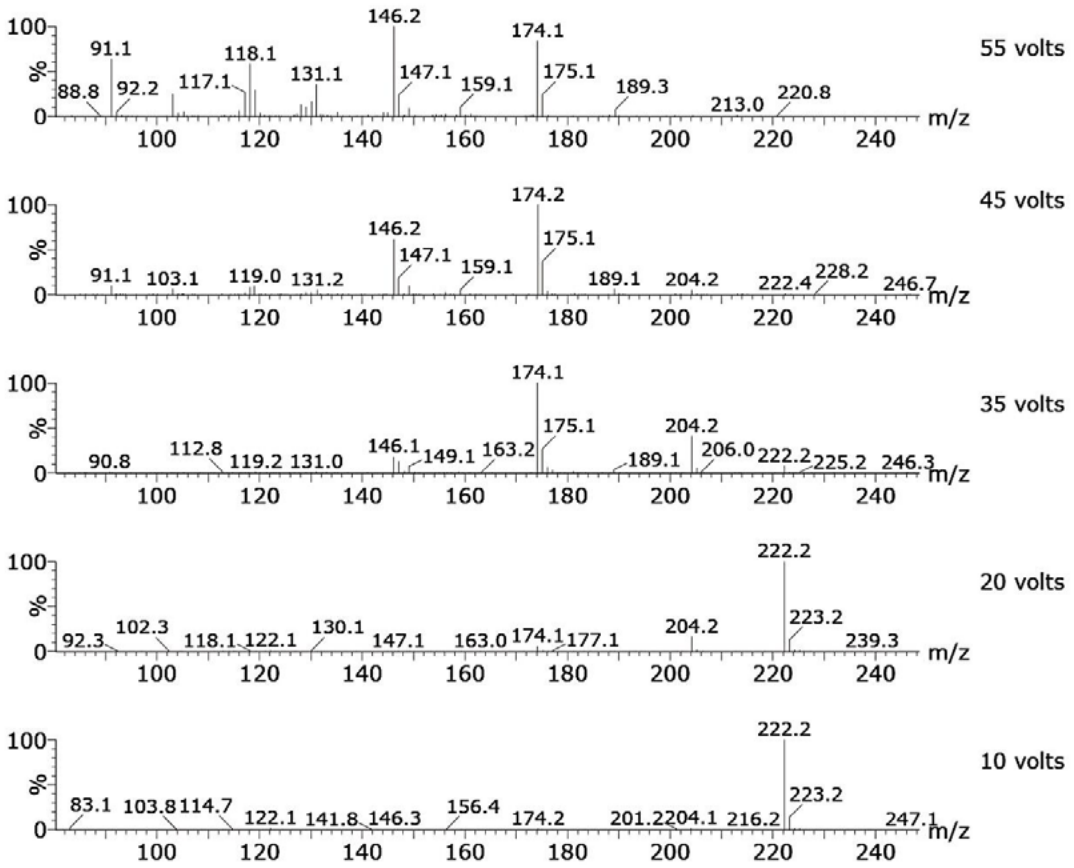

Figure 1. Spectra showing the in-source CID fragmentation pattern obtained for the Novel Psychoactive Substance (NPS) ethylone (bk-MDEA) at 200 ng/mL when different voltages are applied to the cone.

The ACQUITY QDa Mass Detector from Waters requires no specialist training or expertise to set up and all

acquisition and processing methods used here are available free of charge from Waters Marketplace. It is the only mass detector that integrates with, and even fits on top of, your instrument stack. It uses less bench space and less energy than a traditional mass spectrometer. Cleaning and routine maintenance are minimal, thereby maximizing your uptime. It can be integrated with other Waters software platforms and detection techniques, such as UV or PDA.

ChromaLynx is an application manager within MassLynx that allows for deconvolution of the mass spectral data and automatically processes and compares the data to the preconfigured library (containing 1200 compounds), which provides an identification through spectral library matching. The confidence with which a substance is identified is presented as an average match factor, which has a maximum value of 1000. All compounds are required to elute within 0.35 min of the retention time in the supplied library. Customers can create their own libraries from reference material or from authentic material data. Further, it is also possible to re-process previously acquired data, with updated libraries, without the need to prepare fresh samples.

Results

One hundred toxicologically-relevant analytes were spiked into both control human urine and human plasma at 200 ng/mL and prepared using a simple Oasis PRiME HLB µElution method and analyzed using the Waters toxicology STA screening method on a Waters ACQUITY QDa Mass Detector.[7]

In this study, a positive result was achieved by having an average match factor of >700; an example of the results obtained using ChromaLynx of a mixture of compounds spiked into human urine at 200 ng/mL is shown in Figure 2.

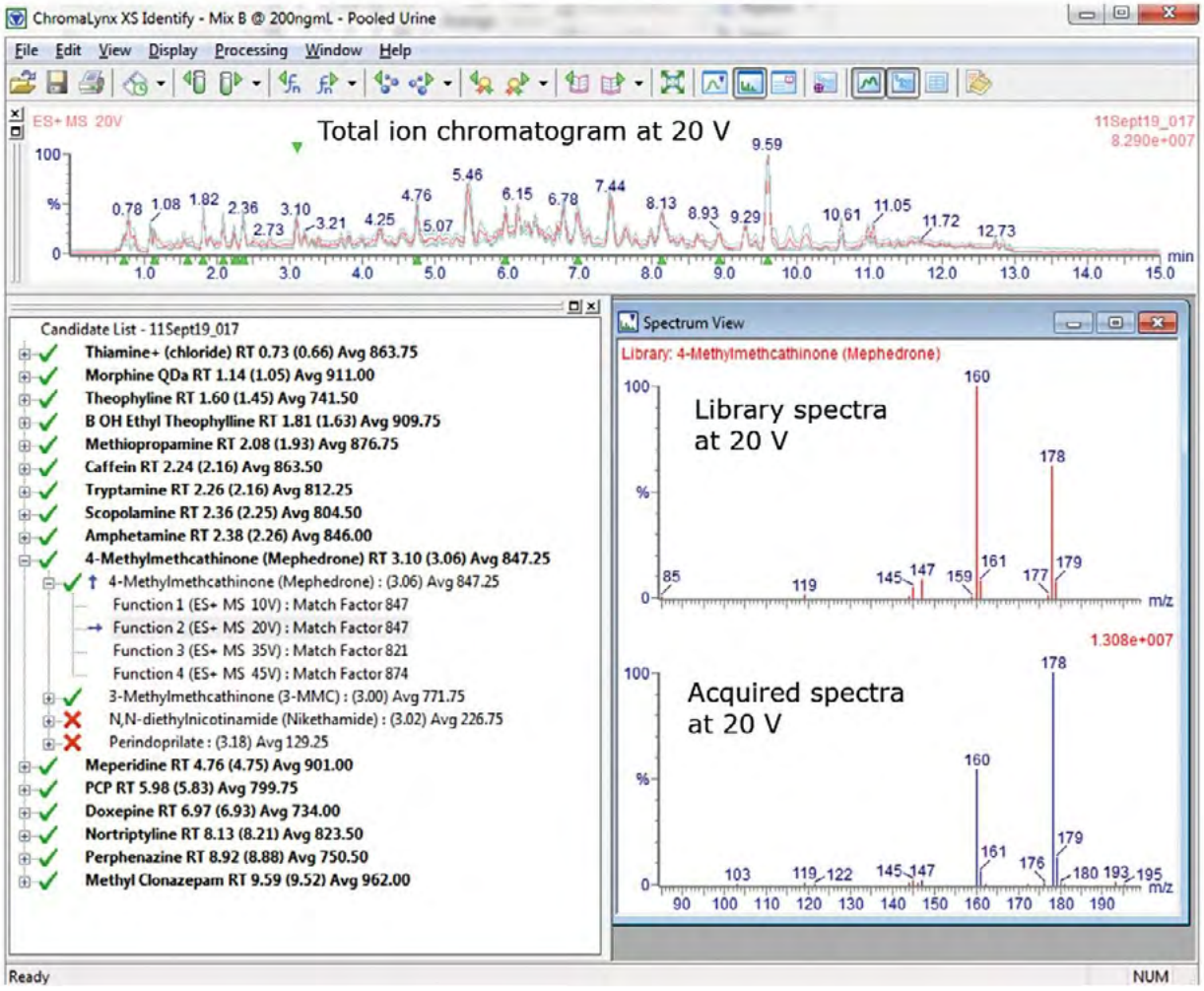

Figure 2. ChromaLynx results browser showing the analysis of a mixture of compounds spiked into human urine at 200 ng/mL, highlighting the identification of 4-methylmethcathinone (mephedrone).

Conclusion

In this technology brief a combination of liquid chromatography and ACQUITY QDa Mass Detection has been shown to provide a quick, simple, and effective way to screen for toxicologically-relevant compounds in various biological matrices. This technique offers benefits of cost savings, speed, and specificity over other established screening techniques such as immunoassay, GC-MS, and LC-UV. The same system can also be used to provide quantitative data as described by Mistry et al.[8]

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Featured Products

ACQUITY QDa Mass Detector <https://www.waters.com/134761404>

ChromaLynx <https://www.waters.com/513759>

720006725, January 2020

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Application Note

Systematic Toxicological Analysis (STA) Using the ACQUITY QDa Mass Detector- Part 2: Evaluation of a fast and simple OASIS PRiME SPE method for xenobiotics in biological samples

Nayan S. Mistry, Robert Lee, Michelle Wood

Waters Corporation

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief describes the application of a simple sample preparation method to allow for the screening of toxicologically-relevant compounds spiked into human urine or plasma using UPLC separation and Waters ACQUITY QDa Mass Detection.

Benefits

In the second of a two-part series, we present a simple solid-phase extraction (SPE) protocol for the preparation of compounds in biological matrices and its use with the Waters STA screening method and the ACQUITY QDa Mass Detector.

Introduction

The success of any LC-MS screening method is dependent on the use of a sample preparation protocol, which is efficient and robust, yet will extract as many analytes as possible from the matrix. Waters Oasis PRiME HLB has been designed to be simple and quick, eliminating the need for sorbent preconditioning and equilibration, while allowing for a more rapid workflow and cleaner extracts than traditional sample preparation solutions.

In this application brief, we describe the use of Oasis PRiME HLB in conjunction with UPLC separation and the ACQUITY QDa Mass Detector using the Waters STA screening application.[1-3] Since its introduction over a decade ago, the associated library has been expanded to contain 1200 toxicologically-relevant substances. The purpose of the current work was to evaluate the feasibility of using the STA methodology with the ACQUITY QDa Mass Detector (Figure 1) to provide a simple, sensitive, low-cost qualitative screening tool for use in forensic toxicology laboratories for the determination of toxicologically-relevant substances in biological matrices.

Figure 1. ACQUITY UPLC H-Class PLUS System and ACQUITY QDa Mass Detector.

Experimental

Materials

Reference material for 100 analytes (Table 1) was obtained from Sigma-Aldrich (Poole, UK); typically, the individual drugs were supplied at a concentration of 1 mg/mL. Six different sources of urine were obtained from volunteers (Wilmslow, UK) and six different sources of plasma (collected using sodium fluoride/potassium oxalate tubes) were purchased from BIOIVT (West Sussex, UK).

