Single Injection Targeted Screening for DUID Testing

Combined Analysis of 98 Acidic and Basic Drugs in Human Whole Blood Using the SCIEX QTRAP® 5500 LC-MS/MS System

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Recently, there has been a steady increase in the number of police officers submitting DUI analysis requests to screen for drugs they suspect the driver may be under the influence of. These requests have dramatically increased since the sale of recreational marijuana in state licensed stores became legal on July 1st, 2017 in Nevada. The DUI testing policy for the forensic toxicology laboratory at the time was that any DUI whole blood sample with a blood alcohol concentration (BAC) above 0.084% (taking uncertainty of measurement into account) would not go onto further testing to determine if there were also any drugs in the driver's blood at the time of the incident. Following a retrospective study on adjudicated whole blood DUI casework in late 2017, the Henderson Police Department's Forensic Toxicology Laboratory found that approximately 60% of the DUI cases from this study that were not previously analyzed due to having a BAC above 0.084% also had drugs in their system at the time of the incident in which they were arrested. These findings prompted the development of a rapid, robust and selective screening method for positive identification of a panel of specific drugs.

In this technical note, a sensitive and reliable workflow using the SCIEX QTRAP 5500 System for sub-nanogram per mL detection of drugs in human whole blood is presented. This targeted screening method is shown to provide a quantitative workflow enabling rapid and confident identification of multiple drugs in a whole blood sample for high-throughput screening.

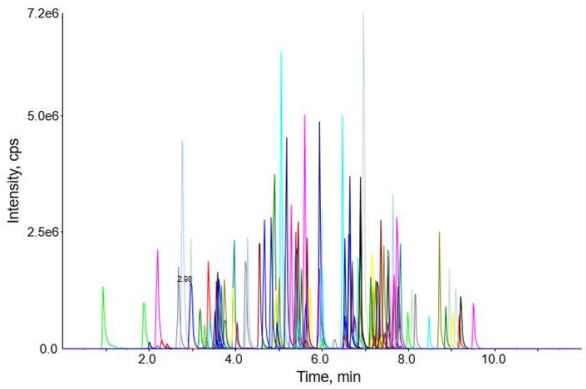


Figure 1: Ultra-Fast Detection of 98 Target Compounds for Confident Drug Identification Using MRM. Total Ion Chromatogram (TIC) resulting from the MRM survey.

Key Features of the QTRAP 5500 LC-MS/MS System

- The SCIEX QTRAP 5500 System is designed to deliver a high level of sensitivity and robustness even for the most complex matrices, making it an ideal fit for forensic screening and confirmation applications.
- Unique QTRAP Technology offers up to 100X more full-scan sensitivity over basic triple quadrupole MS system, making it ideal for non-targeted screening workflows.
- Ultra-fast scan speeds improve precursor ion and neutral loss scan performance allowing the duty cycle to match the time scale required by fast LC.
- *Scheduled* MRM™ algorithm maximizes dwell time without sacrificing quantitative accuracy and precision.
- Optimized chromatography allows separation of 98 target analytes with a high sensitivity of detection (0.5 ng/mL).
- Simultaneous identification and confirmation of drugs of abuse (DOA) can be performed using the full scan MS/MS data (Enhanced Product Ion (EPI) and automated MS/MS library searching).

Methods

Sample Preparation: A total of 98 target analytes were screened in this workflow. They consisted of 35 Rx depressants, 16 benzodiazepines, 17 stimulants, 13 opiates, 12 THC/synthetic cannabinoids, 3 synthetic cathinones and 2 OTC-depressants. The full list of analytes included in this panel is summarized in Table 1. Extraction of whole blood samples was performed using the UCT QuEChERS extraction kits according to procedures shown in Figure 2. Following the SPE procedure, 5 µL of the reconstituted solution were injected for each compound.

