A Modified QuEChERS Approach for the Extraction of Common Prescription and Illicit Drugs from Liver Prior to LC/MS-MS Analysis

UCT Part Numbers

ECQUUS1115CT
15 mL centrifuge tube containing 800mg $MgSO_4$ and 200mg NaCl

CUMC182CT
2 mL dSPE tube containing 150mg MgSO4 and 50mg C18

SLDA50ID21-3UM
Selectra® DA HPLC
50 x 2.1 mm, 3 µm

SLDAGDC21-3UM
Selectra® DA Guard Column
10 x 2.1 mm, 3 µm

SLGRDHLDR
Guard Column Holder

CLINICAL

FORENSICS

Summary:

Blood and urine are by far the primary biological fluids of choice for toxicology analysts to work with in the lab, however, other matrices are often tested to either substantiate the concentrations found in blood and urine or in instances of limited sample. Liver is the primary alternative tissue used for toxicological analysis based on the biological role it plays in the metabolism of drugs and toxicants in the body. Drugs become concentrated in this vital organ and can be found even when there are no detectable quantities present in the blood. This additional information becomes very critical when trying to determine the cause of death.

While the benefits for analyzing liver are clear, the one major drawback is the amount of sample preparation needed in order to get specimens ready for analysis. After liver samples are homogenized, they must undergo further extraction methods such as solid phase or liquid-liquid extractions. While neither of these techniques are particularly difficult, they do have their draw backs. Liquid-liquid extraction methods have the ability to extract several compounds at once, but they can be time consuming and usually require greater quantities of solvents compared to other methods. This increases the overall cost per sample for laboratories. Solid phase extraction methods tend to be quicker and more cost effective, however, if samples are not homogenized properly, column clogging and inconsistent flow rates can lead to inconsistent results for analysts.

Scientists in the forensic community are always looking for methods that will allow for them to achieve the optimum balance between producing reliable results and saving time and cost on analysis. QuEChERS (pronounced "catchers") is an acronym for Quick, Easy, Cheap, Effective, Rugged and Safe. This technique was originally developed for multi-residue pesticides analysis in fruits and vegetables in 2003. This method outlines a modified QuEChERS based procedure that has been optimized to extract various drug compounds from liver prior to analysis by LC-MS.

Sample Pretreatment:

Homogenize liver samples with deionized water. For this work 20g of bovine liver was thoroughly blended with 80g of water in a RobotCoupe® to generate a homogenous sample for use during method development and recovery experiments.

QuEChERS Procedure:

Sample Extraction:

1. Add 2 mL MeCN with 5% NH_4OH to a 15 mL centrifuge tube containing 800 mg $MgSO_4$ and 200 mg NaCl.
2. Add internal standard(s) and appropriate amount of spiking solutions to fortify samples.
3. Add 2 mL liver homogenate, vortex briefly to break up any salt agglomerates.
4. Shake for a minimum of 5 minutes (by hand or mechanically). For this work a Spex 2010 Geno/Grinder® was used (1500 RPM).
5. Centrifuge the sample at ≥ 3000 rcf for 10 minutes.

dSPE Clean-up:

1. Transfer 1 ml of supernatant to a dSPE cleanup tube containing 150 mg $MgSO_4$ and 50mg C18
2. Vortex the sample for 1 minute.
3. Centrifuge the sample at ≥ 3000 rcf for 5 minutes.
4. The purified samples can now be transferred to autosampler vials containing water for analysis. For this work 500ul of the purified sample was dried to completion and reconstituted with mobile phase.

LC-MS/MS Parameters:

Table 1. Instrumentation

HPLC System: Agilent 1200 Series

MS System: AB Sciex 4000 Q Trap

HPLC Column: UCT, Selectra®, DA, 50 x 2.1 mm, 3 µm

Guard column: UCT, Selectra®, DA, 10 x 2.1 mm, 3 µm

Column temperature: 40 °C

Column flow rate: 0.400 mL/min

Auto-sampler temperature: 10 °C

Injection volume: 10 µL

Gradient program:

Time (min)	**A%** (0.1% formic acid in H_2O)	**B%** (0.1% formic acid in MeOH)
0	85	15
0.5	85	15
12	5	95
13	5	95
13.5	85	15
17	85	15