Acetylcodeine	Cocaine	MDMA	Perphenazine
Alprazolam	Codeine	bkMDMA	Phencyclidine (PCP)
Alprenolol	Cotinine	MDEA	Phenmetrazine
Amiloride	Diazepam	bkMDEA	PMMA
Amiodarone	Diazepam, Nor	Meperidine	Prazepam
Amitriptyline	Dihydrocodeine	Meperidine, Nor	Procyclidine
Amphetamine	Doxapram	Mephedrone (4-MMC)	Propranolol
Atenolol	Doxepine	Meprobamate	Propoxyphene
Atropine	Ecgonine Methyl Ester	Methadone	Protriptyline
Benzoylecgonine	EDDP	Methamphetamine	Ramipril
Brompheniramine	Ephedrine	Methiopropamine (MPA)	Ranitidine
Buprenorphine	Fentanyl	Midazolam	Risperidone, Hydroxy
Caffeine	Fentanyl, Nor	Morphine	Scopolamine
Cetirizine	Flunitrazepam	Morphine, 6 Mono Acetyl	Sertraline
Chlordiazepoxide	Fluoxetine	Nadolol	Sildenafil
Chloroquine	Flurazepam	Naltrexone	Sotalol
Chlorpheniramine	Gliclazide	Nitrazepam	Temazepam
Citalopram	Haloperidol	Norbuprenorphine	Thioridazine
Citalopram, Desmethyl	Hydrocodone	Nortriptyline	Timolol
Clobazam	Hydromorphone	Oxazepam	Tramadol
Clonazepam	Ketamine	Oxprenolol	Trazadone
Clonazepam, 7-Amino	Ketamine, Nor	Oxycodone	Triprolidine
Clozapine	Lormetazepam	Oxymorphone	Venlafaxine
Clozapine, Desmethyl	Maprotiline	Paracetamol	Verapamil
Cocaethylene	MDA	Paroxetine	Zopiclone

Table 1. List of 100 compounds evaluated.

Spiked Matrix Preparation

Analytes were combined into several mixtures (each mix containing a maximum of 10 analytes) to yield mixed spiking solutions at a concentration of 25 µg/mL in acetonitrile.

Each mixture was spiked individually into the blank matrices to give final concentrations of 200 and 500 ng/mL.

Sample Preparation- Fast, Simple Solid- phase Extraction (SPE)

Spiked matrix (150 µL) was added to 150 µL de-ionized water and the sample was vortex-mixed for 60 sec and centrifuged. Two-hundred microliters of the spiked matrix/ water mix was loaded onto the Oasis PRiME HLB µElution plate (p/n: 186008052). After loading, the wells were washed with 200 µL of 5% methanol. The analytes were eluted with 2 × 50 µL of acetonitrile/methanol (90/10, v/v) containing 1% formic acid into a 800-µL roundwell collection plate (p/n: 186002481).

The samples were evaporated to dryness under a stream of nitrogen at 50 °C using a Porvair sample concentrator and reconstituted in 40 µL of 5 mM ammonium formate pH 3.0 containing 10% acetonitrile. The

collection plate was covered with a Waters silicone/PTFE-treated cap mat and placed on a multi-tube vortex mixer for 3 min. Figure 2 shows a schematic that illustrates the extraction protocol used for sample preparation.

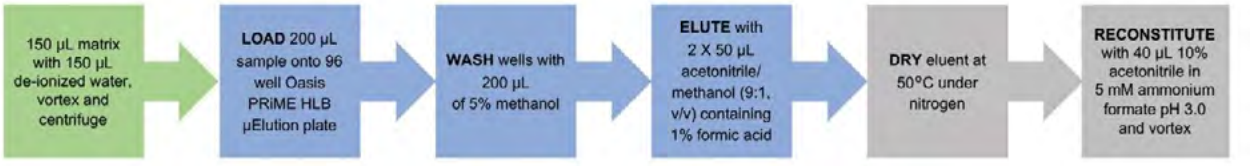

Figure 2. PRiME HLB workflow protocol used for sample preparation.

Screening Method

Samples were analyzed using the previouslydeveloped Waters STA screening method, which had been modified to be used in combination with the Waters ACQUITY UPLC H-Class PLUS System and ACQUITY QDa Mass Detector.[2]

Chromatographic separation was achieved using a 15-min gradient elution. Data were acquired in full scan mode at multiple cone voltages, to generate spectral data in positive mode using in-source collision-induced dissociation (CID). Tables 2 and 3 list the LC conditions and the ACQUITY QDa settings respectively.

Time (min)	Flow (mL/min)	%B	Curve
Initial	0.400	10	Initial
10.00	0.400	50	6
10.75	0.400	95	6
12.25	0.400	95	6
12.50	0.400	10	6
15.00	0.400	10	6

Table 2. LC gradient profile, which involves a 300-µL precolumn volume.

Parameters	Settings
Polarity	ESI+
Acquisition range (*m/z*)	80–650
Acquisition time (min)	15
Probe temperature (°C)	600
Source temperature (°C)	120
Cone voltage (V)	10, 20, 35, and 45
Capillary (kV)	0.8

Table 3. ACQUITY QDa conditions.

Results and Discussion

The spiked samples prepared by the described Oasis PRiME HLB µElution method were analyzed using the Waters STA screening method on an ACQUITY QDa Mass Detector. Acquired data was automatically processed using the ChromaLynx Application Manager (with MassLynx Software) and compared with a prepared library, which provides an identification through spectral library matching. The confidence with which a substance is identified is presented as an average match factor, which has a maximum value of 1000. The average match factor is determined by comparing the acquired and library spectra for each of the four cone voltages. In this study, putative positive identifications had an average match factor greater than 700, while compounds with an average match factor of between 600 and 700 were classed as tentative identifications. In addition, retention time needed to be within 0.35 min of the reference, as specified in the Waters STA library.

In this study, compounds were categorized as detected if they were tentatively or positively identified. At 200 ng/mL, 93% of the investigated analytes could be detected in urine and 98% in plasma. Figure 3 summarizes the total number of detections at 200 and 500 ng/mL in both matrices.

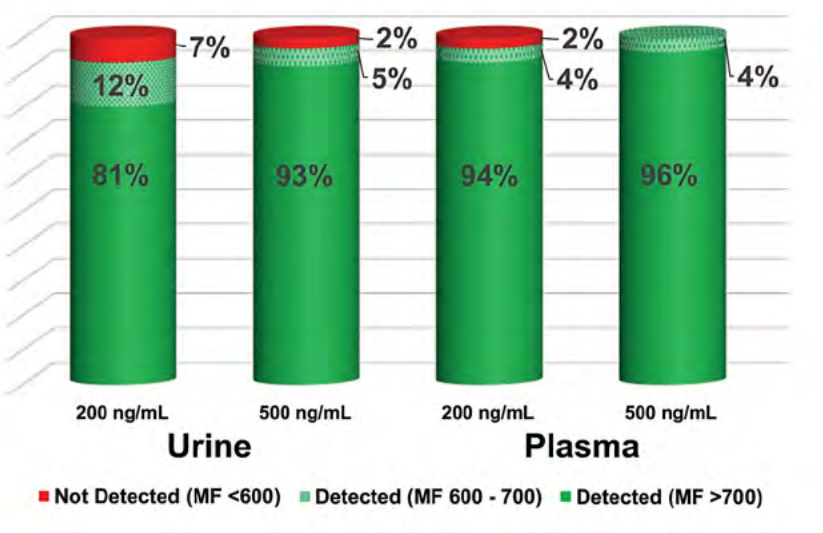

Figure 3. Compound detection summary at 200 and 500 ng/mL in pooled urine and plasma.

Conclusion

In this technology brief, a simple sample preparation method using Oasis PRiME HLB µElution in conjunction with UPLC separation and ACQUITY QDa Mass Detection has been shown to provide a quick, simple, and effective way to screen for toxicologically-relevant compounds in human urine and plasma.

The use of Oasis PRiME HLB µElution in a 96-well plate format will allow for the sample preparation process to be automated and thus increase sample throughput.

The ACQUITY QDa is a low-cost, sensitive, and versatile instrument that can be used to successively detect toxicologically-relevant compounds in biological matrices in combination with UPLC chromatographic separation.

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Featured Products

ACQUITY UPLC H-Class PLUS System <https://www.waters.com/10138533>

ACQUITY QDa Mass Detector <https://www.waters.com/134761404>

ChromaLynx <https://www.waters.com/513759>

MassLynx MS Software <https://www.waters.com/513662>

720006726, January 2020

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Application Note

Evaluation of the ACQUITY UPLC H-Class System with ACQUITY QDa Mass Detector for the Determination of Amphetamine, Methamphetamine, Ketamine, and Norketamine in Human Urine for Forensic Toxicology

Nayan S. Mistry, Robert Lee, Michelle Wood

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

The purpose of this study was to evaluate the ACQUITY UPLC H-Class/ACQUITY QDa System as an alternate technique to potentially replace two commonly applied immunoassays by developing a single, fast analytical method which incorporates a simple sample preparation procedure. Selectivity is achieved by monitoring retention time, precursor and product ion(s), and ion ratios. The method also comprised an analytical range which extended to 2000 ng/mL, offering the user an ability to quantify.

Benefits

The ACQUITY QDa Mass Detector has demonstrated great promise as an alternative technique to immunoassay.

Introduction

Amphetamines (methamphetamine and amphetamine) are among the most commonly abused illicit substances in the world. In 2016, it was estimated that around 35 million adults worldwide used amphetamines.[1] The region with the highest annual use was North America at approximately 2% of the population. In the last few years however, there also appears to be a marked increase in consumption within other geographies, particularly East and Southeast Asia, where the amphetamines have been identified as one of the most worrying threats of drug use. Moreover, a rise in the use of Ecstasy (3,4 methylenedioxymethamphetamine; MDMA) in West Asia has been noted, though it is estimated that less than half of the tablets purported to be Ecstasy, truly contain MDMA, but are commonly found to contain methamphetamine, amphetamine, or ketamine.[2]

Forensic laboratories often utilize immunoassay for routine urine analysis. While the technique is rapid and simple, there can be some disadvantages, often associated with poor selectivity for example, false positive results for amphetamine or ketamine have been reported for a variety of substances.[3-5] As most positive immunoassay results require a confirmatory test (such as GC or LC-MS), the consequence of false positive identifications, as a result of poor assay selectivity, can significantly compromise laboratory efficiency.

The purpose of this study was to evaluate the UPLC-QDa system as an alternate analytical technique for the analysis of two commonly abused drug classes. This technique uses chromatographic separation coupled with a simple mass detector to address the issues of cross-reactivity and laboratory inefficiencies associated with immunoassay.

Figure 1. The ACQUITY UPLC H-Class System and ACQUITY QDa Mass Detector.

Experimental

Materials and experimental

Certified reference standards (1 mg/mL) and their corresponding deuterated internal standards (ISTDs) (100 µg/mL) were purchased from Sigma-Aldrich (Poole, UK; Table 1). A commercial quality control (QC) urine sample (Medidrug WDT Confirm U, -25% cutoff; p/n: 27UQ01KE) containing amphetamine, methamphetamine, and ketamine at 150 ng/mL was purchased from Medichem (Germany). Authentic drug-free urine samples were collected from volunteers and pooled.

To evaluate analytical selectivity and linearity, a series of calibrators ranging from 40 to 2000 ng/mL, were prepared by spiking mixed reference standards into the pooled drug-free urine. A series of in-house QCs (75, 150, 600, and 1600 ng/mL) were prepared from a separate batch of reference material in pooled urine.

Prior to analysis, all samples and QCs were diluted 5-fold in an aqueous solution containing the ISTDs at a concentration of 500 ng/mL.

Chromatographic separation was achieved using a fast 3 min gradient elution on an ACQUITY UPLC BEH C $_{18}$, 1.7 µm, 2.1 × 50 mm Column coupled with an ACQUITY UPLC BEH C_{18}, 1.7 µm, 2.1 x 5 mm VanGuard Pre-Column on the ACQUITY UPLC H-Class System. Data were acquired with the ACQUITY QDa Mass Detector in positive ionization mode applying selected ion recording (SIR). In-source collision-induced dissociation (CID) was applied at specific cone voltages to generate at least one product ion.[6,7]

Compound	Retention time (min)	Precursor and product ions (*m/z*)	Deuterated internal standards	Retention time (min)	Precursor and product ion (*m/z*)
Amphetamine	0.85	**136.1**	Amphetamine - D11	0.82	**147.2**
		91.1			98.1
		119.1			
Methamphetamine	1.03	**150.1**	Methamphetamine - D14	0.99	**164.2**
		91.1			98.1
		119.1			
Ketamine	1.60	**238.1**	Ketamine - D4	1.60	**242.1**
		125.0			129.0
		179.1			
Norketamine	1.50	**224.1**	Norketamine - D4	1.50	**228.1**
		125.0			129.0
		207.1			

Table 1: Analytes and corresponding ISTDs together with target ion(s). Precursor ion is indicated in bold font.