Load to tube	•2 mL CH_3CN/NH_4OH (95:5)
Load to tube	•1 mL whole blood
Load to tube	•100 µL internal standard
Vortex	•5 sec
Centrifuge	•10 min at 2,500 rpm
Transfer	•1 mL of supernatant to UCT spin filter tube
Centrifuge	•5 min at 4,750 rpm
Filter	•Keep purified sample in spin filter, remove cap and filter
Evaporate	•Evaporate to dryness under nitrogen at 35˚C
MeOH addition	•Add 50 µL of HPLC MeOH to tube and vortex
Reconstitute	•Add 450 µL of HPLC-grade H_2O to tube and vortex
Transfer	•Transfer to glass vial and inject 5 µL onto instrument

Figure 2: Solid Phase Extraction (SPE) Workflow of Whole Blood Samples Using the UTC QuEChERS Extraction Kit. A 12-step extraction protocol was used for selectively extracting the internal standards from whole blood samples for analysis with the QTRAP 5500 System.

HPLC Conditions: The separation was performed on a Shimadzu Prominence UFLC system using a Phenomenex Kinetex® phenyl-hexyl column (50x4.6mm, 2.6µm, 00B-4495-E0). Mobile phase A (MPA) and mobile phase B (MPB) were ammonium formate with formic acid in water and formic acid in methanol, respectively. The total HPLC runtime was 12 minutes.

Mass Spectrometry: Analysis was performed on the SCIEX QTRAP 5500 System with IonDrive™ Turbo V source and Electrospray Ionization (ESI) probe. A targeted drug screening method was developed using a highly sensitive and selective Multiple Reaction Monitoring (MRM) survey scan, followed by full scan MS/MS analysis (EPI – Enhanced Product Ion scan) on the detected analytes using the linear ion trap mode. A schematic of the workflow used for this targeted screening experiment is shown in Figure 3.

Data Processing: MS/MS library searching was used for confirmation of detected compounds in the linear ion trap using Analyst® Software Reporter.

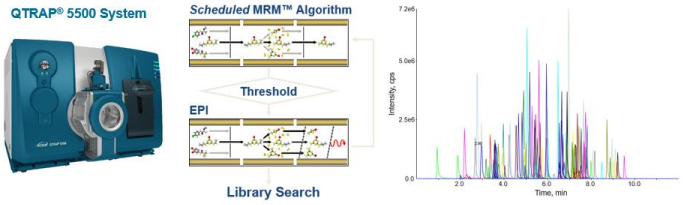
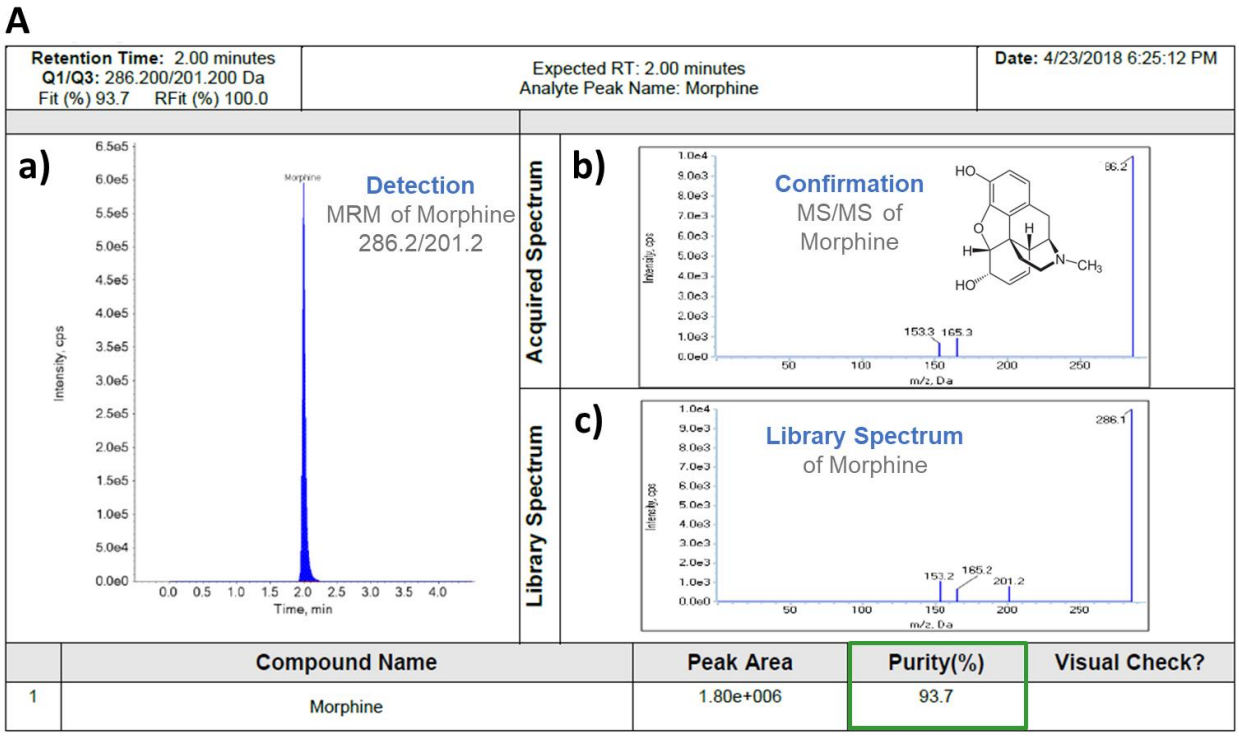


