Evaluating the Potential for Cross Contamination when performing 96-well Sample Preparation prior to LC-MS/MS Analysis.

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Introduction

In all areas of analytical laboratory testing it is vital to ensure proper quality measures are in place to reduce or eliminate cross contamination between samples, which could result in false positive and/or false negative results. In many cases sample carryover in the LC/MS system is checked early on in the method development process. However, one area that can often be overlooked is during the sample preparation stages. This involves all aspects from pipetting, sample transfer, extraction protocol, evaporation and mixing steps. This poster evaluates various stages of the sample preparation process to determine the potential for cross contamination and present approaches to minimize and or eliminate the effect. This will be demonstrated via a series of dye experiments combined with analyte testing using LC-MS/MS.

Experimental

Reagents

Formic acid (FA) hydrochloric acid (HCl), ammonium acetate and Rhodamine B were purchased from Sigma Chemical Co. (Dorset, UK). Drug standards were purchased from LGC Standards (Teddington, UK). All solvents were HPLC grade from Sigma Chemical Co. (Dorset, UK) and Milli-Q (Merck Millipore, Germany) water used throughout.

Dye Cross Contamination

Initial 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) 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.

Analyte Cross Contamination

Evaporation experiments were repeated using various analyte suites (opiates) spiked at high concentrations; typically 2 µg. All surrounding wells were spiked at the assay's limit of quantitation. Evaporated samples were reconstituted in 200 µL mobile phase prior to analysis.

UPLC Conditons

Instrument: Waters Acquity IClass UPLC (Waters Assoc., Milford, MA, USA)
Column: Acquity BEH C18: 50 mm x 2.1 mm id, 1.7 µm, (Waters Assoc., Manchester, UK.)
Mobile Phase: A: 2 mM Ammonium Acetate (aq) with 0.1% FA
Mobile Phase B: 2 mM Ammonium Acetate with 0.1% FA in MeOH
Flow Rate: 0.4 mL/min
Gradient: Various
Injection Volume: 10 µL
Column Temperature: 40 ˚C

Mass Spectrometry

Instrument: Xevo TQS triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface for mass analysis. Positive ions were acquired in the multiple reaction monitoring (MRM) mode using the protonated precursor ion for each analyte.
Desolvation Temperature: 450 °C
Ion Source Temperature: 150 °C
Collision Gas Pressure: 3.6 x 10^{-3} mbar

Results

Initial investigations looked into simple pipetting strategies using both single channel manual and automated 8-channel processing with the Biotage Extrahera™ instrument. The trends were replicated using both strategies. The nature of the solvents and/or matrices differ widely so it is important to use appropriate aspiration and dispensation speeds along with proper saturation of pipette head vapour pressures. These along with pre and post air gaps using automated pipetting systems should alleviate any cross contamination effects.

Sample mixing was investigated using square and round well collection plates. It is common practice to mix samples and/or reconstitute samples after evaporation in 96-well collection plates prior to LC-MS/MS analysis. Subtle differences were observed between the plates and care should be taken when selecting vortex speeds combined with solvent volumes for each. For larger volumes a Vortex Genie was used with setting 3. The 2 mL round plate can tolerate slightly higher mixing volumes due to better vortex action compared to square well plates. ***Figure 1.*** demonstrates the maximum tolerance for 1 and 2 mL square collection plates and 2 mL round well collection plates. The tolerated volumes for each plate was deemed to be approximately: 0.5 mL, 1.5 mL and 1.7 mL respectively. As illustrated, cross contamination into adjacent wells is observed above these volumes.

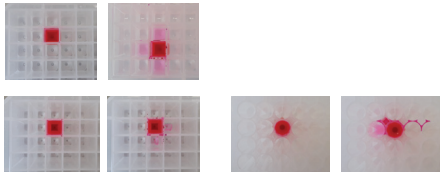

Figure 1. *Illustration of cross contamination using: 0.5 and 0.75 mL in a 1 mL square collection plate (top); 1.5 and 1.75 mL in a 2 mL square collection plate (bottom left); 1.7 and 1.8 mL in a 2 mL round collection plate (bottom right). Solvent MeOH*

Investigations were carried out to determine differences between 96-well SPE processing using positive pressure and vacuum on the occurrence and degree of cross contamination. ***Figure 2.*** demonstrates the effect of luer tip placement with various vacuum spacing and positive pressure processing.

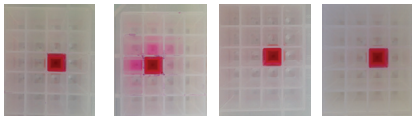

Figure 2. *SPE processing: Vacuum with optimized spacer; Vacuum with non-optimized spacer; Positive pressure; Extrahera™, positive pressure. Solvent MeOH*

Generally the use of positive pressure demonstrates less potential 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.

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. ***Figures 3 and 4.*** demonstrate evaporative cross contamination effects when using 1 and 2 mL plates, respectively. At 750 µL and 1.5 mL cross contamination is observed.

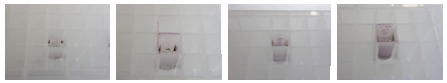

Figure 3. *Evaporation at gas flow 40 L/min and temperature 40 °C. Height of SPEDry nozzles about 10 mm above plate. 500 and 750 µL volumes in a 1 mL collection plate: DCM (left); MeOH (right)*

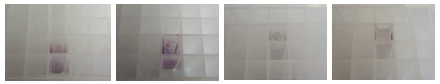

Figure 4. *Evaporation at gas flow 40 L/min and temperature 40 °C. Height of SPEDry nozzles about 5 mm above plate. 1.25 and 1.5 mL volumes in a 2 mL collection plate: DCM (left); MeOH (right)*

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

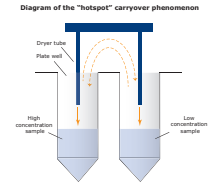

Figure 5. *Schematic of the evaporative carryover phenomenon*

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.

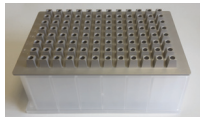

Figure 6. *Biotage® ACT plate adaptor fitted to 96-well collection plate*

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. ***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.

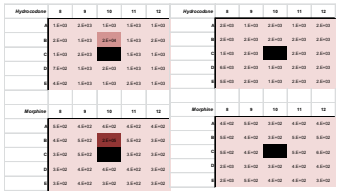

Figure 7. *Opiate cross contamination results evaporating 750 µL MeOH on a 1 mL collection plate: No plate (left); plate adaptor (right)*

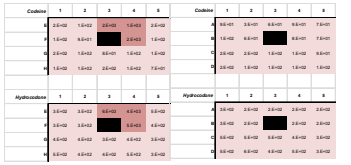

Figure 8. *Opiate cross contamination results evaporating 1.2 mL MTBE on a 2 mL collection plate: No plate (left); plate adaptor (right)*

Conclusion

- This poster illustrates the potential for cross contamination during various parts of 96-well sample preparation
- Steps were taken to negate the phenomenon at all stages of the process
- Pipetting requires careful solvent consideration and adequate tip equilibration
- Sample mixing/reconstitution can be affected by plate design, with round well plates being less susceptible to cross contamination due to better vortex action
- Analyte volatility is a key component to the potential for cross contamination
- Evaporative cross contamination can be affected by gas flow, temperature, nozzel 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

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