Results and Discussion

Data were acquired using MassLynx Software and processed using the TargetLynx Application Manager. The following criteria were used for a positive identification: presence of the precursor ion together with the product ion(s) at the expected retention time (±0.2 min) and with an ion ratio of ±20% of the expected value. Reference ion ratios were based on the ratio of peak-area response of the precursor to the response of the associated product ion(s), and were calculated from the average obtained across the four QC concentrations.

All compounds demonstrated good linearity across the concentration range investigated (40–2000 ng/mL); Figure 2 shows an example of the response for ketamine.

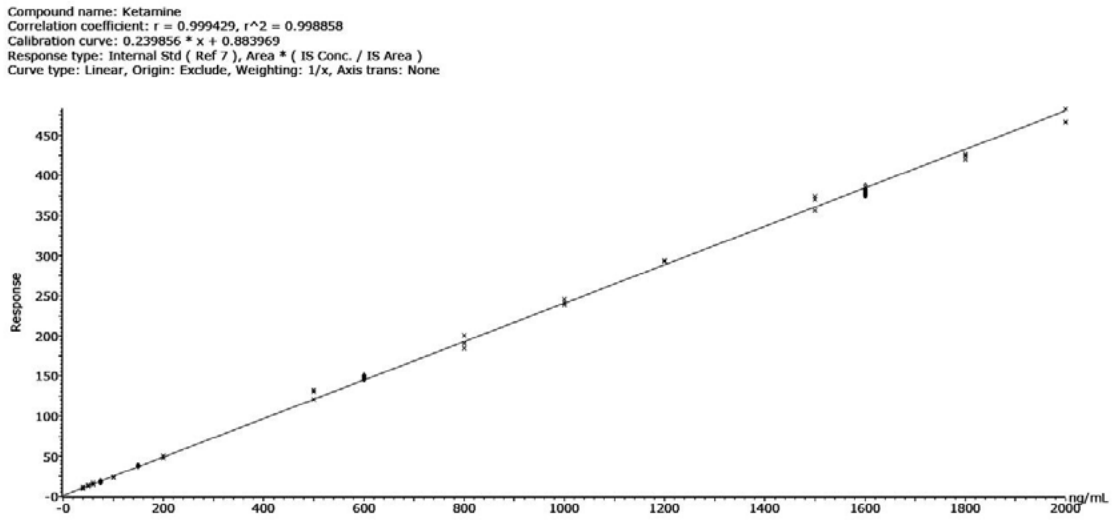

Figure 2: Example of response for ketamine prepared in urine. All compounds were quantified by reference to their ISTDs. Data shows replicate analyses for each calibrator (n=3, -X-) and QC (n=15, -u-).

The analysis of the commercial urine QC (WDT -25%) showed the successful identification and the calculated concentrations for amphetamine, methamphetamine, and ketamine were within 12% of expected concentrations.

To assess the effect of some common analytes that are known to interfere with the amphetamine or ketamine immunoassays, 21 substances (Table 2) were selected for investigation. Blank urine was spiked individually with each of the 21 standards at a concentration of 10,000 ng/mL. None of the investigated analytes resulted in a false positive result for amphetamine, methamphetamine, ketamine, and norketamine. In addition, the potential interference was investigated by spiking a QC urine (600 ng/mL) with the same analytes – the results showed no interference in terms of quantitative data.

A selection of authentic urine samples, previously characterized using an established high resolution mass spectrometry screening technique based on the Xevo G2-XS QTof, were tested.[8] The results showed good agreement between the two approaches.

Although LC-MS/MS is now established for illicit drug screening and quantification, in forensic toxicology laboratories worldwide, this study shows that for some applications, a single quadrupole-based detector can provide sufficient analytical sensitivity and dynamic range. The ACQUITY QDa Mass Detector has demonstrated great promise as an alternative technique to immunoassay.

Brompheniramine	MDEA	Phentermine
Buflomedil	MDMA	Phenylpropanolamine
Buproprion	MDA	Propranolol
Chloroquine	Meberverine	Pseudoephedrine
Ephedrine	Methoxetamine*	Quetiapine*
Fluoxetine	PCP*	Ranitidine
Labetalol	Phenmetrazine	Trazadone

Table 2: Selected number of substances reported to cross-react with amphetamine and ketamine immunoassays.*[3-5]

Conclusion

Drug testing facilities face a perpetual struggle to provide rapid and accurate results. The purpose of this study was to evaluate the ACQUITY UPLC H-Class/ACQUITY QDa System as an alternate technique to potentially replace two commonly applied immunoassays by developing a single, fast analytical method which incorporates a simple sample preparation procedure. Selectivity is achieved by monitoring retention time, precursor and product ion(s), and ion ratios. The method also comprised an analytical range which extended to 2000 ng/mL, offering the user an ability to quantify.

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Featured Products

ACQUITY UPLC H-Class PLUS System <https://www.waters.com/10138533>

ACQUITY QDa Mass Detector <https://www.waters.com/134761404>

MassLynx Mass Spectrometry Software <https://www.waters.com/513164>

TargetLynx <https://www.waters.com/513791>

Xevo G2-XS QTof Quadrupole Time-of-Flight Mass Spectrometry <https://www.waters.com/134798222>

Available for purchase online

ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 50 mm, 1/pkg <
https://www.waters.com/waters/partDetail.htm?partNumber=186002350>

ACQUITY UPLC BEH C18 VanGuard Pre-column, 130Å, 1.7 µm, 2.1 mm X 5 mm, 3/pkg <
https://www.waters.com/waters/partDetail.htm?partNumber=186003975>

720006595, June 2019

NPS/Designer Drugs

Application Notes

Application Note

Simple, Fast, and Clean Extraction of Synthetic Cannabinoids from Whole Blood Using Oasis PRiME HLB

Xin Zhang, Jonathan P. Danaceau, Erin E. Chambers

Waters Corporation

For forensic toxicology use only.

Abstract

A novel reversed-phase SPE sorbent is described in this application note, Oasis PRiME has been used to achieve consistent, high recoveries of synthetic cannabinoids and metabolites with low matrix effects while virtually eliminating endogenous phospholipids from whole blood samples. The enabled excellent quantitiative results, even without the use of deuterated internal standards.

Benefits

- A simple SPE protocol for that eliminates conditioning and equilibration steps.
- Efficient recoveries and low matrix effects for all tested compounds.
- Rapid, universal extraction method for analysis of synthetic cannabinoids and metabolites from whole blood.
- >95% reduction in phospholipids compared to protein precipitation.
- Excellent quantitative accuracy andprecision for a wide variety of synthetic cannabinoids and metabolites.

Introduction

Oasis PRiME HLB is a novel reversed phase solid phase extraction (SPE) sorbent developed to enable simpler and faster SPE protocols, while at the same time generating cleaner extracts than other sample preparation methods. With Oasis PRiME HLB, a 3-step load-wash-elute SPE protocol eliminating conditioning and equilibration was successfully employed to extract 22 synthetic cannabinoids and metabolites from whole blood samples. Excellent analyte recoveries and modest matrix effects (ME) were achieved across the entire panel of compounds. These results were consistent, with low variability for all compounds. In addition, Oasis PRiME HLB removed more than 95% phospholipids from the whole blood samples compared to protein precipitation (PPT). The 22 synthetic cannabinoids and metabolites were extracted using Waters Oasis PRiME HLB 30 mg plates. Calibration curves for all compounds ranged from 0.2–100 ng/mL. Quantitative results from quality control samples were accurate and precise across the entire calibration range. The analysis of several different classes of these drugs and metabolites, which includes neutral molecules, acids and bases, demonstrates the utility of this method across the different chemotypes and should render this method applicable to newly developed related compounds with little, if any, modification necessary.

Experimental

LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	CORTECS UPLC C_{18}, 90Å, 1.6 µm; 2.1 x 100 mm (p/n 186007095)
Column temp.:	30 °C
Injection volume:	5 µL
Flow rate:	0.6 mL/min

Mobile phase A:	0.1% formic acid in MilliQ water
Mobile phase B:	0.1% formic acid in ACN
Gradient:	Initial conditions started at 30% B. The %B was increased to 50% over 2 minutes, and held at 50% B for 1 minute, increased to 90% B over 4 minutes and then returned to 30% over 0.2 minutes. The system was allowed to re-equilibrate for 1.3 min. The entire cycle time was 8.5 min. The solvent gradient is listed in Table 1.

Gradient

Time (min.)	Flow (mL/min.)	%A	%B
0	0.6	70	30
2.0	0.6	50	50
3.0	0.6	50	50
7.0	0.6	10	90
7.2	0.6	70	30
8.5	0.6	70	30

Table 1. Mobile phase gradient. The compositions of MPA and MPB are listed in the Methods section.

MS conditions

MS system:	Xevo TQD
Ionization mode:	ESI Positive
Acquisition mode:	MRM (See Tables 2 and 3 for transitions)
Capillary voltage:	1 kV
Collision energy (eV):	Optimized for individual compounds (See Table 2)
Cone voltage (V):	Optimized for individual compounds (See Table 2)

Data management

All data were acquired and analyzed using Waters MassLynx software v.4.1 (scn 855) and quantified using TargetLynx Software. MS conditions were optimized using Intellistart.

Materials

AM2233, JWH-015, RCS-4, JWH-203, RCS-8, JWH-210, JWH-073, and JWH-018 were purchased from Cerilliant (Round Rock, TX). All other compounds and metabolites were purchased from Cayman Chemical (Ann Arbor, MI)

Individual stocks (1 mg/mL) were prepared in methanol, DMSO, or 50:50 DMSO: methanol. A combined stock solution of all compounds (10 µg/mL) was prepared in methanol. Working solutions were prepared daily in 40% methanol.

Calibrators and quality control (QC) samples were prepared by spiking working standards at various concentrations and into matrix (whole blood). Calibrator concentrations ranged from 0.2–100 ng/mL for all analytes. Quality control samples were prepared at 2.5, 7.5, and 75 ng/mL, in whole blood.

The 22 compounds analyzed are listed in Table 1 and constitute a panel that includes various classes of forensically relevant synthetic cannabinoids. These include adamantoylindoles (AM 1248 and AKB48), napthoylindoles (JWH 022), phenylacetyl indoles (RCS-4 and RCS-8), and tetramethylcyclopropylindoles (UR-144 and XLR11). Major metabolites of JWH-073 and JWH-018 were also included, as some of these compounds are structural isomers with identical mass spectral fragments that require adequate chromatographic separation for accurate quantitation.

Sample preparation

Samples were extracted using Oasis PRiME HLB 30 mg Plates. 0.1 mL of a solution of 0.1 M zinc sulfate/ammonium acetate was added to 0.1 mL whole blood, and vortexed for 5 seconds to lyse the cells. All samples were then precipitated by adding 400 µL ACN. The entire sample was vortexed for 10 seconds and centrifuged for 5 min at 7000 rcf. The supernatant was then diluted with 1.2 mL water prior to loading. The sample was directly loaded on the Oasis PRiME 30 mg Plate without conditioning or equilibration. All wells were then washed with 2 x 500 µL 25:75 MeOH:water, and eluted with 2 x 500 µL 90/10 ACN/MeOH. The eluate was then evaporated under Nitrogen and reconstituted with 100 µL 30% ACN. 5 µL was injected onto the UPLC system.

Analyte recovery was calculated according to the following equation:

Analyte recovery was calculated according to the following equation:

$$\% \text{ Recovery} = \left(\frac{\text{Area } A}{\text{Area } B}\right) \times 100\%$$

Where A equals the peak area of an extracted sample and B equals the peak area of an extracted matrix sample in which the compounds were added post-extraction.

Matrix effects were calculated according to the following equation:

$$\text{Matrix Effects} = \left(\frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}}\right) \times 100\%$$

The peak area in the presence of matrix refers to the peak area of an extracted matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to analytes in a neat solvent solution.