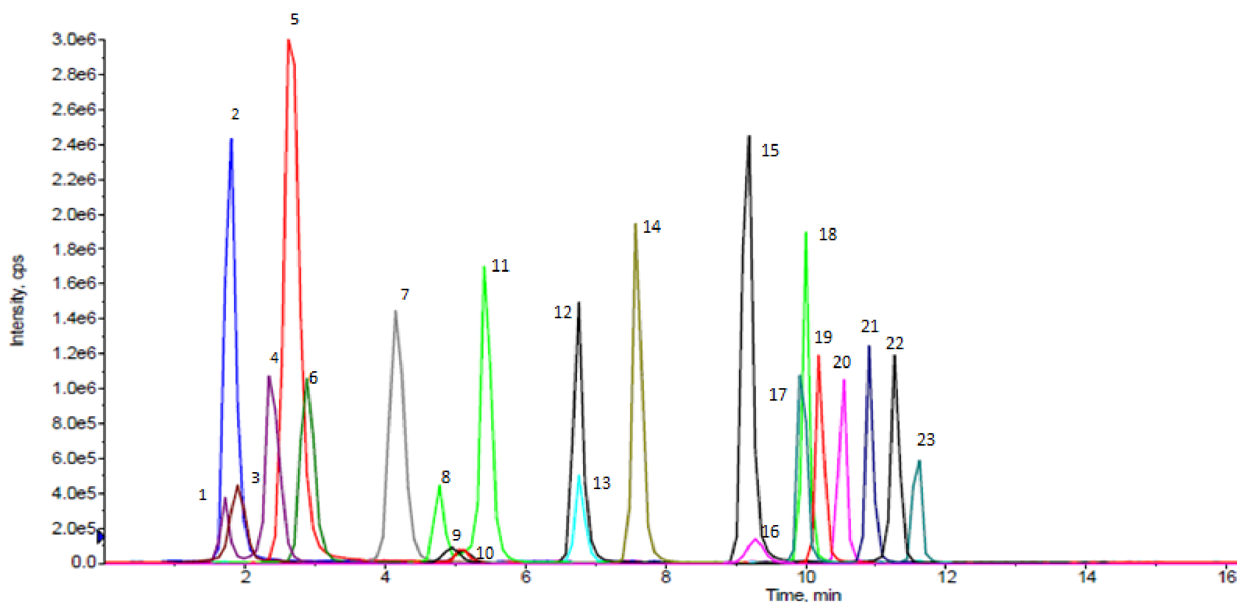

Figure 1: Chromatogram of an extracted liver sample spiked at 100 ng/g
1.Morphine 2.Amphetamine 3.Oxymorphone 4.Hydromorphone 5.Methamphetamine 6.MDA 7.MDMA 8.Codeine 9.6-MAM 10.Oxycodone 11.Hydrocodone 12.BE 13.7-amino Clonazepam 14.Cocaine 15.PCP 16.Midazolam 17.Lorazepam 18.Oxazepam 19.Clonazepam 20.Nordiazepam 21.Temazepam 22.Alprazolam 23.Diazepam

Table 2. MRM Transitions

Analyte	Retention Time (min)	Q1	Q3	Analyte	Retention Time (min)	Q1	Q3
Morphine	1.63	286.0	152.0	**7-Amino Clonazepam**	6.76	286.1	222.3
Amphetamine	1.79	136.1	91.2	**Cocaine**	7.59	304.1	182.0
Oxymorphone	1.82	302.0	227.0	**PCP**	9.21	244.0	86.1
Hydromorphone	2.31	286.0	185.0	**Midazolam**	9.30	326.0	291.0
Methamphetamine	2.64	150.0	91.1	**Lorazepam**	9.93	321.0	275.0
MDA	2.85	180.2	105.0	**Oxazepam**	10.00	287.0	241.3
MDMA	4.14	194.2	105.1	**Clonazepam**	10.19	316.1	270.2
Codeine	4.77	300.0	152.0	**Nordiazepam**	10.53	271.1	140.1
6-MAM	4.95	328.0	165.0	**Temazepam**	10.91	301.1	255.2
Oxycodone	5.12	316.0	256.0	**Alprazolam**	11.27	309.1	281.2
Hydrocodone	5.43	300.0	199.0	**Diazepam**	11.60	285.1	193.2
Benzoylecgonine	6.72	290.1	168.0				

