Optimized Sample Preparation for Low Level Determination of Alcohol Marker EtG from Hair Using UPLC-MS/MS Analysis

Lee Williams[1], Zainab Khan[1], Lucy Lund[1], Thomas Smith[1], Adam Senior[1], Helen Lodder[1], Russell Parry[1], Geoff Davies[1], Alan Edgington[1], Claire Desbrow[1] & Dan Menasco[2]

Biotage GB Limited, Distribution Way, Dyffryn Business Park, Ystrad Mynach, Cardiff, CF82 7TS, UK
Biotage AB, 751 03 Uppsala, Sweden

Introduction

Hair analysis is growing in popularity due to the non-invasive nature of the sample collection. Hair testing can also provide advantages over other more widely used matrices, such as determination of prolonged exposure and potential timelines. However, as a solid matrix, sample preparation can often be lengthy and labor intensive. Minor ethanol metabolites such as ethyl glucuronide (EtG) and ethyl palmitate (EtPa) can be measured in hair as direct markers of alcohol consumption. As suggested by the Society of Hair Testing (SoHT) in 2019, EtG determination from hair is the preferred marker for the assessment of abstinence. This poster aims to demonstrate improved workflow and clean-up of hair matrix to allow low level EtG determination required for alcohol abstinence testing.

Experimental

Reagents

Drug standards were purchased from LGC Standards (Teddington, UK) and Sigma-Aldrich Company ltd. (Gillingham, UK). Analytical reagents were from Sigma-Aldrich. All solvents were HPLC grade from Rathburn Chemical Ltd (Scotland, UK) and Milli-Q (Merck Millipore, Germany) water used throughout. Hair samples were donated by healthy human volunteers.

Sample Preparation

Matrix Preparation:

Weigh 10 mg of hair into 2 mL Biotage® Lysera tubes containing 4 x 2.4 mm stainless steel beads. Dry matrix pulverization and "in-solution" options using 1 mL of H_2O added to each hair sample were compared. Internal standard EtG-d_5 added at a concentration of 50 pg/mg of hair.

Micropulverization (MPE) Procedure:

Biotage® Lysera: 3 x 1-minute cycles at 5.3 m/sec with 20s dwell between cycles (dry and in-solution pulverization). Dry pulverized hair was treated with 1 mL H_2O and mixed for 1 further cycle.

Centrifuge extracts for 10 minutes at 13,300 rpm.

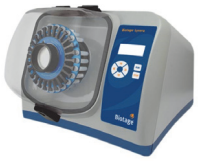

Figure 1. *Biotage® Lysera Bead Mill Homogenizer equipped with 24-position 2 mL tube carriage.*

Solid Phase Extraction: EVOLUTE® EXPRESS SPE 30 mg 96-well plates; AX (603-0030-PX01) and WAX (602-0030-PX01) were screened for method development purposes. SPE miniaturization was performed using the corresponding 10 mg 96-well plate options and finally the Biotage® Mikro AX 2 mg low volume 96-well plates (603-0002-LVP) for improved workflow. Final protocols demonstrated in ***Table 1.*** All SPE processing was performed using a positive pressure 96 manifold.

Table 1. *SPE Protocols for 10 mg and 2 mg Mikro SPE Formats.*

Step	EVOLUTE AX	EVOLUTE WAX	10 mg Volumes	Mikro Volumes
Condition	MeOH		500 µL	100 µL
Equilibration	H_2O		500 µL	100 µL
Sample load	Hair Extract		800 µL	400 µL
Wash 1	H_2O		500 µL	100 µL
Wash 2	MeOH		500 µL	100 µL
Dry	2 minutes			
Elution	5% Acetic Acid in MeOH OR *****0.5% Formic acid in 95/5 H2O/MeOH***	0.5% NH_4OH in MeOH	200 µL	50 µL

Post extraction: Samples were evaporated at 40 °C and reconstituted in 100-200 µL of 0.1% formic acid (aq) depending on format. Elimination of evaporation was investigated for 10 mg 96-well and 2 mg Low Volume Mikro plate formats, comparing **direct injection or dilution prior to injection, respectively.

UPLC/MS Conditions

Instrument: Waters ACQUITY Premier UPLC (Waters Assoc., Manchester, UK).
Column: Acquity UPLC HSS T3 1.8 µm (100 x 3 mm) (Waters Assoc., Manchester, UK).
Mobile phase: 0.1% formic acid aq. (A) and Acetonitrile (B).
Flow rate: 0.4 mL/min.
Column temp: 40 °C. **Injection volume:** 10 µL.
Gradient: Conditions available on request.

Mass Spectrometry

Instrument: Waters Xevo TQ Absolute triple quadrupole mass spectrometer equipped with an ES interface for mass analysis (Waters Assoc., Manchester, UK). Negative ions were acquired in the MRM mode.

Desolvation Temp: 500 °C **Ion Source Temp:** 150 °C

Table 2. *MRM Parameters (Qual Ions in parenthesis).*

Analyte	Polarity	MRM	Cone Voltage (V)	Collision Energy (eV)
EtG	-	221.1 > 74.8 (221.1 > 84.8)	10 10	15 18
EtG-d_5	-	226.1 > 74.8	10	15

Results

Chromatographic performance demonstrated subtle differences between reversed phase and HILIC chromatography with respect to Tr, peak shape and band broadening, and ESI sensitivity related to mobile phase composition (data not shown). However, better overall sensitivity was observed using RPLC.