Results and Discussion

Chromatography

The design of the solid-core CORTECS Particle, combined with optimal packing in the column, results in excellent chromatographic performance. A representative chromatogram of all compounds from a 20 ng/mL calibration standard is shown in Figure 1. Using a CORTECS UPLC C_{18} Column (90Å, 1.6 µm, 2.1 x 100 mm), all analytes were analyzed within 7.5 minutes with a total cycle time of 8.5 minutes. Peak shape was excellent for all compounds, with no significant tailing or asymmetries, and all peak widths were under 3 seconds at 5% of baseline.

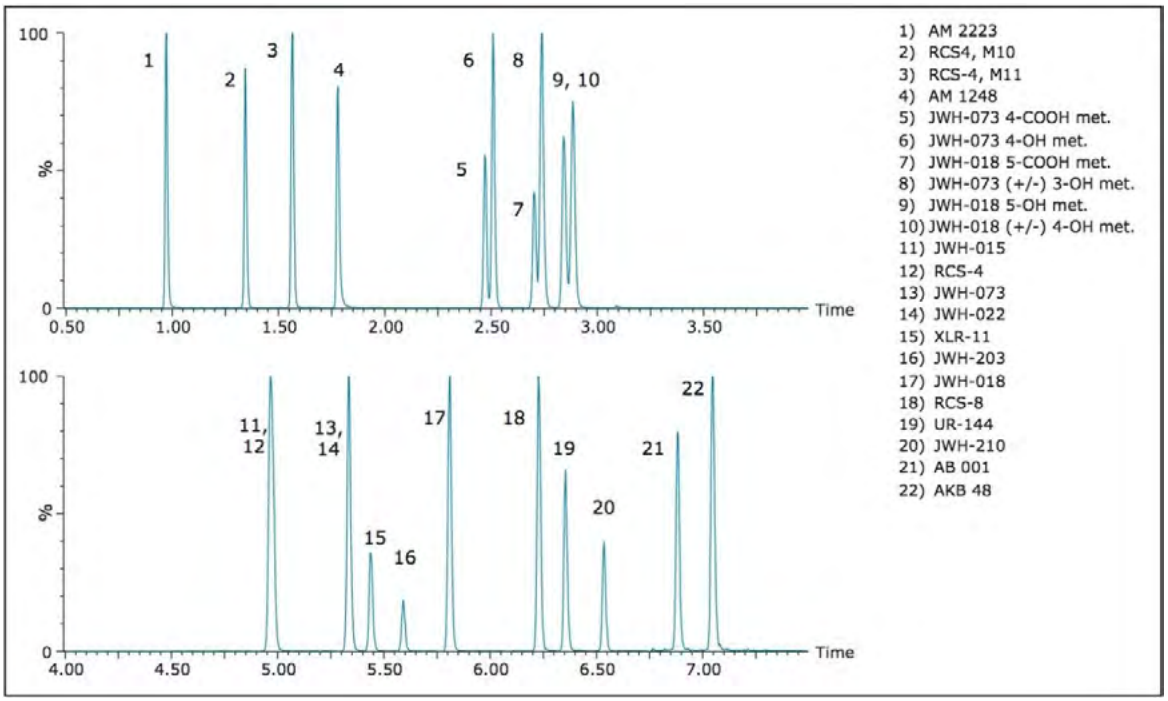

Figure 1. UPLC-MS/MS chromatogram for 22 synthetic cannabinoids and metabolites.

Recovery and matrix effects

The synthetic cannabinoids and metabolites in this application include compounds that are neutral, acidic and basic. Use of the Oasis PRiME HLB Sorbent enabled the simultaneous extraction of all of the compounds and metabolites tested, regardless of their functionality. Recoveries and matrix effects (ME) were calculated according to the equations described in the experimental section and the results are shown in Figure 2. This extraction protocol results in nearly complete recovery for all compounds and minimizes matrix effects for the majority of analytes. All but one compound had recoveries of 80% or greater with an overall average recovery of 91%. Recoveries were consistent with an average %RSD at 5% across all compounds. Matrix effects across the panel were excellent. Only two compounds had matrix effects that slightly exceeded 40%, and all remaining compounds had matrix effects less than 25%. The average magnitude of matrix effects was only 17%. The high recoveries and minimal matrix effects for this panel of synthetic cannabinoids indicate that Oasis PRiME HLB should give similar results for other related compounds with a simple load-wash-elute protocol.

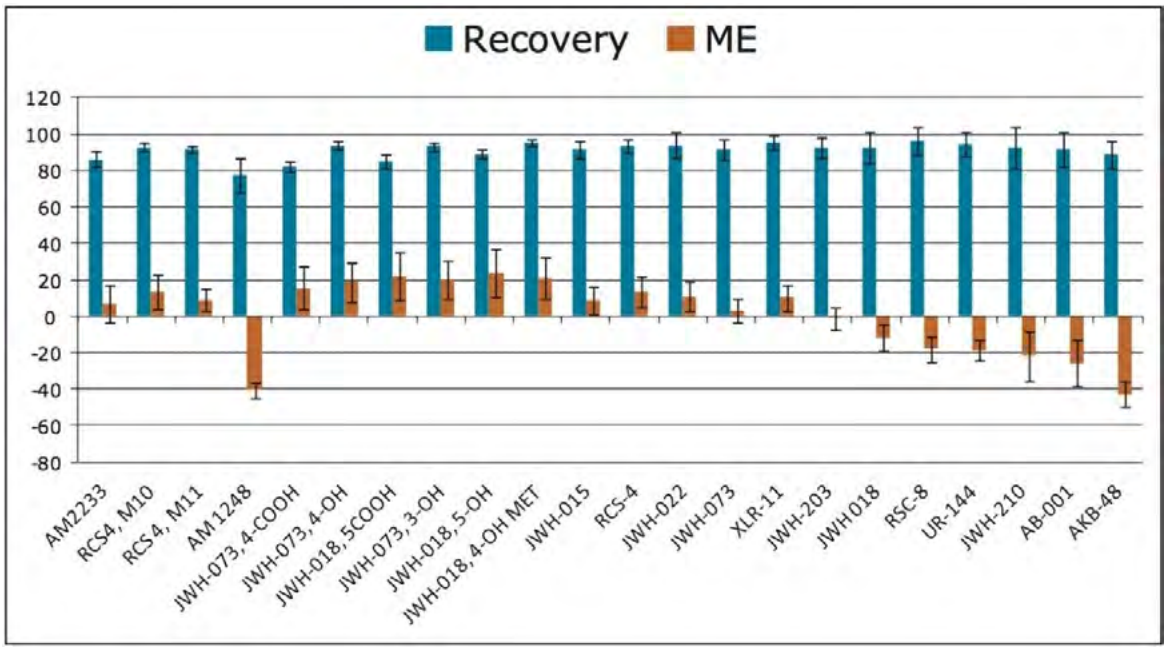

Figure 2. Recovery and matrix effects of synthetic cannabinoid compounds from whole blood following extraction with Oasis PRiME HLB µElution plates. Bars and error bars represent means and standard deviations (N=6), respectively.

Phospholipid removal

One of the key attributes of Oasis PRiME HLB, is its ability to deliver cleaner extracts than other sample preparation methods. One way that this is achieved is by removing endogenous phospholipids. Figure 3 shows chromatograms of combined phospholipid traces from an Oasis PRiME HLB extract and an identical sample subject to protein precipitation. Compared with protein precipitation (PPT), Oasis PRiME HLB removes over 95% phospholipids (Figure 3) resulting in a much cleaner extraction. This can translate to reduced matrix effects, longer column lifetimes, and less mass spectrometer source maintenance.

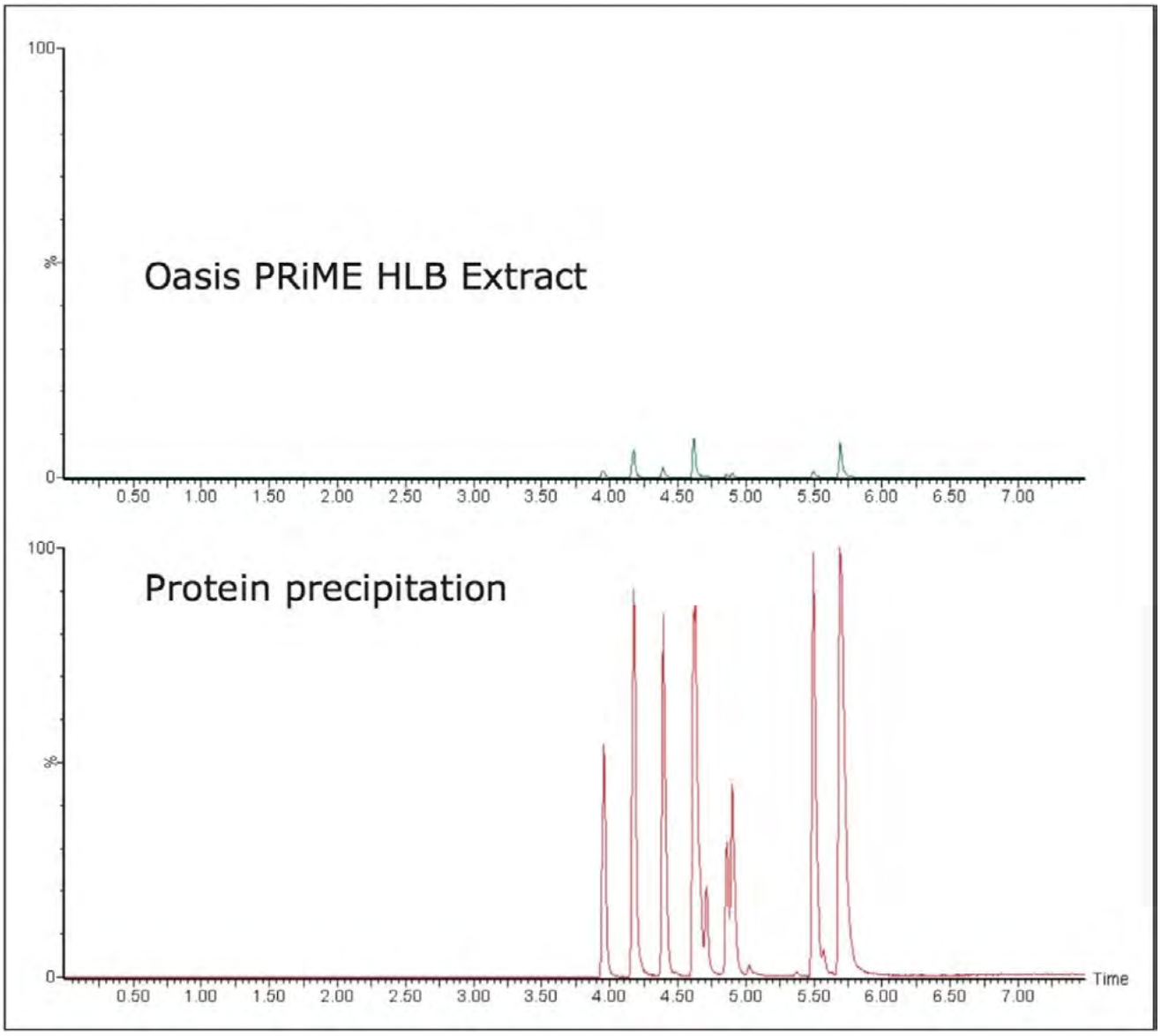

Figure 3. Chromatograms of phospholipids remaining in Oasis PRiME HLB extraction vs. protein precipitation. Scales are linked.

