Evaluation of Novel Automated Sample Preparation Compared to Manual Processing in Forensic Toxicology

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Introduction

Data reliability in forensic toxicology is of paramount importance. The possibility of false positives and/or false negatives can have wide ranging consequences and impacts decisions on various parts of the analytical method development process. This poster compares the performance of manual processing to a novel automated sample preparation system prior to GC/MS or LC-MS/MS analysis. Emphasis will also be placed on the potential for 96-well cross contamination and strategies for its elimination.

Experimental

Reagents

Drug standards were purchased from LGC Standards (Teddington, UK). Ammonium hydroxide, ammonium acetate, formic acid, hydrochloric acid and Rhodamine B were purchased from Sigma-Aldrich (Dorset, UK). Blank whole blood was purchased from Sera Labs International (Sussex, UK). Blank urine was kindly donated by healthy individuals. 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.*)

96-well Plate: ISOLUTE® SLE+ 400 µL capacity; 820-0400-P01.
Columns: ISOLUTE® SLE+ 1 mL capacity 'C' columns; 820-0140-C.

Matrix Pre-treatment:
Whole blood and urine was pre-treated 1:1 with 1% NH_4OH (aq).

Sample Application:
1 mL of pre-treated urine was applied to the columns.
300 µL of pre-treated whole blood was applied to the fixed well plates.

Analyte Extraction:
Following urine sample application to columns, elution was with 2 x 2.5 mL aliquots of DCM/isopropanol 95/5.
Following whole blood sample application to fixed well plates, elution was with 900 µL DCM/ACN 99/1, followed by 900 µL MTBE. Each aliquot was allowed to flow under gravity for 5 minutes. A pulse of positive pressure for 10-20 seconds allowed complete removal of the final aliquot.

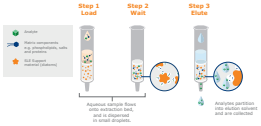


***Figure 1**. Schematic of ISOLUTE® SLE+ Supported Liquid Extraction Procedure.*

Post Extraction:
All extracts were evaporated at 40 °C in the presence of 100 µL 50 mM HCl in MeOH, to avoid analyte losses of amphetamines.

Manual Sample Preparation

All extraction protocols were developed using a vacuum manifold or semi-automated postitive pressure manfold.

Biotage® Extrahera™ Automated Sample Preparation Platform

The optimized extraction protocols were transferred to an automated sample preparation platform equipped with an 8 channel pipetting head and positive pressure processing functionality. The system is interconvertible between 4 and 8 channel pipetting into 24 (6 x 4 arrangement) columns or 96-well plates, respectively. The Extrahera™ platform is shown in ***Figure 2***.

***Figure 2.** Biotage® Extrahera™ automated sample preparation platform.*

Dye Cross Contamination

Experiments were based on the use of a dye, Rhodamine B, dissolved in multiple solvents in order to provide a visible means of determining the potential and degree of cross contamination. All work was performed using the 96-well collection plate format (1 and 2 mL capacity) due to the close proximity of samples. Occurrence of cross contamination was investigated in the pipetting, sample transfer, extraction protocol, evaporation and mixing steps.

Rhodamine Dye Sample Preparation: Approximately 1 mg/mL dissolved in multiple solvents with differing characteristics; MTBE, DCM and MeOH.

LC-MS/MS Conditons

Instrument: Waters Acquity UPLC or IClass interfaced via electrospray ionization to a Quattro Premier XE triple or XEVO TQS quadrupole mass spectrometer, respectively (Waters Assoc., Manchester, UK). Positive ions were acquired in the multiple reaction monitoring (MRM) mode.
Column: ACE Excel C18: 10**0 mm x 2.1 mm id, 1.7 µm, (**Hichrom Ltd., Reading, UK.)
Mobile Phase: A: 5 mM Ammonium Acetate (aq)
Mobile Phase B: 5 mM Ammonium Acetate (MeOH)
Flow Rate: 0.3 mL/min **Gradient:** Various
Injection Volume: 10 µL **Column Temperature:** 40 °C
Desolvation Temp: 450 °C **Ion Source Temp:** 150 °C

Results

When assessing cross contamination sample carryover in the LC/MS system is usually investigated early in the method development process. However, one area often overlooked is sample preparation. This involves multiple aspects: pipetting, mixing steps, sample transfer, extraction, and evaporation. The first parameters can be instrument or operator dependent so particular attention was put on the extraction and evaporation steps. ***Figure 3.*** illustrates the effect of luer tip placement into the collection plate when using various vacuum and positive pressure processing. Generally the use of positive pressure demonstrates less potential for cross contamination due to better penetration of the luer tips (outlet nozzles) into the collection plate. For vacuum processing it is important to ensure adequate penetration of the luer tips into the collection plate, due to different manifold spacing and SPE plate design in terms of luer tip length.