Results:

Table 3. Recovery and R^2 Data

Analyte (n=4)	Recovery 75ng/g	Recovery 300 ng/g	R^2 0-500ng/g
Morphine	93%	82%	0.97
Hydromorphone	99%	83%	0.98
Oxymorphone	93%	88%	0.96
Codeine	86%	84%	0.95
Hydrocodone	101%	88%	0.97
Oxycodone	83%	92%	0.96
6-MAM	75%	84%	0.95
7-Amino Clonazepam	61%	79%	0.96
Alprazolam	86%	82%	0.95
Clonazepam	86%	95%	0.97
Diazepam	71%	79%	0.93
Lorazepam	78%	84%	0.94
Midazolam	72%	73%	0.98
Nordiazepam	71%	77%	0.95
Oxazepam	77%	73%	0.99
Temazepam	89%	88%	0.97
Amphetamine	86%	84%	0.97
Methamphetamine	94%	85%	0.99
MDA	77%	80%	0.97
MDMA	82%	83%	0.98
Cocaine	86%	86%	0.95
Benzoylecgonine	61%	41%	0.98
PCP	86%	83%	0.98

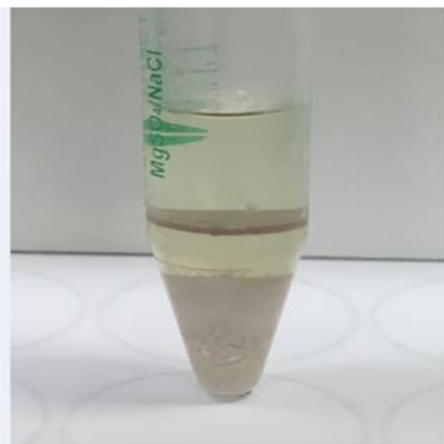

Figure 2: Separation of aqueous and organic layers after initial extraction

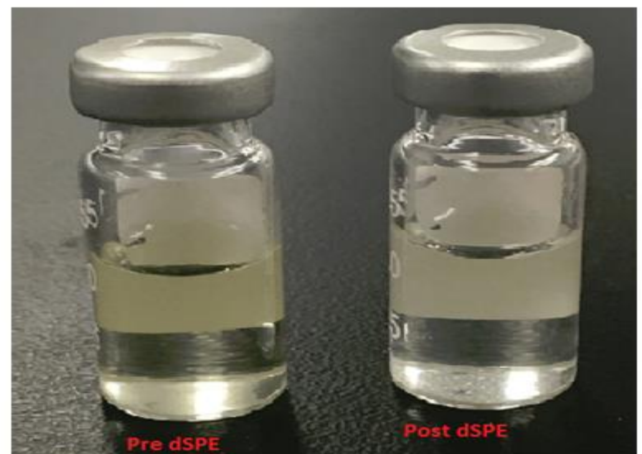

Figure 3: Samples before (left) and after (right) dSPE

CLINICAL
FORENSICS

Discussion:

Excellent recoveries were achieved for the range of analytes included in this study. Recoveries were evaluated by fortifying samples at two varying concentrations. On average, the recovery for samples spiked at 75 ng/g was 81 % and for samples spiked at 300 ng/g it was 83 %. Recoveries were calculated by dividing the chromatographic peak area of samples spiked prior to extraction by the peak area produced by samples that were spiked into a pre-extracted blank matrix.

As laboratories, all over the world explore the uses of QuEChERS, several modifications from the traditional food safety approach will need to be made to improve analyte recovery when applying the method to a forensic toxicological setting. One of the obvious changes that needs to be accounted for is the adjustment of salt and solvent amounts due to smaller sample sizes. Food testing laboratories are accustomed to working with large volumes (the original method started with 10 g of starting material), while in the forensic toxicology realm, sample amount is often limited and once it is consumed, there is rarely, if ever a chance to obtain more. For this reason, the 4:1 $MgSO_4$: NaCl salt blend ratio was maintained from the original method, however since the amount of starting sample was reduced five-fold, the salt ratio was reduced from 4 g $MgSO_4$: 1 g NaCl to 800 mg $MgSO_4$: 200 mg NaCl. The volume of acetonitrile was also minimized to account for the reduction. The compounds of interest explored in this method are also quite diverse in regards to polarity. In further studies, Lehotay et al. found that buffering the extraction to lower the pH greatly improved the recovery of several compounds. [6] Various extraction pH values were evaluated during method development. It was found that increasing the pH greatly improved the overall extraction. At this higher pH most of the compounds being evaluated were promoted into their mostly unionized form, and thus making it easier for them to partition into the organic phase of the initial extraction.