Standard curve performance, accuracy, precision, and sensitivity

In order to assess linearity and analytical sensitivity, calibration curves were extracted at concentrations ranging from 0.2–100 ng/mL for all components. Quality control samples (N=4) at 2.5, 7.5, and 75 ng/mL were also extracted and analyzed. Table 2 summarizes R^2 values from the calibration curves and QC summary data for all

compounds. Quality control (QC) results were accurate and precise at low, medium and high concentrations. Accuracies for low level QC samples (2.5 ng/mL) ranged from 95–110% (except one compound, AM2233) with an average of 102%. The results for the medium and high QC levels were excellent for all analytes except one, with all accuracies within 15% of expected values. Analytical precision was excellent with most % RSDs less than 10% and none greater than 15%. When accuracy was assessed over all levels (low, medium, and high), the means ranged from 93% to 104%. Limits of quantification of 0.1 ng/mL were reached for most of the analytes and were no greater than 1 ng/mL. These results were achieved without the use of deuterated internal standards, once again demonstrating the consistency associated with Oasis PRiME HLB.

		QC concentrations (ng/mL)						
		2.5		7.5		75		
	R^2	%Acc	%RSD	%Acc	%RSD	%Acc	%RSD	Mean
AM2233	0.994	74.53	7.78	85.52	5.29	93.18	4.96	84.41
RCS4, M10	0.995	107.75	6.91	95.72	5.43	95.93	3.33	99.80
RCS 4, M11	0.993	106.60	3.15	92.52	5.09	92.92	3.34	97.34
JWH-073, 4-COOH	0.998	108.00	2.44	96.00	5.43	107.07	3.20	103.69
JWH-073, 4-OH	0.996	106.90	5.01	95.88	3.76	94.12	2.15	98.97
JWH-018, 5COOH	0.996	107.58	5.98	97.98	2.32	99.30	2.65	101.62
JWH-073, 3-OH	0.996	110.00	6.22	94.43	2.24	91.95	2.38	98.79
JWH-018, 5-OH	0.996	103.17	9.39	83.55	4.01	101.77	3.90	96.16
JWH-018, 4-OH MET	0.996	106.40	5.45	85.15	2.87	104.70	4.17	98.75
JWH-015	0.998	108.85	6.19	99.57	3.49	100.95	2.48	103.12
RCS-4	0.998	109.80	4.06	94.63	3.47	94.62	5.25	99.68
JWH-022	0.999	107.32	3.23	97.27	4.95	96.90	5.65	100.50
JWH-073	0.999	111.28	3.11	97.38	4.96	99.62	7.47	102.76
XLR-11	0.998	101.32	8.07	98.90	4.12	103.78	5.71	101.33
JWH-203	0.999	101.60	4.34	99.72	6.55	101.24	7.13	100.85
JWH 018	0.998	102.30	9.31	99.08	8.86	102.00	7.62	101.13
RSC-8	0.995	95.92	8.61	93.02	9.10	100.98	11.55	96.64
UR-144	0.997	99.22	9.81	99.58	9.55	104.60	7.24	101.13
JWH-210	0.993	110.60	10.42	94.02	12.92	100.35	8.71	101.66
AB-001	0.995	107.02	7.82	92.54	11.04	101.98	6.22	100.51
AKB-48	0.974	97.07	11.76	89.95	13.76	107.63	13.50	98.21
	Mean	**102.43**		**92.27**		**98.24**		

Table 2. R^2 values and quality control results for all compounds. Mean values at the bottom indicate averages of all compounds at particular concentrations. Values to the right indicate averages of individual compounds across all QC concentrations.

No.	Compound	RT	Mol. Formula	Cone voltage	MRM Transitions	Coll. energy
1	AM2233	0.97	$C_{22}H_{23}IN_2O$	48	459.2⇒98.05	50
				48	459.2⇒112.1	40
2	RCS-4, M10	1.34	$C_{20}H_{21}NO_3$	40	324.2⇒121.0	36
				40	324.2⇒93.0	72
3	RCS-4, M11	1.57	$C_{20}H_{19}NO3$	42	322.2⇒121.0	32
				42	322.2⇒93.0	60
4	AM 1248	1.78	$C_{26}H_{34}N_2O$	62	391.4⇒135.1	42
				62	391.4⇒112.1	50
5	JWH-073 4-butanoic acid met.	2.47	$C_{23}H_{19}NO_3$	52	358.2⇒155.1	32
				52	358.2⇒127.1	70
6	JWH-073 4-hydroxybutyl met.	2.51	$C_{23}H_{21}NO_2$	52	344.2⇒155.1	32
				52	344.2⇒127.1	70
7	JWH-018 5-pentanoic acid met.	2.71	$C_{24}H_{21}NO_3$	54	372.2⇒155.1	32
				54	372.2⇒127.1	72
8	JWH-073 (+/-) 3-hydroxybutyl met.	2.74	$C_{23}H_{21}NO_2$	54	344.2⇒155.1	36
				54	344.2⇒127.1	64
9	JWH-018 5-hydroxypentyl met.	2.84	$C_{24}H_{23}NO_2$	50	358.2⇒155.1	24
				50	358.2⇒127.1	48
10	JWH-018 (+/-) 4-hydroxypentyl met.	2.89	$C_{24}H_{23}NO_2$	50	358.2⇒155.1	34
				50	358.2⇒127.1	64
11	JWH-015	4.97	$C_{23}H_{21}NO$	48	328.2⇒155.1	32
				48	328.2⇒127.1	62
12	RCS-4	4.98	$C_{21}H_{23}NO_2$	48	322.2⇒135.1	40
				48	322.2⇒92.0	68
14	JWH-022	5.34	$C_{24}H_{21}NO$	52	340.2⇒155.1	34
				52	340.2⇒127.1	60
13	JWH-073	5.34	$C_{23}H_{21}NO$	48	328.2⇒155.1	36
				48	328.2⇒127.1	56
15	XLR-11	5.44	$C_{21}H_{28}FNO$	52	330.3⇒125.1	34
				52	330.3⇒83.0	42
16	JWH-203	5.59	$C_{21}H_{22}ClNO$	44	340.2⇒188.1	32
				44	340.2⇒125.0	42
17	JWH-018	5.82	$C_{24}H_{23}NO$	50	342.2⇒155.1	34
				50	342.2⇒127.1	60
18	RCS-8	6.23	$C_{25}H_{29}NO_2$	44	376.3⇒121.1	36
				44	376.3⇒91.0	66
19	UR-144	6.36	$C_{21}H_{29}NO$	48	312.3⇒214.2	35
				48	312.3⇒125.1	34
20	JWH-210	6.54	$C_{26}H_{27}NO$	54	370.2⇒214.2	34
				54	370.2⇒183.1	36
21	AB 001	6.88	$C_{24}H_{31}NO$	62	350.3⇒135.1	44
				62	350.3⇒93.0	62
22	AKB 48	7.05	$C_{23}H_{31}N_3O$	36	366.3⇒135.1	28
				36	366.3⇒93.1	68

Table 3. Molecular formulae, retention times, and MS/MS conditions for the synthetic cannabinoid compounds and metabolites in this application. Quantification transitions are listed first, followed by confirmatory transitions

Precursor Ion (*m/z*)	Product Ion (*m/z*)	Cone Voltage (V)	Collison Energy (eV)
496.40	184.40	35	30
520.40	184.40	35	30
522.40	184.40	35	30
524.40	184.40	35	30
704.40	184.40	35	30
758.40	184.40	35	30
760.40	184.40	35	30
784.40	184.40	35	30
786.40	184.40	35	30
806.40	184.40	35	30
808.40	184.40	35	30

Table 4. MS/MS conditions for the Phospholipids.

Conclusion

This application note highlights the use of Oasis PRiME HLB, a novel reversed-phase SPE sorbent which is designed to enable simple and fast SPE protocols while nearly eliminating endogenous phospholipids. Employing a simple load-wash-elute strategy, without any sorbent conditioning or equilibration, a panel of 22 synthetic cannabinoids was extracted from whole blood samples. Extraction recoveries averaged 91% across the entire panel, with an average matrix effect magnitude of only 17%. These results were consistent with mean %RSDs of 5% for all compounds. In addition, greater than 95% of phospholipids were removed vs. protein precipitation. Quantitative results were also excellent. Even without the use of deuterated internal standards, calibration curves were linear, with R^2 values of 0.99 for 21/22 compounds. 97% of QC results were within 15% of target values and all %RSDs were less than 15%. In conclusion, Oasis PRiME has been used to achieve consistent, high recoveries with low matrix effects while virtually eliminating endogenous phospholipids from whole blood samples. The enabled excellent quantitiative results, even without the use of deuterated internal standards.

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720005417, May 2015

Sports Doping

Application Notes

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Application Note

Analysis of Doping Agents by UPC^2-MS/MS

Jonathan P. Danaceau, Ivana Gavrilović, Peter Christensen, Michelle Wood

Waters Corporation, Drug Control Centre, King's College London

For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

UPC^2-MS/MS is a chromatographic technique orthogonal to GC and LC. A UPC^2-MS/MS method was developed for testing a variety of doping agents. Extremely polar compounds such as meldonium, amiloride, and ethyl glucuronide were well retained, and most other compounds displayed excellent chromatographic performance. Retention times were stable for all compounds within and between batches, with %RSDs <0.6%. The method had the analytical sensitivity and selectivity to accurately detect all compounds at, or below, WADA's Minimum Required Performance Levels (MRPL). This technique represents a valuable addition to GC and LC to more fully cover the chromatographic space required for anti-doping analysis.

Benefits

- Orthogonal selectivity and retention, allowing retention of compounds that perform poorly by GC or LC
- Rugged, reproducible chromatography for a variety of doping agents
- Analytical sensitivity required to meet Minimum Required Performance Levels (MRPL) of doping agents

Introduction

The Prohibited List of the World Anti-Doping Agency (WADA) [WADA, 2021] currently contains hundreds of specifically banned substances, as well as performance enhancing agents which are not explicitly named, but belong to banned classes of drugs. One of the greatest challenges for anti-doping labs is the physicochemical diversity of compounds that require analytical testing. Many of these are currently addressed by LC-MS (LC-HRMS and LC-MS/MS) and GC-MS (GC-HRMS and GC-MS/MS). However, there remain many substances for which the current technologies are challenged for reliable identification and confirmation. Many of these substances are polar, with minimal retention on traditional chromatographic platforms, or have poor peak shape due to their chemistry. UPC^2-MS/MS is a separation technique that is orthogonal to both GC and LC, often providing separation, resolution and selectivity that is not attainable by the other chromatographic techniques [Nováková, 2015; Losacco, 2020]. This application brief details the chromatographic method development and analysis of a wide variety of banned substances with a diversity of physicochemical properties, by UPC^2-MS/MS. These included substances such as stimulants, steroids, drugs of abuse, glucocorticoids, diuretics, beta-blockers, and other banned substances. Using the UPC^2-MS/MS method it was possible to retain and resolve compounds such as meldonium, amiloride, and ethyl

glucuronide, which are challenging to analyze by other chromatographic techniques, as well as dozens of other test compounds. Analysis of 1000 anonymized anti-doping samples showed no adverse analytical findings. Retention times were stable for all analytes within and between batches, and the method had the analytical sensitivity to accurately identify all compounds at WADA's Minimum Required Performance Levels (MRPL). [WADA, 2019]

Experimental

Materials

Reference material for all analytes and internal standards were generously provided by the Drug Control Centre (DCC), King's College London (London, UK). Eight compounds were used for initial column screening and method development. These are listed in Table 1 together with their specific MS conditions.

For the second phase of the work, a larger group of compounds (also provided by the DCC at King's College London) were investigated (see Appendix). Individual reference materials were combined to yield two mixed solutions – QC1 and QC2. These were prepared in methanol for method development and retention time verification, and in blank urine as a spiked reference sample, for inclusion when analyzing the batches of authentic samples. The Appendix lists the compounds, relevant concentrations, retention times, and specific MS conditions.

An internal standard (IS) solution contained mefruside, ephedrine-d_3, and salbutamol-d_3 at a concentration of 10 µg/mL.

Authentic Samples

One-thousand authentic, anonymized anti-doping urine samples were generously supplied by the DCC and analyzed using the final conditions listed.