Figure 3: Workflow Used for Targeted Screening of 98 Target Compounds in Whole Blood Using the QTRAP 5500 System. a) A comprehensive drug screening method was performed on a QTRAP 5500 System, b) A sensitive and selective *Scheduled* MRM Algorithm survey scan was initially performed followed by the collection of full scan MS/MS analysis (EPI – Enhanced Product Ion scan) on each the 98 detected analytes for ID confirmation, and c) Resulting Total Ion Chromatogram (TIC) for the MRM survey scan resulting from the separation and detection of the 98 target analytes.

Also listed in Table 1 is the cutoff concentration set for each analyte in the screening method. This cutoff is based on therapeutic potency for each drug, and a positive result in the drug screen is any result that is above that cutoff concentration for a particular analyte. The cutoff concentration for each analyte in the screening method also corresponds with the LOQ for the quantitative method used for each analyte, such that any drug detected below the LOQ is excluded from quantitative analysis.

Compound Identification Using Full Scan MS/MS and Spectral Library Searching

The QTRAP 5500 System is a hybrid triple quadrupole linear ion trap mass spectrometer which allows acquisition of a selective MRM survey scan followed by an EPI scan (full scan MS/MS) which is triggered when signal is detected for each specific MRM transition. The use of MRM selected screening and MS/MS library searching allows for great selectivity even among structurally related analytes eluting off the column at similar retention times. The acquired full scan MS/MS spectra contain the complete molecular fingerprint of the compounds and can be searched against relevant spectral libraries for confident identification using spectral library database searching. This approach provides accurate compound confirmation which significantly reduces the risk of false positives in the unknown samples. Using this comprehensive screening method, 98 different compounds were readily detected and identified in a 12-minute analysis. Figure 4 shows extracted ion chromatograms (XICs), MS/MS spectra with MS/MS matched library spectra for morphine (A) and amphetamine (B). The MS/MS spectra allowed the positive identification of these compounds through spectral library searching.