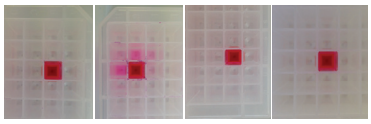


***Figure 3.** SPE processing: (L-R) Vacuum with optimized spacer; Vacuum with non-optimized spacer; Positive pressure; Biotage® Extrahera™, positive pressure. Solvent MeOH*

Investigations into evaporation were conducted using a SPEDry 96 sample evaporation unit. These units have adjustable nozzle height positions, gas flow and gas temperature. Variation of these parameters along with solvent and volumes were monitored for potential impact on cross contamination. ***Figure 4.*** demonstrates evaporative cross contamination effects when using the 2 mL capacity collection plate. At volumes of 1.5 mL or above cross contamination was observed.

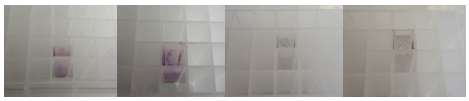


***Figure 4.**Evaporation at gas flow 40 L/min and temperature 40 °C. Height of SPE Dry nozzles about 5 mm above plate. 1.25 and 1.5 mL volumes in a 2 mL collection plate: DCM (left); MeOH (right)*

Figure 5. theorizes the phenomenon occurring to cause cross contamination during the evaporation process.

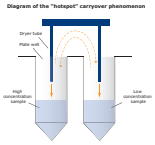


***Figure 5.** Schematic of the evaporative carryover phenomenon*

A 96-well plate accessory has been developed In order to negate the evaporative carryover effect. ***Figure 6.*** illustrates the Biotage® ACT plate adaptor used in subsequent experiments. In order to assess the effectiveness of the Biotage® ACT plate adaptor, we performed LC-MS/MS experiments spiking high concentration of analyte into selected wells and observing concentrations at the LOQ in surrounding wells.

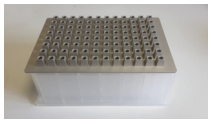


***Figure 6.** Biotage® ACT plate adaptor fitted to 96-well collection plate*

Figures 7 and 8. illustrate analyte peak areas with and without the plate adaptor. Levels far above the LOQs were returned in multiple adjacent wells when not using the plate adaptor. This could result in potential false positives.

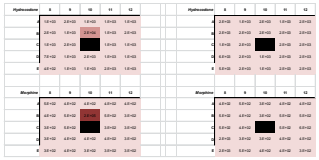


***Figure 7.** Opiate cross contamination: 750 µL MeOH evaporation on a 1 m L collection plate: No plate (left); plate adaptor (right)*

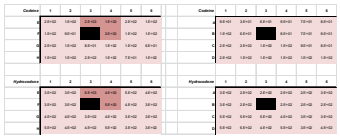


***Figure 8.** Opiate cross contamination: 1.2 mL MTBE evaporation on a 2 mL collection plate: No plate (left); plate adaptor (right)*

Supported liquid extraction protocols were previously developed for multiple drugs of abuse classes from urine and whole blood matrices. These were adapted to the Extrahera sample preparation platform. ***Figures 9 and 10.*** demonstrate the recovery profiles of multiple drug panels using manual and automated protocols in urine and whole blood respectively.

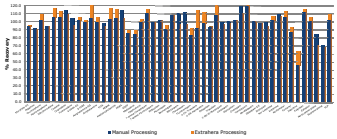


***Figure 9.** Extraction recovery profile from urine using the 1 mL columns.*

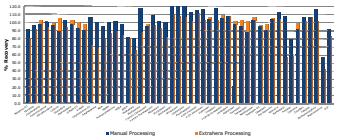
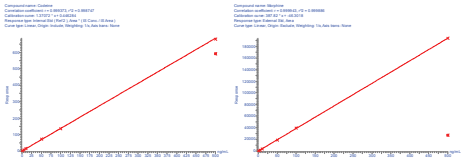


***Figure 10.** Extraction recovery profile from whole blood using the 400 µL fixed well plates.*

Figures 11 and 12 demonstrate calibration curves for representative analytes in the drugs of abuse panel, following Extrahera processing from 1-500 ng/mL. Quadratic function was observed at high concentrations for a number of analytes. However, dilution and internal standards helped and ultimately demonstrated excellent linearity and coefficients of determination ($r^2 > 0.99$).



***Figures 11-12.** Calibration curves of codeine extracted from urine by the 1 mL format and morphine extracted from whole blood by fixed well plate, respectively. Both were produced following Extrahera sample processing.*

Conclusions

- This poster describes effective use of a novel sample preparation system for both column and 96-well plate format and strategies for the elimination of cross contamination.
- Analyte volatility is a key component to the potential for cross contamination.
- Evaporative cross contamination can be affected by gas flow, temperature, nozzle height and solvent properties.
- The Biotage® ACT plate has proved effective for reducing or eliminating evaporative cross contamination as shown by the analyte "hot spot" experiments.
- The Extrahera automated sample preparation platform functions with 24-column and 96-well arrangement for the adaptation of manual extraction methods.

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