Sample Preparation

Sample preparation was adapted from Nováková ***et al.*** [Nováková, 2015]. Two hundred microliters of urine was diluted with 790 µL ACN and 10 µL of IS mixture (10 µg/mL) and centrifuged at 5000 rcf for 10 min; 2 µL of the supernatant were injected onto the column.

LC Conditions

UPC^2-MS/MS

LC system:	ACQUITY UPC^2 System
Detection:	Xevo TQ-XS
Column(s):	Torus Diol (OH) Column, 130 Å, 1.7 µm, 3.0 x 100 mm.
Column temp.:	35 ^{o}C
Sample temp.:	10 ^{o}C
Injection volume:	2 µL
Flow rate:	1.2 mL/min
Mobile phase A:	CO_2
Mobile phase B:	Methanol with 0.1% strong ammonia
Make up flow:	Methanol at 0.2 mL/min

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Init	1.2	90	10	6
1.0	1.2	90	10	6
4.0	1.2	50	50	6
4.5	1.0	43.3	56.7	6
5.0	1.0	43.3	56.7	6
5.1	1.2	90	10	6
7.0	1.2	90	10	6

MS Conditions

MS system:	Xevo TQ-XS
Ionization mode:	ESI+ and ESI-
Capillary voltage:	2.0 kV (-2.0 kV)
Collision energy (CE):	Compound dependent (see Appendix)
Cone voltage (CV):	Compound dependent (see Appendix)

Initial Column Testing Conditions

Two chromatographic dimensions were screened in order to find the optimal conditions; these were the organic modifier composition and the column chemistry. The following mobile phase B (MPB) modifiers were screened: no modifier, 0.1% formic acid, 0.1% strong ammonia, and 10 mM ammonium formate. Each was added to methanol and used as MPB.

Four columns were also screened, all with the same dimensions and particle size (130 Å, 1.7 µm, 3.0 x 100 mm). Stationary phases included: the Viridis BEH 2-Ethylpyridine (2-EP), Torus 2-PIC, the Torus 1-AA, and the Torus Diol (OH) Column. All columns used the solvent ramp detailed in final method, except that the flow rate for all columns other than the Diol Column was 1.5 mL/min.

Compound	RT (min)	Ionization mode	CV (V)	$[M+H]^+$/ $[M-H]^-$	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	CE1 (eV)	CE2 (eV)
Danazol	1.46	Pos	20	338.2	303.3	321.3	15	15
Fluticasone propionate	1.77	Pos	20	501.2	293.2	313.1	20	15
Probenecid	3.25	Neg	20	284.1	240.1	140.1	20	20
GW 1516	3.33	Pos	20	454.2	188.1	256.1	45	45
Bumetanide	4.10	Neg	20	363.1	207.1	80.0	20	20
Meldonium	4.26	Pos	25	147.1	59.1	132.1	20	15
Ethyl glucuronide (EtG)	4.62	Neg	25	221.1	85.0	75.0	15	15
3' OH stanozolol glucuronide	5.28	Neg	20	519.3	343.2	175.1	40	20

Table 1. Compounds used for initial UPC^2 testing.

Results and Discussion

Column and Modifier Testing

Four UPC^2 Columns and four modifiers were initially evaluated using a limited test mix of compounds. Initial testing using the Viridis 2-EP Column revealed that using 0.1% strong ammonia resulted in superior peak shape and retention compared with 0.1% formic acid, 10 mM ammonium formate, or no modifier at all. Further testing with the additional columns showed that the Torus Diol Column outperformed the other three with regards to retention, peak shape, reduced tailing, and analytical sensitivity, particularly for 3-OH stanozolol-glucuronide and meldonium. An example of the chromatography on the Diol column is shown in Figure 1. Excellent chromatographic performance was seen for the eight initial doping compounds tested. Ethyl glucuronide and meldonium, both of which are difficult to retain by reversed-phase LC or GC were well retained and exhibited very good peak shape with minimal tailing. The other compounds also demonstrated good retention and symmetrical peak shape, despite their chemical variety. The Diol Column was therefore used to analyze the larger panel of doping substances as well as 1000 authentic anonymized athlete samples.

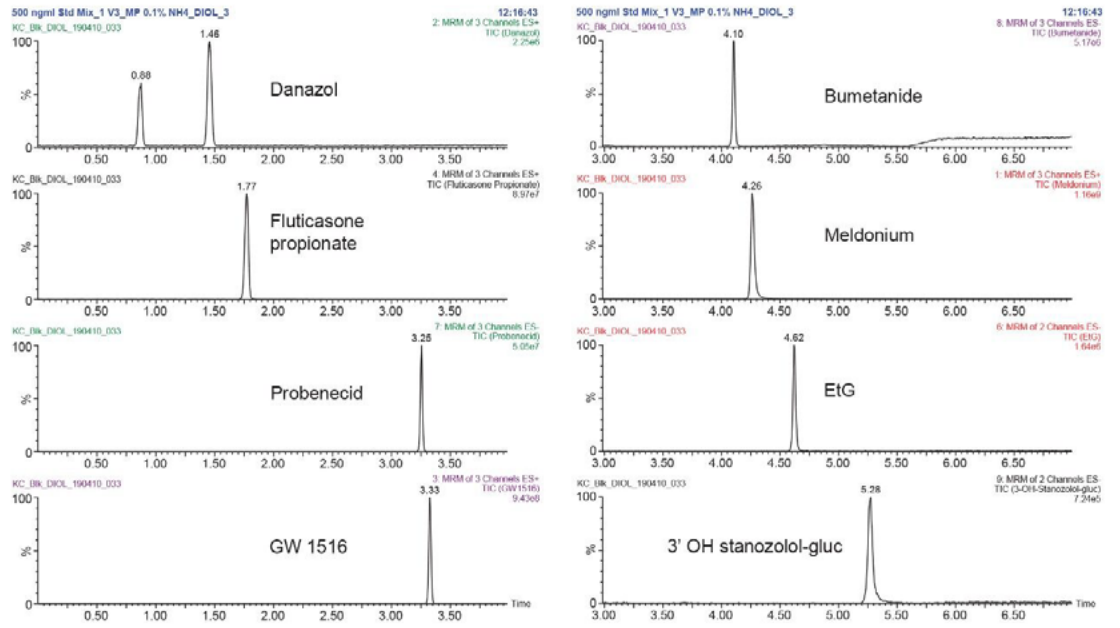

Figure 1. Final chromatography of doping compounds from the initial screening experiments. This separation was achieved on a Torus Diol (OH) Column (130 Å, 1.7 µm, 3.0 x 100 mm). The concentration of all analytes was 500 ng/mL.

Analysis of Authentic Samples

The expanded panel of compounds listed in the Appendix was used to screen 1000 authentic anti-doping samples. This list of substances was compiled by scientists from anti-doping laboratories; compounds were selected to ensure representation for several key drug classes from the WADA prohibited list. The chromatography of these can be seen in Figure 2. Most compounds demonstrated good chromatographic performance regarding retention, peak shape and selectivity. Nikethamide, for example, eluted early, but displayed good peak shape and retention time stability, unlike some of the retention time stability issues described by Losacco ***et al***. [2020] when using a BEH Column with ammonium formate as a mobile phase modifier. Most of the other peaks displayed excellent chromatographic characteristics. Some exceptions were compounds such as fentanyl, which consistently displayed a peak doublet. Octopamine had significant tailing and minor tailing was seen for oxymorphone and cathine. Many of the sulfated steroids either co-eluted or were not fully baseline resolved from their structural analogs. Nevertheless, most compounds representing a wide variety of chemotypes displayed excellent chromatographic performance.

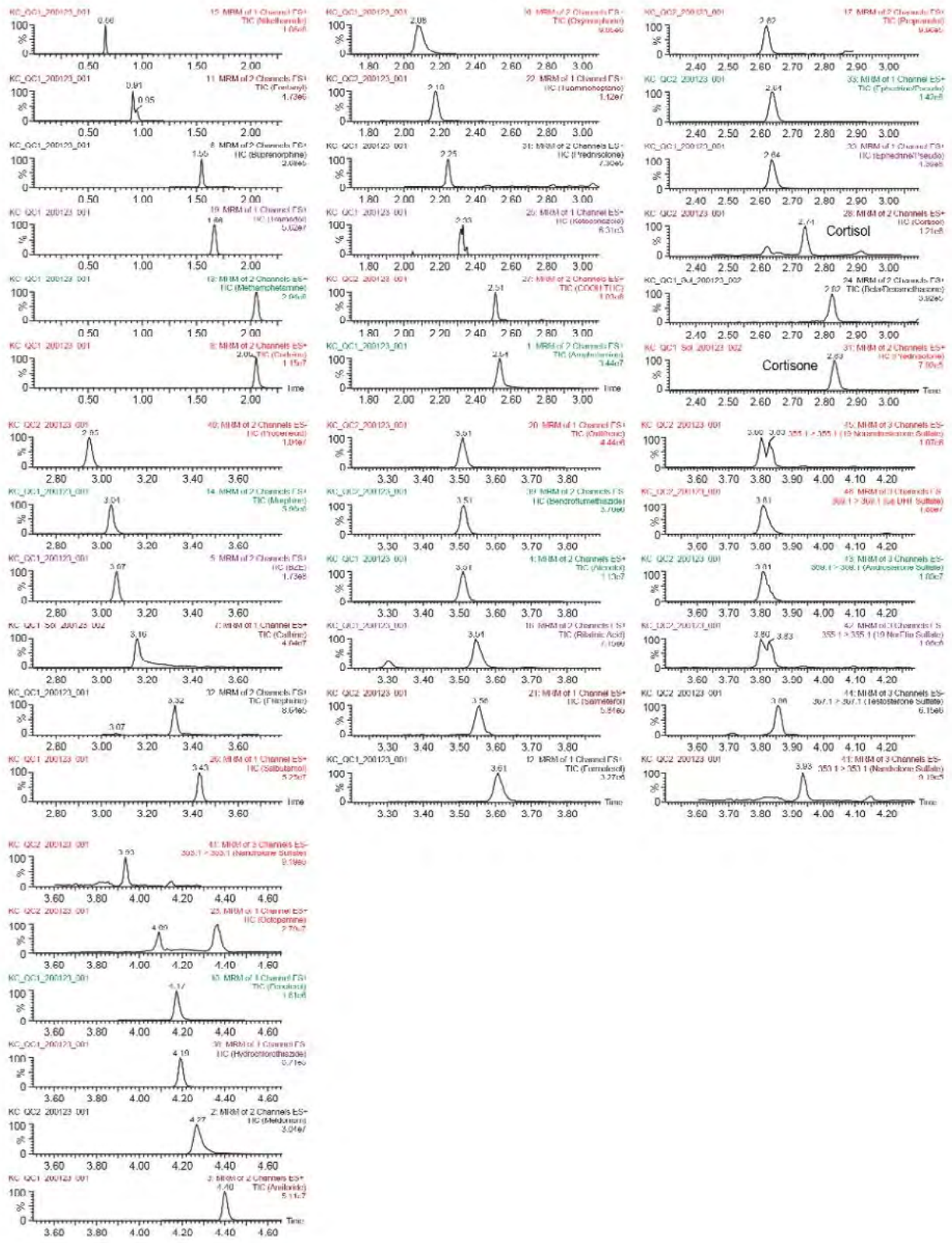

Figure 2. Chromatography of the compounds in the expanded panel used for the second phase of experiments. Cortisol is specifically labelled at 2.74 min and cortisone is labelled in the prednisolone MRM channel at 2.83 min. All other compounds are named in the upper right corner of their traces. All retention times are listed in the Appendix.

One key advantage of UPC^2 is its orthogonality to other chromatographic separation techniques. This can be seen in the retention and selectivity for extremely polar compounds such as ethyl glucuronide, amiloride, and meldonium. Other polar and moderately polar compounds such as morphine, salmeterol, etilefrine, and amphetamine were also very well retained and resolved, demonstrating the overlap between UPC^2 and LC. This broad, alternative selectivity should allow UPC^2 to be an important complementary method, expanding the reach of traditional chromatographic methods such as LC and GC and offering confirmation by an alternative chromatographic technique.

