Screening of Drugs of Abuse and Pesticides in Liver Following Homogenization and Supported Liquid Extraction (SLE) Prior to GC/MS Analysis

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Introduction

In postmortem cases, where drugs or pesticides have been used for their poisonous properties, traditional matrices such as urine and whole blood may be inappropriate for qualitative and quantitative analysis. As the site of metabolism for most drugs and toxins, the liver may provide more insight to cause of death than other bodily fluids.

Screening drugs of abuse can be complicated due to the wide variation of functional groups associated with different analyte classes. Most extraction techniques cannot extract all analytes using a single procedure without using non-optimal extraction protocols resulting in compromised extract cleanliness. Supported liquid extraction is a 96-well plate or individual column based extraction technique analogous to traditional liquid-liquid extraction. The technique allows for the simultaneous analysis of cross functional analytes in a single extraction protocol without forfeiting extract cleanliness.

Experimental

Reagents

Drug standards were purchased from LGC Standards (Teddington, UK). Ammonium hydroxide, formic acid, hydrochloric acid and GC derivatizing agents were purchased from Sigma-Aldrich (Dorset, UK). Blank liver tissue was purchased locally. All solvents were HPLC grade from Fisher Scientific (Loughborough, UK) and Milli-Q (Merck Millipore, Germany) water used throughout.

Sample Preparation

ISOLUTE® SLE+ Procedure (*Figure 1.*)

Columns: ISOLUTE® SLE+ 1 mL capacity 'C' columns; 820-0140-C.

Matrix Preparation: Using a Biotage® Lysera weigh 200 mg of liver into a 7 mL Lysera tube containing 2.8 mm ceramic beads. Add 1.8 mL of 50:50 (v:v) methanol:water and 500 ppb internal standard solution, cap and load the tubes into the instrument.

Homogenization Procedure: Program the Lysera: One cycle at 5.3 m/sec for 30 seconds. Transfer the homogenized liver into 2 mL Eppendorf tubes, cap and place in a micro centrifuge to operate at 13,300 rpm for 10 minutes.

Sample Application: 500 µL of liver homogenate was applied to the columns.

Analyte Extraction: 2 x 2.5 mL aliquots of DCM.

Each aliquot was allowed to flow under gravity for 5 minutes in an appropriate glass tube with 100 µL HCl in methanol (0.2 M). A pulse of positive pressure for 10-20 seconds was applied to completely remove the final aliquot.

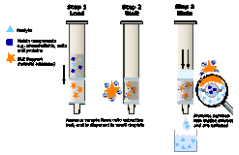


Figure 1. *Schematic of ISOLUTE® SLE+ Supported Liquid Extraction Procedure.*

Post Extraction:

The extracts were evaporated to dryness at ambient temperature, reconstituted with 200 µL ethyl acetate and vortexed. The samples were transferred to high recovery autosampler vials and evaporated again at ambient temperature. The dry samples were reconstituted with 25 µL ethyl acetate and 25 µL MSTFA prior to vortex mixing, capping and heating for 30 minutes at 80 °C on a heat block.

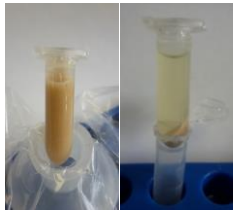
GC/MS Conditions

GC: 7890A GC with QuickSwap (Agilent Technologies Inc.)
Column: Agilent J&W DB-5MS, 30 m x 0.25 mm ID x 0.25 µm
Carrier Gas: Helium 1.2 mL/min (constant flow)
Inlet: Split 5:1, Temp: 300 °C
Injection volume: 1 µL
Oven: Initial: 55°C, ramp 25 °C/min to 325 °C, hold for 3.2 minutes.
Backflush: 2 void volumes (1.6 mins)
Transfer Line: 300 °C
MS: 5975C MSD (Agilent Technologies Inc.).
Source Temperature: 230 °C
Quadrupole Temperature: 150 °C
Monitored Ions: EI signals were acquired using selected ion monitoring (SIM) mode. See Biotage.com application notes section for monitored ions for each analyte.

Results

Initial pre-treatment optimization was necessary in order to load samples of liver onto the supported liquid extraction columns. The Lysera instrument facilitated this by allowing fast sample homogenization of solid liver tissue into a pipettable aliquot amenable to sample handling. Liver samples were prepared at masses of 100-1000 mg to determine the amount of tissue that could be homogenized with an appropriate volume of solvent. Homogenization took place in 7 mL tubes with 2.8 mm ceramic beads.

Homogenization was performed with water and various mixtures with isopropanol, methanol and acetonitrile. ***Figure 2.*** shows a sample of 200 mg of liver and 1.8 mL of 50/50 water/methanol following homogenization. ***Figure 3.*** shows the sample following homogenization and a centrifugation step at 13,300 rpm for 10 minutes.



