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

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Traditional sample extraction methods tend to focus on a small number of compounds or a single drug class to achieve optimal results. Unfortunately, with enhanced specificity comes an increase in overall sample preparation time and cost. The high demands placed on toxicology laboratories to produce accurate results quickly has fuelled a transition towards universal sample preparation techniques. These broad approaches are fast and efficient as they are able to encompass more analytes and drug classes within a given panel; however, since many of these methods are not as selective, analysts may be forced to compromise on analyte recovery and sample cleanliness.

Preliminary studies have shown that the QuEChERS technique should be considered as a reliable alternative when extracting multiple drug classes from challenging biological matrices. The project described here, explores a modified universal QuEChERS approach for sample clean-up in post-mortem liver, coupled with LC-MS detection, for the analysis of several common prescription and illicit drugs.

Introduction

The sensitivity and resolving power of analytical instrumentation being used in the forensic toxicology community has vastly evolved in the past decade. Once samples are received, analysts are tasked with identifying and quantitating a wide variety of drugs and toxicants from an assortment of matrix types. Even with improvements to instrumentation, this is no easy feat. The sample preparation technique on these given matrices largely dictates the quality and validity of the final results. Many new instruments feature enhanced sensitivity and regulatory reporting limits as a result are being lowered. The number of compounds needing to be identified and quantitated is also steadily on the rise. Unfortunately, if samples are not handled appropriately, it can curtail the ability for analysts to achieve reliable results, often times leading to a compromise on the sample preparation approach in order to save time and money.

One possible solution toxicology laboratories are beginning to explore that provides an optimum balance between producing reliable results and saving time and cost on analysis is the QuEChERS approach. Crossing over from the food safety industry, QuEChERS (pronounced 'catchers') is an acronym for Quick, Easy, Cheap, Effective, Rugged and Safe. This technique was originally developed for multi-residue pesticide analysis in fruits and vegetables in 2003 by Anastassiadies et al [1,2]. When QuEChERS were first developed the typical procedure for sample clean-up in the food safety industry was time consuming and required large amounts of solvents.

With the QuEChERS approach, these arduous methods are reduced to three simple steps:

1. A liquid micro-extraction
2. Solid phase clean-up
3. GC-MS or LC-MS analysis.

Initially, QuEChERS salts (magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl)) are added to an aqueous based matrix that has been mixed with acetonitrile. The use of acetonitrile makes final extracts amenable to both GC-MS and LC-MS analysis. The magnesium sulphate serves to bind large amounts of water while the sodium chloride increases the ionic strength of the aqueous phase in addition to inducing phase separation. Following this step, dispersive SPE (dSPE) can then be executed to provide additional matrix removal if this is required. This secondary clean-up also serves to eliminate any residual water that remains from step one and also allows for extraction salts to diffuse homogenously throughout the entire sample. The end result is a more thorough, condensed overall extraction when compared to traditional SPE protocols [3].

Since its conception, this technique has been utilised for sample clean up on a wide variety of matrices to include animal-based products (meat, fish, kidney, chicken, milk, honey), cereals and grains, and other food produce market sectors (wines, juices, fruit and vegetables) [2,3]. The QuEChERS method has not only proven to be simple to perform, but also is rugged enough to withstand any necessary modifications that make it amenable to complex matrices such as those commonly encountered in a forensic toxicological setting.

Various types of specimens are acquired during an autopsy for toxicological analysis. While blood and urine are by far the more desirable biological fluids of choice for analysts to work with, other matrices are often tested to either substantiate the concentrations found in blood and urine or in instances of limited sample [4]. 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 analysing 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 homogenised, 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 homogenised properly, column clogging and inconsistent flow rates can lead to inconsistent results for analysts.