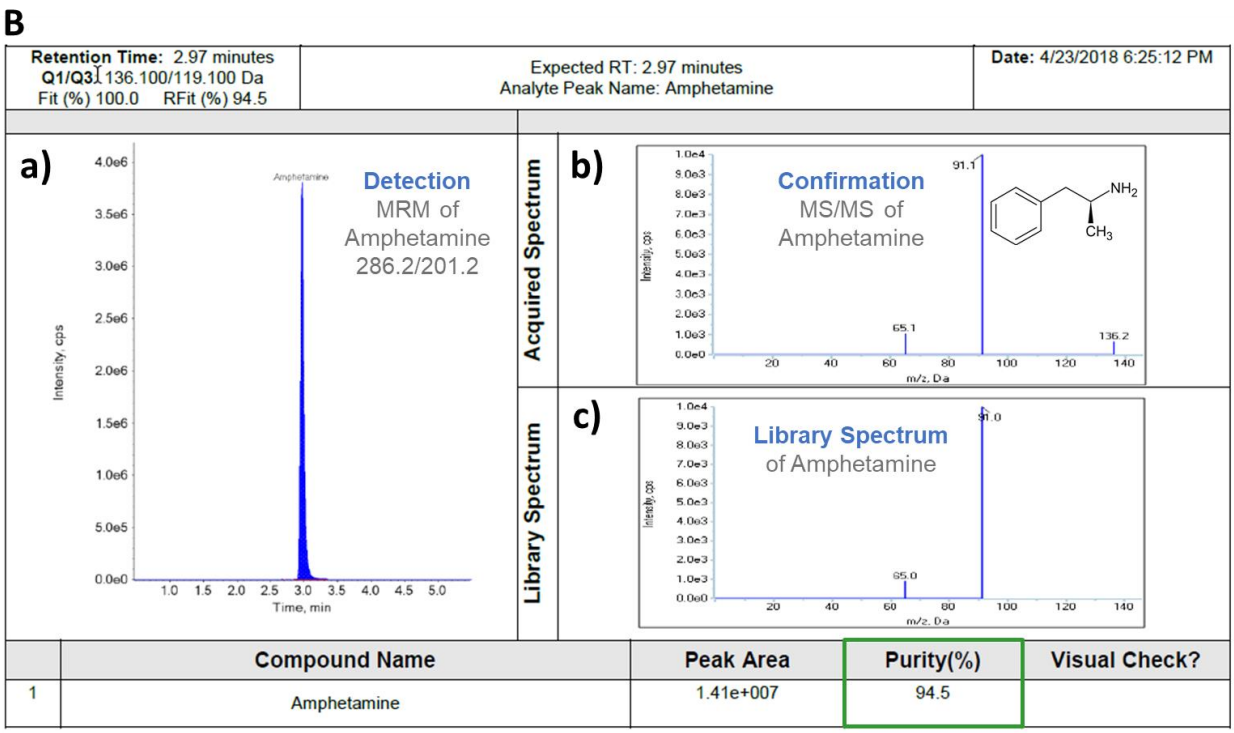


Figure 4: Automatic MS/MS Library Search for Confident Compound Identification. Example of the detection of (A) morphine and (B) amphetamine from a case sample. a) Extracted ion chromatograms, b) MS/MS spectra used for confirmation and c) MS/MS library spectra used to confirm identification. MRM XIC provides quantitative data and the full scan MS/MS is matched to the library spectrum for confident confirmation. Purity scores of 93.7% (morphine) and 94.5% (amphetamine) provide for a definitive detection of the drugs.

Development of a Robust Processing Method Using MultiQuant™ Software

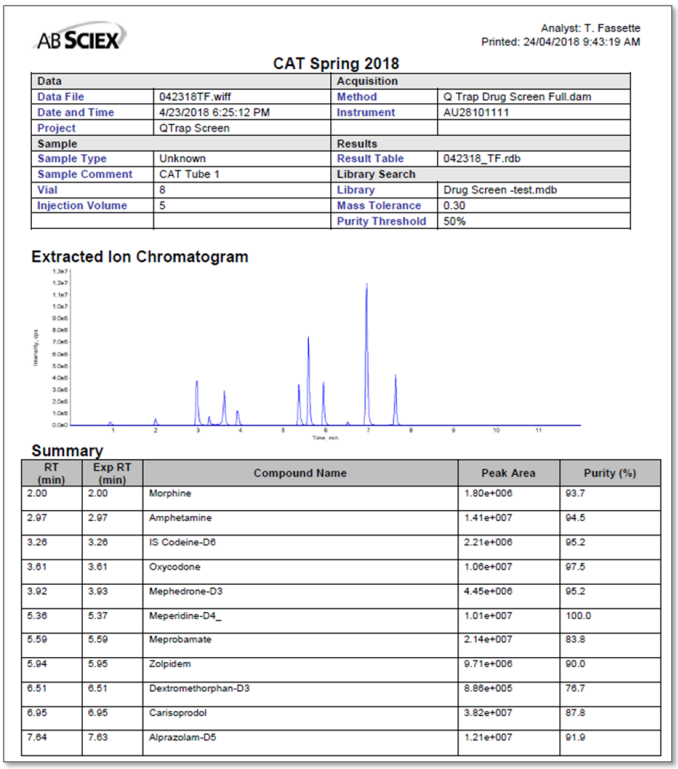
Data was acquired with Analyst 1.7 and processed with Multiquant Software 3.0.3. A processing method for the 98-analyte panel was developed to analyze the time-scheduled MRM data. Detection and integration of the peaks from the background was easily accomplished using MultiQuant Software. The correct peak was automatically selected within the viewing window based on the MRM transition of each compound, reducing the chance of calling peaks from other closely eluting compounds.