Evaporative effects were investigated using a range of traditional SPE elution solvents, as shown in ***Figure 2.*** Results demonstrate up to 30% signal reduction depending on exact evaporation conditions.

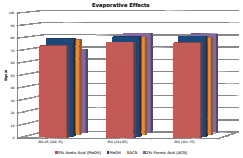

Figure 2. *Evaporation profiles.*

SPE was performed using polymer-based mixed-mode strong (AX) and weak (WAX) anion exchange chemistries. Due to the polar nature of EtG, extraction efficiency improved without ionic strength and pH control during the conditioning, equilibration and wash steps. ***Figure 3***. demonstrates recovery profiles obtained using various pH conditions in the wash step.

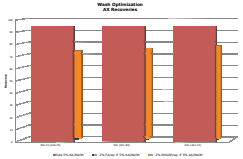

Figure 3. *AX Wash Recovery Investigation.*

Elution solvent composition was key to obtaining optimum recovery and matrix factors. ***Figure 4***. demonstrates effect of acid and elution solvent (LHS), and aq/organic ratio for improved cleanliness (RHS).

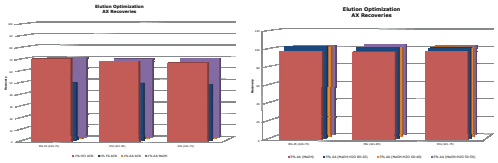

Figure 4. *AX Elution Recovery Investigation.*

Elution with 95/5% aq/MeOH was achieved using appropriate acid/concentration. Minimum elution volumes were investigated for recoveries and matrix factors using both 30 and 10 mg bed masses, as demonstrated in ***Figure 5.***

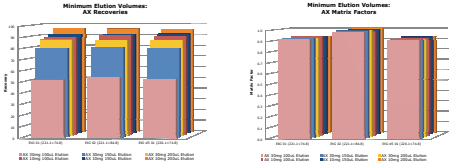

Figure 5. AX minimum elution volume *recovery and matrix factor profiles (30 > 10 mg).*

Likewise, the optimized EVOLUTE® EXPRESS WAX SPE protocol was subjected to miniaturization, moving from 30 > 10 > 2 mg bed masses. Recovery and matrix factor results are presented in ***Figure 6.***

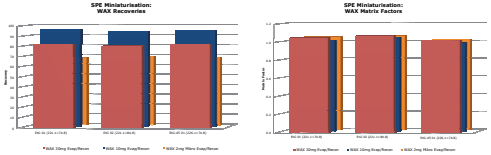

Figure 6. WAX SPE miniaturization (30 > 10 > 2 mg) recovery and *matrix factor* profiles.

Due to high aq elution content and mobile phase compatibility of the EVOLUTE® EXPRESS AX protocol, further miniaturization to the 2 mg Mikro AX format was evaluated in combination with direct injection for workflow improvements. This format allowed excellent extraction with elution volumes as low as 50 µL, as demonstrated in ***Figure 7.***

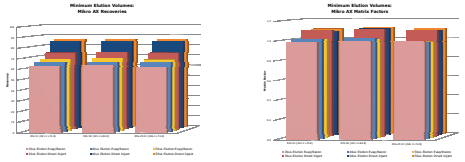

Figure 7. Mikro AX minimum elution volume *recovery and matrix factor profiles: Data includes evap/recon vs dilution/direct inject.*

Due to autosampler limitation, 50 µL of H_2O was added prior to injection. This approach yields higher recoveries due to elimination of signal loss on evaporation.

Figure 8. demonstrates calibration curve (2-100 pg/mg) performance for the EVOLUTE® EXPRESS AX protocol in 10 mg and 2 mg AX plate formats. All procedures delivered excellent performance, coefficients of determination, $r^2 > 0.99$ and low pg/mL levels as required for abstinence testing.

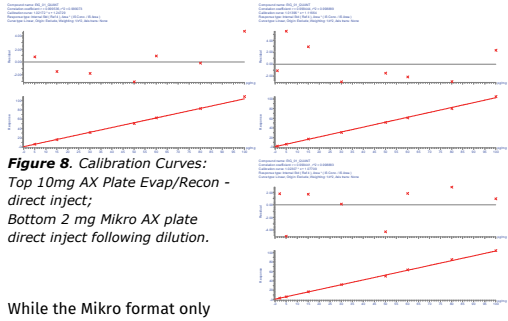

Figure 8. *Calibration Curves:*
Top 10mg AX Plate Evap/Recon - direct inject;
Bottom 2 mg Mikro AX plate direct inject following dilution.

While the Mikro format only allows 400 µL load volumes compared to 800 µL using standard SPE format, the sensitivity is bridged by lower elution volumes.

Conclusion

- This poster presents simplified approaches for the low-level detection of EtG in hair matrix, as required in abstinence cases.
- Scalability of SPE from 30mg > 2 mg formats demonstrating high reproducible recoveries and low matrix factors.
- EVOLUTE® AX chemistry, along with optimized elution solvents provide an excellent extraction option allowing the elimination of evaporation steps for improved workflow when using 10 mg or 2 mg Mikro SPE formats.

Disclosure: The authors declare no competing financial interest. However, salaries of all authors are paid by Biotage.

ASMS 2024, Anaheim, CA
Part Number: P260.V.1