Retention Time Stability

Reference standards (QC1 and QC2) injected in the beginning, middle, and end of each batch revealed stable retention times for all analytes. All compounds had between batch retention time %RSDs <0.6%. The majority had %RSDs under 0.5% and 63% were under 0.3%. This easily meets WADA's retention time criteria for positive identification [WADA, 2015]. In addition, the internal standards included in each sample were monitored and were found to all have retention time %RSDs <0.3% within a batch.

Sensitivity

WADA defines analytical thresholds as Minimum Required Performance Levels (MRPL). These values are listed in the Appendix and were the concentrations used in the urine QC standards (QC1 and QC2) with the exception of hydrochlorothiazide, propranolol, and bendroflumethiazide, which were spiked at 50% of MRPL. Ketoconazole and tramadol have no established MRPL and were spiked at the concentrations listed in the Appendix. With the exception of ketoconazole, all the compounds investigated could easily be identified by the system at the noted concentrations. Responses for ketoconazole were close to the detection limit at 50 ng/mL, but it was still detected in all spiked QC samples in all 23 batches. Buprenorphine was easily detected at 5 ng/mL as was fentanyl at 2 ng/mL.

Conclusion

Waters UPC^2-MS/MS System using the Xevo TQ-XS has been demonstrated to be a reliable, orthogonal alternative to GC and LC-MS assays, especially for polar compounds that do not retain well by other chromatographic methods. Retention times were stable across 23 batches (>1200 injections) for all analytes. Method development revealed that of those investigated, the Torus Diol Column combined with a mobile phase modifier of 0.1% strong ammonia resulted in the best chromatography for nearly all the compounds. Even using a simple dilute and inject method, the system has the sensitivity and selectivity to positively identify all spiked compounds at the MRPL and in many cases, even at 50% MRPL in both positive and negative ESI.

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Acknowledgements

Jonathan P. Daneaceu, Peter Christensen and Michelle Wood - Waters Corporation

Ivana Gavrilović - Drug Control Centre, King's College London

Appendix. Retention times (RT), concentrations and MS Conditions for all analytes.

Compound	RT (min)	Conc. (ng/mL)	Ionization mode	M+H]+/ [M-H]-	CV (V)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	CE1 (eV)	CE2 (eV)
Amiloride	4.40	100	Pos	230.0	4	171.0	116.1	18	30
Amphetamine	2.54	100	Pos	136.1	10	91.1	119.0	10	10
19 nor-Androsterone sulfate	3.81	50	Neg	355.1	30	355.1	231.1	25	40
19 nor-Etiocholanolone sulfate	3.83	50	Neg	355.2	30	355.2	231.1	25	40
5a-DHT-sulfate	3.81	50	Neg	369.2	30	369.1	285.2	25	40
Androsterone-sulfate	3.81	200	Neg	369.2	30	369.2	259.1	25	40
Atenolol	3.51	100	Pos	267.2	10	116.0	190.2	18	20
Bendroflumethiazide	3.51	100	Neg	420.1	56	289.0	328.2	22	30
Benzoylecgonine	3.07	100	Pos	290.2	2	168.1	105.1	30	18
Betamethasone	2.82	30	Pos	393.2	20	355.0	279.0	20	20
Buprenorphine	1.55	5	Pos	468.3	58	414.2	55.0	32	4
Cathine	3.16	100	Pos	134.1	44	117.1		25	
Codeine	2.05	50	Pos	300.1	28	215.1	165.0	24	38
Cortisol	2.74	30	Pos	363.2	25	121.1	91.1	22	50
Cortisone	2.83	30	Pos	361.2	25	343.2	325.2		
Dexamethasone	2.79	30	Pos	393.2	20	355.0	279.0	20	20
DHEA-sulfate	3.81	200	Neg	369.2	30	369.1	285.2	25	40
Ephedrine-d3 (IS)	***2.65***	***100***	***Pos***	***169.1***	***2***	***151.1***	***115.1***	***10***	***25***
Ephedrine	2.64	100	Pos	166.1	2	148.1		10	
Etilefrine	3.32	100	Pos	182.1	20	135.0	164.1	12	30
Fenoterol	4.17	20	Pos	304.2	38	135.1		16	
Fentanyl	0.92	2	Pos	337.2	30	188.2	105.1	20	35
Formoterol	3.61	20	Pos	345.1	10	149.1		18	
Hydrochlorothiazide	4.19	100	Neg	295.9	62	269.0		20	
Ketoconazole	2.33	50	Pos	531.3	20	489.0		20	
Mefruside (IS)	***2.21***	***100***	***Pos***	***383.3***	***20***	***285.0***	***190.0***	***10***	***25***
Meldonium	4.26	200	Pos	147.1	25	59.1	132.2	20	15
Methamphetamine	2.05	100	Pos	150.1	18	91.1	119.1	16	9
Morphine	3.04	50	Pos	286.1	25	201.1	165.1	25	35
Nandrolone sulfate	3.94	50	Neg	353.2	30	353.1	271.1	25	40
Nikethamide	0.66	100	Pos	179.1	44	108.1		18	
Octopamine	4.09	1000	Pos	136.0	40	91.1		16	
Oxilifrine	3.51	100	Pos	182.0	20	105.0		20	
Oxymorphone	2.08	50	Pos	302.1	34	227.1	242.1	25	25
Prednisolone	2.25	30	Pos	361.2	25	343.2	325.2	20	20
Probenecid	2.95	100	Neg	284.1	50	240.1	139.9	16	24
Propranolol	2.62	50	Pos	260.2	10	116.1	183.2	16	16
Pseudoephedrine	2.64	100	Pos	166.1	2	148.1		10	
Ritalinic acid	3.54	100	Pos	220.1	25	84.0	56.0	40	40
Salbutamol-d3 (IS)	***3.43***	***500***	***Pos***	***243.2***	***20***	***225.1***	***151.1***	***7***	***25***
Salbutamol	3.43	500	Pos	240.1	20	222.0	166.0	7	10
Salmeterol	3.56	20	Pos	416.2	50	380.2		18	
Testosterone-epi-sulfate	3.87	50	Neg	367.2	30	367.2	351.1	25	30
Testosterone-sulfate	3.90	50	Neg	367.2	30	367.2	351.1	25	30
THC-COOH	2.51	150	Pos	345.2	25	193.0	299.2	25	25
Tramadol	1.67	50	Pos	264.2	25	58.0		15	
Tuaminoheptane	2.18	100	Pos	116.0	18	57.1		10	

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720007172, February 2021

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Application Note

Simultaneous Analysis of Diuretics and Beta-Blockers by Mixed Mode SPE and UPLC-MS/MS for Anti-Doping Analysis

Jonathan P. Danaceau, Michelle Wood, Lisa J. Calton

Waters Corporation

For forensic toxicology use only.

Abstract

The goal of this study is to simultaneously identify banned diuretics and beta-blockers in urine samples to support anti-doping laboratories.

The use of mixed-mode SPE and the ACQUITY UPLC I-Class/Xevo TQ-S micro System enabled clean, fast, and efficient extraction and analysis of urinary diuretics and beta-blockers for anti-doping purposes. The Oasis MAX µELution plates enabled efficient and reproducible extraction of 20 beta-blockers and 20 diuretic compounds. Matrix effects were significantly decreased compared to diluted samples. The Xevo TQ-S micro had the speed and sensitivity to accurately identify all analytes in under four minutes, meeting WADA's strict ion ratio criteria even at 20% of the MRPL. The ability to perform rapid inter-scan polarity switching enabled the analysis in a single injection, saving both time and instrument wear compared to sequential injections of positive and negative ionizing compounds. This will enable rapid, accurate analysis of these compounds, while maintaining a cleaner UPLC-MS/MS system.

Benefits

Solid phase extraction and rapid polarity switching enable clean, fast, and efficient analysis of banned diuretics and beta-blockers.

Introduction

Diuretics and beta-blockers are both banned by the World Anti-Doping Agency (WADA).[1] Despite their chemical differences, many anti-doping laboratories prefer to analyze these two classes of molecules together for workflow considerations.[2-4] Beta-blocking agents are bases that ionize under positive electrospray ionization (ESI) and diuretics are mostly acids that ionize under negative ESI conditions. This often necessitates sequential analyses via LC-MS; one in positive mode and a second in negative mode. In addition, these chemical differences make simultaneous extraction a challenge, as any SPE sorbent must be able to accommodate a wide range of polarities and chemotypes.

Results and Discussion

The challenges described above have been solved using Oasis MAX µElution plates to cleanly and efficiently extract diuretics and beta-blockers from urine samples. This was followed by UPLC-MS/MS analysis on a Waters ACQUITY UPLC I-Class System and Xevo TQ-S micro Mass Spectrometer. Separation was achieved on a Waters ACQUITY UPLC CSH C_{18} Column (1.7 µm, 2.1 x 100 mm). Mobile phases consisted of 0.01% formic acid (MPA) and acetonitrile (MPB).

The compounds and their retention times are listed in Table 1. All compounds eluted within four minutes and the entire UPLC cycle was five minutes (Figure 1). Two to three MRM transitions were acquired for all compounds. The rapid polarity switching of the Xevo TQ-S micro enabled this fast, yet efficient chromatography, despite simultaneous analysis of 24 positive and 18 negative ionizing compounds.

	ESI positive			ESI negative	
	Name	**R.T.**		**Name**	**R.T.**
1	Sotalol	0.56	24	Acetazolamide	1.13
2	Atenolol	0.58	25	Chlorthiazide	1.22
3	Amiloride	0.57	26	Hydrochlorothiazide	1.31
4	Carteolol	0.93	27	Hydroflumethiazide	1.66
5	Pindolol	0.94	28	Dichlorphenamide	1.90
6	Nadolol	0.97	29	Chlorthalidone	1.94
7	Triamterine	1.01	30	Trichlormethiazide	2.22
8	Timolol	1.20	31	Methyclothiazide	2.33
9	Acebutolol	1.23	32	Metolazone	2.53
10	Metoprolol	1.24	33	Furosemide	2.67
	Metoprolol-d7 (IS)	1.24		Furosemide-d5 (IS)	2.66
11	Levobunolol	1.30	34	Indapamide	2.73
12	Esmolol	1.37	35	Benzthiazide	2.77
13	Celiprolol	1.47	36	Cyclothiazide	2.84
14	Oxprenolol	1.48	37	Bendroflumethiazide	2.94
15	Labetolol	1.57	38	Bumetanide	3.33
16	Bisoprolol	1.58	39	Probenecid	3.52
17	Metipranolol	1.68	40	Ethacrynic Acid	3.64
18	Propranolol	1.69			
19	Alprenolol	1.73			
20	Betaxolol	1.79			
21	Clopamide	2.21			
22	Carvedilol	2.08			
23	Canrenone	3.50			

Table 1. Names and retention times of beta-blockers and diuretics, sorted by ionization mode.