Analytical Performance of the QTRAP 5500 System

Following the creation of a robust processing method, calibration curves were generated to determine the limits of quantification (LOQ). The method showed great sensitivity as it was able to readily detect ions down to 0.5 ng/mL. The limit of detection for the concentration of each cutoff control sample in the method was easily discernable from negative samples that were run. The *Scheduled* MRM algorithm used in this workflow aided in the acquisition of many more compounds by collecting MRM transitions just around the expected elution time for each compound, resulting in an increase of data quality and confidence in forensic analyte detection at sub ng/mL concentration levels. The use of the QTRAP 5500 ystem provided speed and sensitivity to the assay while allowing to streamline the identification of drugs present in the case samples at different concentrations in a single analytical workflow. Combining this high quality quantitation data with the library matching, this screening method enables improved robustness without the false positive and false negative results that are commonly seen with other screening methods used in forensic toxicology laboratories.

Generation of Case Sample Reports

The data analysis component of MultiQuant Software is designed to provide a centralized results grid for streamlined review and efficient case sample report processing. One of the advantageous features of the software is that a case sample report can easily be generated for each sample at the click of a button. The report is highly customizable and contains detailed information on sample, instrument and other relevant information such as experimental conditions and data processing parameters. In addition, retention time errors, peak area and purity scores can be automatically calculated and visualized in the Analyst Software Reporter.



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Analyst: T. Fassette
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CAT Spring 2018

Data		Acquisition	
Data File	042318TF.wiff	Method	Q Trap Drug Screen Full.dam
Date and Time	4/23/2018 6:25:12 PM	Instrument	AU28101111
Project	QTrap Screen		
Sample		**Results**	
Sample Type	Unknown	Result Table	042318_TF.rdb
Sample Comment	CAT Tube 1	**Library Search**	
Vial	8	Library	Drug Screen -test.mdb
Injection Volume	5	Mass Tolerance	0.30
		Purity Threshold	50%

Extracted Ion Chromatogram

Summary

RT (min)	Exp RT (min)	Compound Name	Peak Area	Purity (%)
2.00	2.00	Morphine	1.80e+006	93.7
2.97	2.97	Amphetamine	1.41e+007	94.5
3.26	3.26	IS Codeine-D6	2.21e+006	95.2
3.61	3.61	Oxycodone	1.06e+007	97.5
3.92	3.93	Mephedrone-D3	4.45e+006	95.2
5.36	5.37	Meperidine-D4_	1.01e+007	100.0
5.59	5.59	Meprobamate	2.14e+007	83.8
5.94	5.95	Zolpidem	9.71e+006	90.0
6.51	6.51	Dextromethorphan-D3	8.86e+005	76.7
6.95	6.95	Carisoprodol	3.82e+007	87.8
7.64	7.63	Alprazolam-D5	1.21e+007	91.9

Figure 5. Generation of Case Sample Reports Using the Analyst® Reporter Component of the Software. (Top) Detailed summary of sample, instrument, data processing parameters, etc. (Middle) Extracted Ion Chromatogram (XIC) of the detected analytes. (Bottom) Summary table listing all the detected drugs, their peak areas as well as the purity scores obtained from the library matching of the MS/MS spectra.