Figures 2-3. *Liver sample following homogenization: before and after centrifugation.*

Extraction of non-centrifuged homogenate resulted in unacceptable flow characteristics. Centrifugation was necessary to assist flows and improved extract cleanliness.

Derivatization options were investigated prior to extraction evaluation. Silyation (BSTFA with TMCS) and acetylation (PFPA) options were compared to samples without derivatization. Selected analytes and their peak heights are demonstrated with this early testing in ***Figure 4.***, alongside a simple ethyl acetate reconstitution. The silyation approach offered optimal results for a broad range of analytes. MSTFA replaced BSTFA following this testing due to improved signal response.

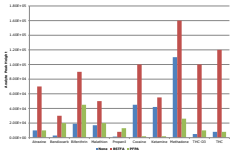


Figure 4. *Peak height data following derivatization evaluation.*

Figure 5. demonstrates the analyte recoveries following ISOLUTE SLE+ 1 mL column extraction. This testing was performed in the absence of liver homogenate to prove adequate analyte partitioning into the water-immiscible solvent, in this instance DCM. Various pre-treatment options were evaluated were water/methanol, water/ acetonitrile and compared. Water-immiscible solvents evaluated were DCM, MTBE, ethyl acetate, hexane and heptane. DCM offered the cleanest extraction of all solvents, while still partitioning the required analytes.

Figure 5. *Recovery profile chart for initial water-based loading options.*

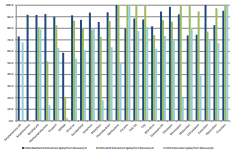


Figure 6. demonstrates the analyte extraction recovery from liver homogenate with the optimized method.

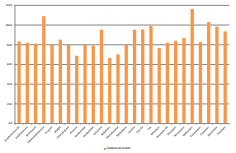


Figure 6. *Recovery profile chart for homogenized liver.*

Figure 7. Illustrates chromatography from an extracted sample spiked with 1000 ppb of analyte mix.

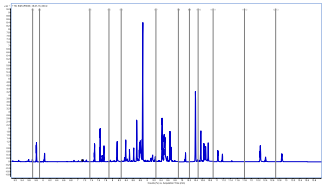


Figure 7. *Representative chromatography for analytes spiked at 1000 ppb.*

Investigation of extract cleanliness in the form of phospholipids was conducted using LC-MS/MS experiments. ***Figure 8.*** demonstrates phospholipid levels from a protein precipitated blood sample compared to a blank solvent injection and optimized liver extract.

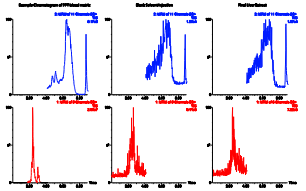
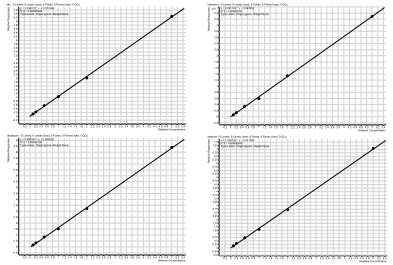


Figure 8. *Phospholipid content comparison TICs.*

Calibration lines were constructed from 50ppb to 2500 ppb with this optimized method. The internal standards were spiked at 500 ppb. ***Figures 9-12.*** demonstrate the linearity of some representative analytes. The coefficient of determination (r^2) for each analyte was greater than 0.99.



Figures 9-12. *Calibration lines for THC, bifenthrin, diazepam and atrazine.*

The calibration lines also highlight the lower limit of quantitation (LLOQ). These are summarized for the optimized method in ***Table 1.***

Table 1. *Analyte LLOQ values.*

Drug Analyte	LLOQ (ppb)	Drug Analyte	LLOQ (ppb)
Amphetamine	50	Cocaine	50
Bendiocarb	50	Methadone	50
Methamphetamine	50	THC	50
Propanil	50	Bifenthrin	50
MDMA	50	Diazepam	50
Chlorothalonil	50	Nitrazepam	50
Atrazine	50	Midazolam	50
Butabarbital	50	Clonazepam	250
Secobarbital	50	Estazolam	100
Ketamine	50	Alprazolam	250
Malathion	100	Triazolam	250
Phenobarbital	50		

Conclusion

- Liver tissue (n=24) was quickly and efficiently homogenized within 30 seconds using the Lysera system.
- ISOLUTE® SLE+ enables a fast, reliable protocol to extract pesticides and drugs of abuse panels from liver matrix using a single methodology.
- Extract cleanliness demonstrated good removal of endogenous matrix components allowing simple method transfer to LC-MS/MS methodology.
- This poster illustrates multiple benefits to laboratory workflow saving both worker hours and consumable costs.

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