Experimental Sample Preparation

Blank bovine liver samples were homogenised using a Robot-Coupe Blixer ® at a 1:4 ratio by weight with deionised water. Blank liver homogenate (2 mL) was fortified with appropriate amounts of working standards prior to being added to 15 mL centrifuge tubes containing QuEChERS salts (800 mg magnesium sulphate ($MgSO_4$) and 200 mg sodium chloride (NaCl)) and 2 mL of acetonitrile containing 5% ammonium hydroxide. Samples were briefly vortexed to break up any salt agglomerates prior to shaking for 5 minutes at a rate of 1000 strokes/minute using a Spex Geno/Grinder®. After shaking, samples were placed into a centrifuge and spun for 10 minutes at a speed of 3000 rcf. Further sample cleanup was performed by adding 1 mL of the centrifuged supernatant to 2 mL micro-centrifuges tubes containing 150 mg of $MgSO_4$ and 50 mg, silica based C_{18} endcapped sorbent (UCT LLC, Bristol PA, USA. Part Number CUMC182CT). Samples were vortexed at a rate of 100 strokes/minute for 1 minute, then placed into a centrifuge and spun for 5 minutes at a rate of 3000 rcf. A 500 µL aliquot of the final extract was then dried to completion for concentration purposes and reconstituted in 100 µl of the appropriate mobile phase for instrumental analysis; however, a simple dilution of the final extract with water may be performed dependant on the sensitivity of the analytical instrument being used.

Chromatographic Analysis

Analysis was performed using an Agilent 1200 HPLC system combined with an ABSciex 4000 Q Trap. UCT's Selectra® DA 50 mm x 2.1 mm, 3 µm column was used for separation. The column temperature was kept at 40° C and the injection volume was 10 µL. The mobile phases consisted of solvent A: water containing 0.1% formic acid and solvent B: methanol containing 0.1% formic acid with a flow rate of 0.4 mL/min. Analytes were chromatographically separated using the following gradient: A 0.5 minute initial hold at 15% solvent B followed by a linear ramp to 95% solvent B in 12 min. The gradient was held at 95% B for 1 minute after which it was lowered to 15% B in 0.1 min and held at this level for 4 minutes. The entire cycle time was 17 minutes (Figure 1).

The MS was operated in positive ESI mode, analyte MRM transitions can be found in Table 1. Data was acquired and analysed using Analyst Software (version 1.5.2).

Results and 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. A full list of recoveries can be found in Table 2. The responses for the representative compounds were linear with R2 values ranging from 0.93 to 0.99 over a concentration range of 0-500 ng/g, with the lowest spiked calibrator being 25 ng/g for all analytes. Internal standards were not used in this study; however, implementing isotopically labelled internal standards always aid in compensating for any analyte loss and remaining matrix components that were unable to be removed via the extraction. It is important to account for any residual matrix components which may lead to analyte enhancement or suppression upon analysis.

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 being applied 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.

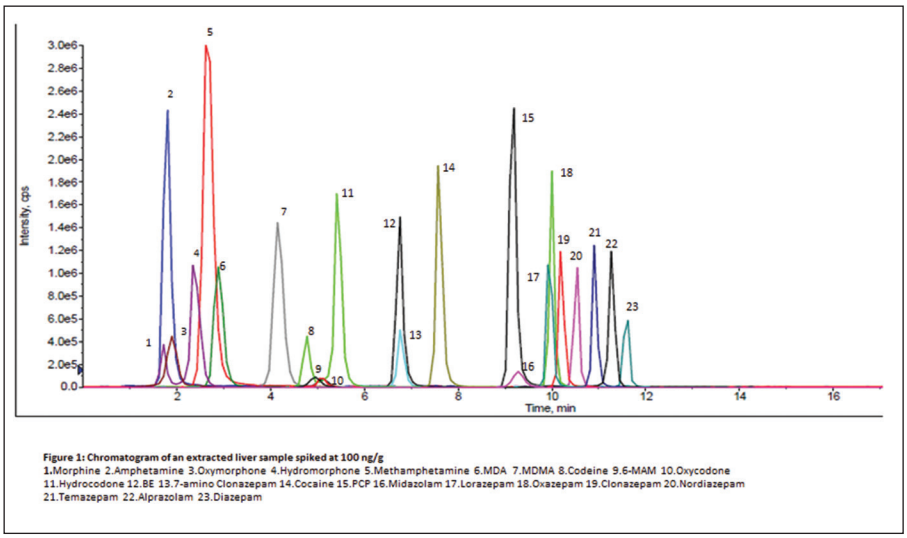

Figure 1: Chromatogram of an extracted liver sample spiked at 100 ng/g
1.Morphine 2.Amphetamine 3.Oxymorphone 4.Hydromorphone 5.Methamphetamine 6.MDA 7.MDMA 8.Codeine 9.6-MAM 10.Oxycodone 11.Hydrocodone 12.BE 13.7-amino Clonazepam 14.Cocaine 15.PCP 16.Midazolam 17.Lorazepam 18.Oxazepam 19.Clonazepam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