Figure 5 shows a customized sample report generated following the analysis of a case sample using the *Scheduled* MRM algorithm previously described. Eleven drugs were accurately identified in the case sample based on the MRM transition peak areas, retention times and purity scores from matched MS/MS. The streamlined data processing made possible by Analyst Software Reporter provided a simplified interface for fast data analysis and quick sample report generation to maximize the forensic laboratory operational efficiency. A comprehensive sample report containing all the relevant case sample report information can be easily created at the click of a button, increasing throughput and efficiency in the toxicology laboratory. The results generated provide both qualitative data and quantitative confirmation in a single and comprehensive report.

Table 1. List of Analytes Used in This DUID Panel Along with Cutoff Concentration (LOD).

Analytes	Cutoff (ng/mL)	Analytes	Cutoff (ng/mL)	Analytes	Cutoff (ng/mL)
Rx Depressants		**Benzodiazepines**		**Opiates**	
Gabapentin	200	7-Aminoclonazepam	20	Morphine	10
Ketamine	20	7-Aminoflunitrazepam	20	Oxymorphone	2
Meperidine	20	Midazolam	20	Hydromorphone	2
Normeperidine	20	Clonazepam	20	Codeine	10
Zopiclone	20	Lorazepam	20	Dihydrocodeine	10
Zolpidem	20	Chlordiazepoxide	20	Oxycodone	2
Zaleplon	20	Alpha-Hydroxyalprazolam	10	6-MAM	10
Citalopram	20	Oxazepam	20	Hydrocodone	2
Norcitalopram	20	Norflurazepam	20	Norfentanyl	1
Methadone	20	Flunitrazepam	20	Tramadol	20
EDDP	20	Temazepam	20	Fentanyl	0.5
Doxepin	20	Alprazolam	10	Acetyl Fentanyl	1
Carisoprodol	200	Etizolam	20	Mitragynine	1
Meprobamate	200	Nordiazepam	20	**THC/Synthetic Cannabinoids**	
Fluoxetine	20	Diazepam	20	THC	2
Norfluoxetine	20	Hydroxyzine	10	11-Hydroxy-THC	2
Levorphanol	10	**Stimulants**		Carboxy-THC	4
Pheniramine	10	Amphetamine	20	AB-PINACA	10
Bupropion	10	Methamphetamine	20	AB-FUBINACA	10
Mirtazapine	10	MDMA	20	AB-CHIMINACA	10
Venlafazine	10	MDA	20	AM-2201	10
Trazodone	10	Anhydroecgonine Methyl Ester	20	JWH-018	10
Carbamazepine	10	Phentermine	20	JWH-073	10
Quetiapine	10	Benzoylecgonine	20	PB-22	10
Amoxapine	10	MDEA	20	UR-144	10
Paroxetine	10	Methylphenidate	10	RCS-4	10
Triazolam	10	Cocaine	10	**Synthetic Cathinones**	
Buprenorphine	1	Cocaethylene	10	Ethylone	10
Cyclobenzaprine	10	Benzylpiperazine	10	Alpha-PVP	10
Desipramine	20	Psilocin	5	MDPV	10
Amitriptyline	20	N,N-Dimethyltryptamine	10	**OTC-Depressants**	
Nortriptyline	20	TFMPP	10	Diphenhydramine	20
Sertraline	20	PCP	2	Dextromethorphan	10
Clomipramine	20	LSD	1		
Tapentadol	20				

Conclusions

The combination of a solid phase extraction (SPE) procedure and optimized chromatography with the highly sensitive QTRAP 5500 System allowed quick and reliable identification of 98 target compounds for DUID testing. This workflow enabled sub-nanogram per mL detection of drugs in a complex biological matrix such as human whole blood while maintaining linearity and precision for all compounds across the calibration range.

- A 12-step extraction protocol was rapidly implemented to the screening workflow to extract the analytes from human whole blood.
- A targeted drug screening method using the MRM survey scans followed by data dependent triggering of full scan MS/MS was developed for fast, selective and sensitive detection and identification of drugs in a single analysis.
- The method showed great sensitivity as it was able to readily detect ions down to 0.5 ng/mL.
- The use of Analyst Software Reporter provided a streamlined data review process and allowed generation of a quick sample report, increasing throughput and efficiency.
- The chromatographic separation was optimized for this MRM assay but can also be used on the SCIEX X500R QTOF for additional screening or confirmation techniques.

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