Three dSPE sorbent combinations were explored for maximum clean-up: $MgSO_4$ + C18, $MgSO_4$ + Primary Secondary Amine sorbent (PSA), and $MgSO_4$+ C18 +PSA. PSA and C18 were included in the evaluation due to the high anticipated lipid content of the liver samples and both sorbent's enhanced ability to irreversibly retain such interferences. To evaluate the varying combinations in question, a small experiment was done by preforming the dSPE step of the procedure utilizing the basified acetonitrile extraction solvent spiked with the drugs in question. The final "extracts" were then compared to neat standards that were spiked into the same extraction solvent. Eliminating the matrix factor from this investigation allowed for observations to be made in regards to how the analytes would respond to the sorbents in question by removing any bias that may have occurred do to analyte enhancement or suppression upon analysis. While some analyte loss was noted with all of the sorbent combinations, $MgSO_4$+C18 only demonstrated minimal analyte loss, where any combinations featuring PSA were affected at a much greater extent. Many of the analytes compromised in the presence of PSA featured two ionizable groups and contained partial negative charges at the basified pH. The loss is most likely attributed to the PSA forming ionic bonds with the analytes that possess that negative charge.

Conclusion:

This application note outlines a modified QuEChERS-based method for the extraction of prescription and illicit drugs from postmortem liver. Samples are extracted with unbuffered QuEChERS salts followed by dSPE cleanup of the supernatant using C18, yielding a clear extract. Analysis is performed by LC-MS/MS using a Selectra® DA HPLC column. Absolute recoveries using this method ranged from 41% to 101% with a majority being 80% or higher. No internal standards were used in this study; however, it is strongly recommended to use matrix-matched calibration curves, along with isotopically labeled internal standards to compensate for any analyte loss or remaining matrix that is not removed via the extraction procedure. While the QuEChERS method itself has not proven to be a silver bullet, it has begun to offer new advantages to the forensic toxicology community when overcoming complex matrices.

References:

1. Anastassiades M, Lehotay SJ, Stajnbaher D, Schenck FJ. *Fast and easy multiresidue method employing acetonitrile extraction/partitioning and dispersive solid-phase extraction for the determination of pesticide residues in produce.* J AOAC Int. 86 (2003) 412.
2. Quechers.com
3. sampleprep.unitedchem.com/products/quechers
4. Levine, Barry, Ph.D.,DABFT,DABCC-T, ed. *Principles of Forensic Toxicology*. 3rd ed. Washington DC: ACC, 2009. Print.
5. Westland, Jessica L., and Frank L. Dorman. *QuEChERS Extraction of Benzodiazepines in Biological Matrices*. Journal of Pharmaceutical Analysis 3.6 (2013): 509-17.
6. Steven J.Lehotay,Kyung AeSon, Hyeyoung Kwon, Urairat Koesukwiwat, Wusheng Fu, Katerina Mastovska, Eunha Hoh, Natchanun Leepipatpiboon. *Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables*. Journal of Chromatography A – 2010
7. Dulaurent, Sylvain, Souleiman El Balkhi, Lauranne Poncelet, Jean-Michel Gaulier, Pierre Marquet, and Franck Saint-Marcoux. *"QuEChERS Sample Preparation Prior to LC-MS/MS Determination of Opiates, Amphetamines, and Cocaine Metabolites in Whole Blood."* Analytical and Bioanalytical Chemistry 408.5 (2016): 1467-474.
8. Plössl F, Giera M, Bracher F. *Multiresidue Analytical Method Using Dispersive Solid-phase Extraction and Gas Chromatography/ion Trap Mass Spectrometry to Determine Pharmaceuticals in Whole Blood.* Journal of Chromatography A 1135.1 (2006): 19-26.

UCT, LLC • 2731 Bartram Road • Bristol, PA 19007 800.385.3153 • 215.781.9255
www.unitedchem.com Email: methods@unitedchem.com

©UCT, LLC 2017 • All rights reserved