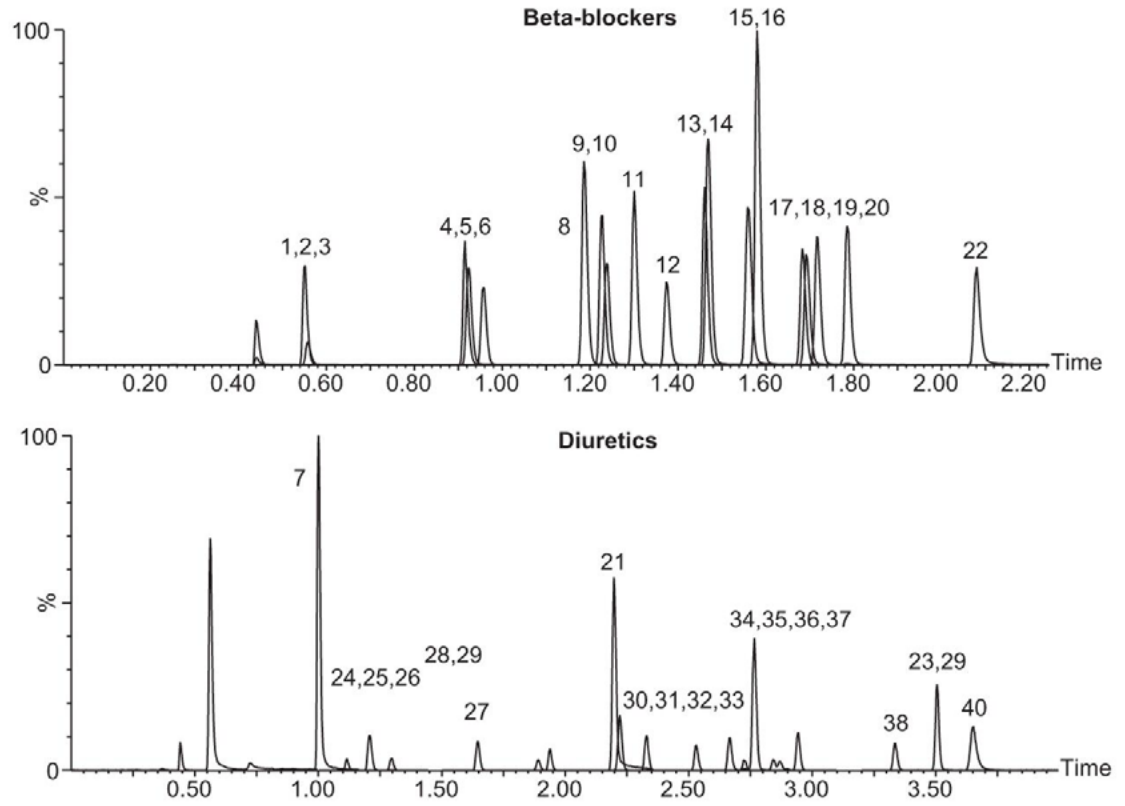

Figure 1. Chromatography of beta-blockers and diuretics. All compounds eluted within four minutes. Analyte labels are listed in Table 1.

Solid phase extraction using the Oasis MAX µElution Plate was performed as follows: 50 µL urine samples were pretreated 1:1 with 5% strong ammonia, then loaded onto the SPE plate, washed with 5:95 MeOH:H2O, eluted with 50 µL of 75:25 MeOH:ACN containing 2% formic acid, and diluted with 200 µL of water. Recovery was efficient for all compounds. Figure 2 shows the average recovery from 12 unique lots of urine. Amiloride, clopamide, and canrenone were grouped with the beta-blockers, as they are bases that ionize by positive ESI. Mean recovery for beta-blockers (and basic diuretics) was 85%, with all but one at 80% or higher; %RSDs were all <20%. Diuretic recovery ranged from 65–94%; all %RSDs <20% with the exception of acetazolamide. The use of the Oasis MAX µElution Plate substantially reduced matrix effects associated with simple sample dilution. As Figure 3 shows, matrix effects, particularly ion suppression, increased from negligible levels to up to over 60% for many of the beta blockers and from 20–40% up to 60–90% for many of the diuretics, even at a 1:10 dilution. Single-factor Anova analysis revealed that matrix effects were significantly increased for all compounds except for acebutolol, metolazone, bumetanide, probenecid, and ethacrynic acid.

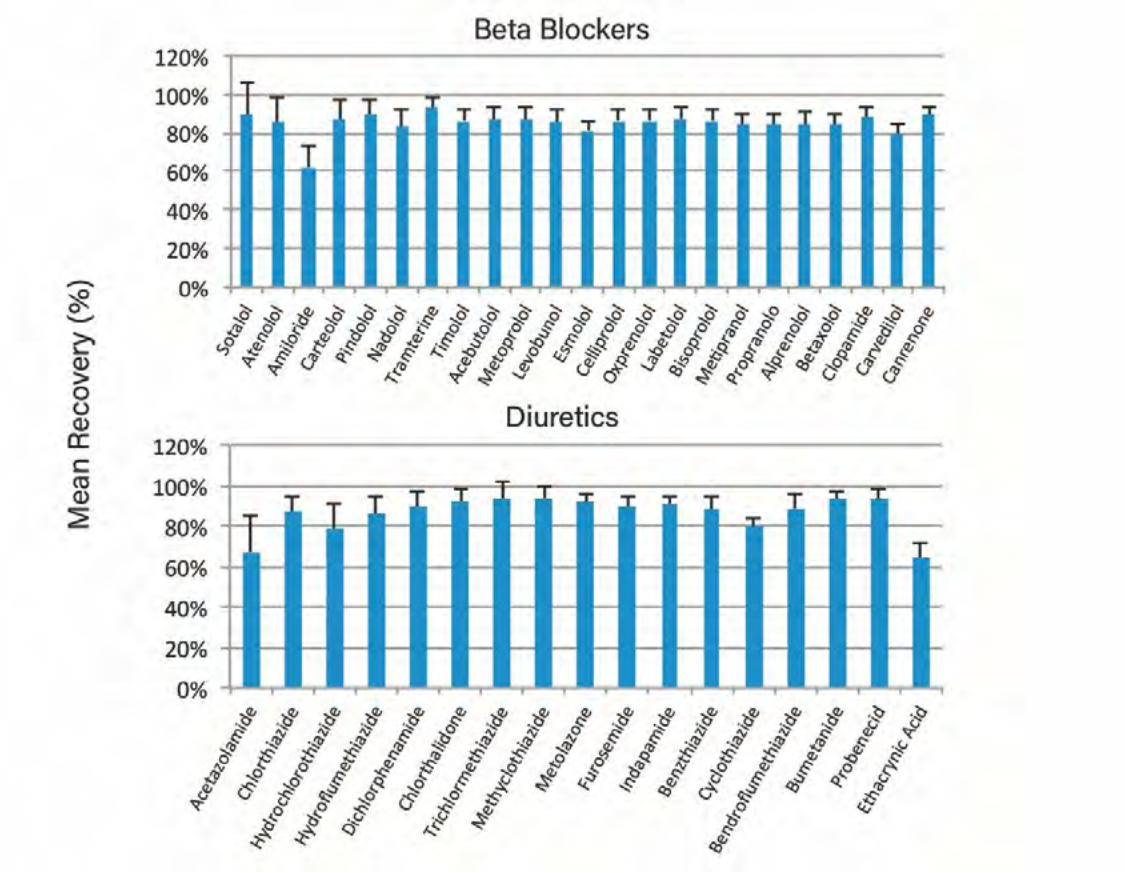

Figure 2. Mean recovery of beta-blockers and diuretics from 12 unique lots of urine matrix. Beta-blocker recovery averaged 85% and all %RSD's <20%. Diuretic (negative ESI) recovery ranged from 64-94%. All %RSD's <20% with the exception of acetazolamide (26%). Bars and error bars represent mean +/- S.D. (N=12).

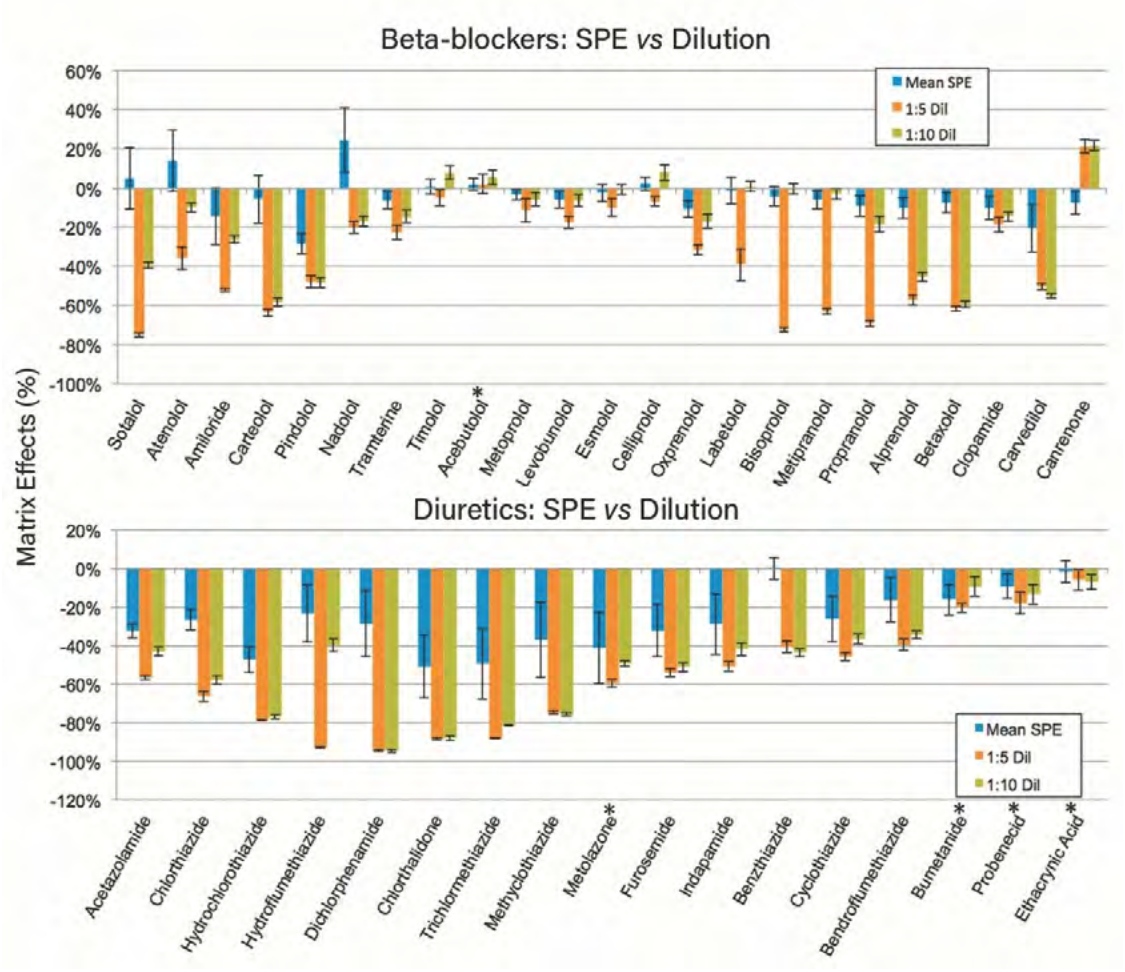

Figure 3. Matrix effects for beta-blockers and diuretics. Mean matrix effects from 12 lots of urine were compared to matrix effects from 1:5 and 1:10 dilution of pooled blank urine in 97:2:1 H_2 O:ACN:formic acid. Even at 1:10 dilution, ion suppression was significantly increased in the diluted samples compared to those prepared by SPE with the MAX µElution plate (N=12 for mean SPE matrix effects and N=4 for the diluted samples). Asterisks indicate compounds in which matrix effects were NOT different between SPE prepared and diluted samples.

All compounds were readily detectable, even at 20% of WADA's Minimum Required Performance Level (MRPL) of 100 ng/mL for beta-blockers and 200 ng/mL for the diuretic compounds.[5] Retention time tolerances were well within WADA requirements as well.[5] Despite the speed of the analysis and the need for polarity switching, WADA ion ratio criteria for confirmation were met for all compounds, even at 20% of the MRPL.[6]

Conclusion

The use of mixed-mode SPE and the ACQUITY UPLC I-Class/Xevo TQ-S micro System enabled clean, fast, and efficient extraction and analysis of urinary diuretics and beta-blockers for anti-doping purposes. The Oasis MAX µELution plates enabled efficient and reproducible extraction of 20 beta-blockers and 20 diuretic compounds. Matrix effects were significantly decreased compared to diluted samples. The Xevo TQ-S micro had the speed and sensitivity to accurately identify all analytes in under four minutes, meeting WADA's strict ion ratio criteria even at 20% of the MRPL. The ability to perform rapid inter-scan polarity switching enabled the analysis in a single injection, saving both time and instrument wear compared to sequential injections of positive and negative ionizing compounds. This will enable rapid, accurate analysis of these compounds, while maintaining a cleaner UPLC-MS/MS system.

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