Figure 1

Analyte	Retention Time (min)	Q1	Q3
Morphine	1.63	286	152
Amp	1.79	136.1	91.2
Oxymorphone	1.82	302	227
Hydromorphone	2.31	286	185
Methamp	2.64	150	91.1
MDA	2.85	180.2	105
MDMA	4.14	194.2	105.1
Codeine	4.77	300	152
6 Mam	4.95	328	165
Oxycodone	5.12	316	256
Hydrocodone	5.43	300	199
BE	6.72	290.1	168
7-amino Clonazepam	6.76	286.1	222.3
Cocaine	7.59	304.1	182
PCP	9.21	244	86.1
Midazolam	9.3	326	291
Lorazepam	9.93	321	275
Oxazepam	10	287	241.3
Clonazepam	10.19	316.1	270.2
Nordiazepam	10.53	271.1	140.1
Temazepam	10.91	301.1	255.2
Alprazolam	11.27	309.1	281.2
Diazepam	11.6	285.1	193.2

Table 1

The 4:1 $MgSO_4$: NaCl salt blend ratio was maintained, 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 minimised 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]. With this approach, samples were extracted at a higher pH to promote analytes into their unionised form, and thus making it easier for them to partition into the organic phase of the initial extraction.

Several studies have been done to introduce QuEChERS to the forensic community [5,7-8]. In a recent investigation by Dulaurent et al. [7] a single step QuEChERS approach was taken in order to reduce the amount of time for the extraction and increase throughput. The authors produced sound results, even with the elimination of the dSPE step. For the purposes of this investigation, dSPE was executed to further clean up samples (Figures 2 and 3) and introduce a higher threshold of sample purity to the instrumentation at hand. Three dSPE sorbent combinations were explored for maximum clean-up: $MgSO_4$ + C_{18}, $MgSO_4$ + Primary Secondary Amine (PSA) sorbent, and $MgSO_4$ + C_{18} + PSA. PSA and C_{18} 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 utilising 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, $MgSO_4$+C_{18} only demonstrated minimal analyte loss, where any combinations featuring PSA were

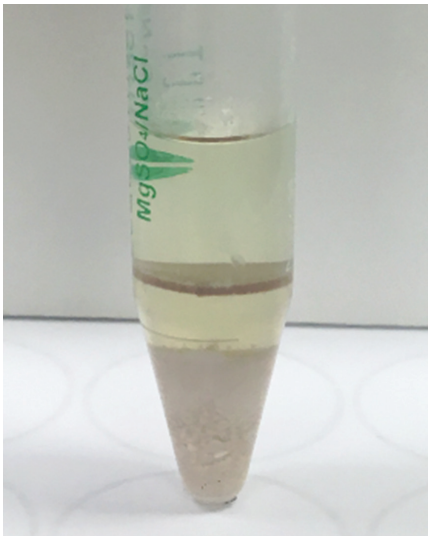

Figure 2

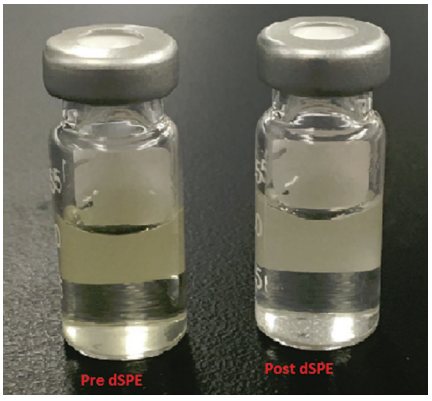

Figure 3

affected at a much greater extent. Many of the analytes compromised in the presence of PSA featured two ionisable 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

The QuEChERS method was conceptualised around the need to extract various types of compounds from a diverse group of matrices in a single, universal method. The expansion of the QuEChERS methodology outside of the food safety industry not only demonstrates its superior ability for sample extraction and clean-up, but also signifies how rugged the technique is. While the method itself has not proven to be a guaranteed, single solution to all of the challenges analysts face, it has begun to offer new advantages to the forensic toxicology community when overcoming complex matrices.

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