# Assessment of Extract Cleanliness Using Different On-Line Clean Up Selectivities for a Turbulent Flow Chromatography (TFC) – MS/MS Method for the Determination of a Common Decongestant Agent in Human Plasma

**Lars Neudert, MSc, Senior Scientist Method Development**
**Simon Wood, BSc, Associate Director, Method Development**
**Petra Struwe, PhD, Senior Director, Global Bioanalytical Services**

## Introduction

Turbulent flow chromatography (TFC) is an on-line extraction procedure allowing clean up of biological samples (e.g. plasma, serum, urine) prior to the analysis of target molecules by LC-MS/MS. Extract cleanliness is considered to be important for high sensitivity bioanalytical assays with typical LLOQs in the low pg/mL range. It is reflected by the ionisation recovery where matrix components present in the biological sample influence the response of analyte under investigation. Therefore, sensitivity and good method performance are especially related to the balance between extraction recovery and ionisation recovery and consequently influenced by the mode of selectivity chosen for the on-line extraction system. Xylometazoline is a drug which is used as a nasal decongestant. Due to its intranasal administration, potentially low plasma concentrations may need to be measured in order to generate pharmacokinetic data. Xylometazoline was therefore chosen to illustrate a comparison of extract cleanliness and extraction recovery for an on-line extraction system using different modes of selectivity.

## Method Summary

Xylometazoline and its d4-labeled IS were extracted from human plasma using a Cohesive Turboflow® on-line extraction system. Different extraction columns were tested allowing for different modes of selectivity. The Turboflow® Cyclone column has a styrene-divinylbenzene copolymer as stationary phase and therefore provides a selectivity that is based purely on hydrophobic interactions. The Turboflow® Cyclone MCX mixed mode column provides a styrene-divinylbenzene copolymer with sulfonic acid modifications and therefore combines strong cation exchange with reverse phase binding capacity. Since its stationary phase is negatively charged across the entire operating pH range, a combination of high pH and solvent strength is required for the elution of weak bases. Finally, the Turboflow® WCX column is bonded with carboxylic acid, a negatively charged ionic moiety providing weak cation exchange mechanism.

The Cohesive Turboflow® on-line extraction system was set up as described in Figure 2, using focus (dual column) mode. Pump conditions for each extraction column were optimised as follows:

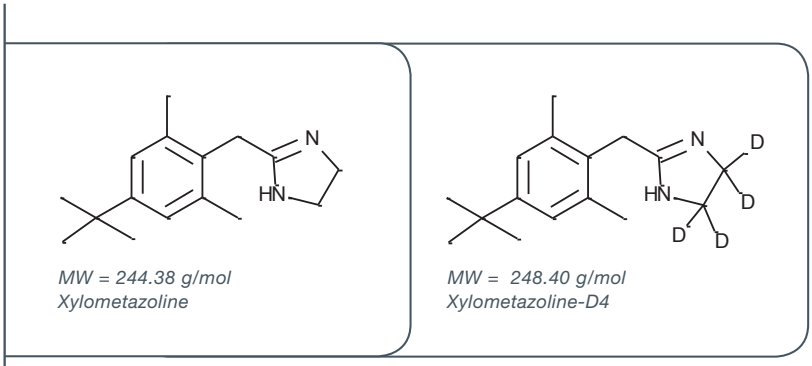


***Figure 1.*** *Chemical structures of Xylometazoline and Xylometazoline-D4*

### TURBOFLOW® CYCLONE (Reverse phase)

Extraction column: Turboflow® Cyclone, 50 x 1.0 mm (Thermo Scientific)

Loading solution A: Water/Formic acid, 100:0.1, v/v

Loading solution B: Acetonitrile/Formic acid, 100:0.1, v/v

Loading solution C: Acetonitrile/2-Propanol/Acetone, 45:45:10, v/v/v

Loading flow rate: 4.0 mL/min

### TURBOFLOW® CYCLONE MCX (Mixed mode)

Extraction column: Turboflow® Cyclone MCX, 50 x 1.0 mm (Thermo Scientific)

Loading solution A: Water/Formic acid, 100:0.1, v/v (pH 3)

Loading solution B: Acetonitrile/25% Ammonia solution (aq.), 100:1, v/v (pH 12)

Loading solution C: Acetonitrile/2-Propanol/Acetone, 45:45:10, v/v/v

Loading flow rate: 4.0 mL/min

### TURBOFLOW® WCX (Weak cation exchanged)

Extraction column: Turboflow® ¬WCX, 50 x 1.0 mm (Thermo Scientific)

Loading solution A: Water/Formic acid, 100:0.1, v/v (pH 3)

Loading solution B: Methanol/25% Ammonia solution (aq.), 100:0.1, v/v (pH 9)

Loading solution C: Acetonitrile/2-Propanol/Acetone, 45:45:10, v/v/v

Loading flow rate: 4.0 mL/min

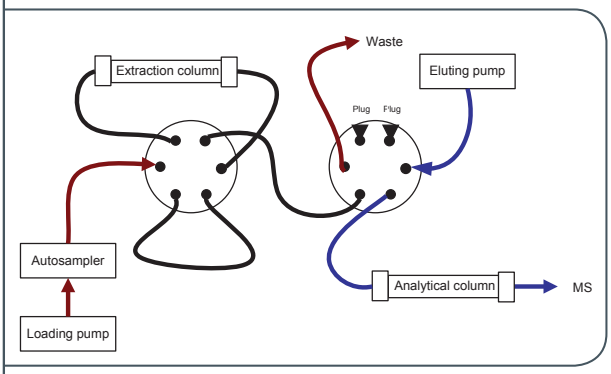


***Figure 2.*** *Turboflow® configuration for on-line extraction (using focus (dual column) mode)*

For additional analytical separation a C18 reverse phase column was added after the extraction system using following conditions:

Analytical column: Onyx Monolithic C18, 50 x 2.0 mm (Phenomenex)

Eluting solution A: Water/Formic acid, 100:0.1, v/v

Eluting solution B: Acetonitrile/Formic acid, 100:0.1, v/v

Eluting flow rate: 1.0 mL/min, linear gradient

Total run time: 5.0 min

Human plasma samples were spiked with analyte at intended concentration levels, covering an analytical range from 30.0 – 5000 pg/mL. Samples were subsequently diluted in a ratio 1:2 with 0.1% Formic acid (aq.) containing IS at an appropriate concentration level. These solutions were injected onto the system at a constant autosampler loop size of 20 µL. Compound detection was carried out on a AB SCIEX API 4000 using ESI in positive MRM mode and unit/unit resolution. Transitions measured were 245.2/189.2 amu for Xylometazoline and 249.1/193.1 amu for Xylometazoline-D4. Example chromatograms obtained for Xylometazoline are shown in Figure 3, comparing each extraction mode at identical LLOQ concentration.

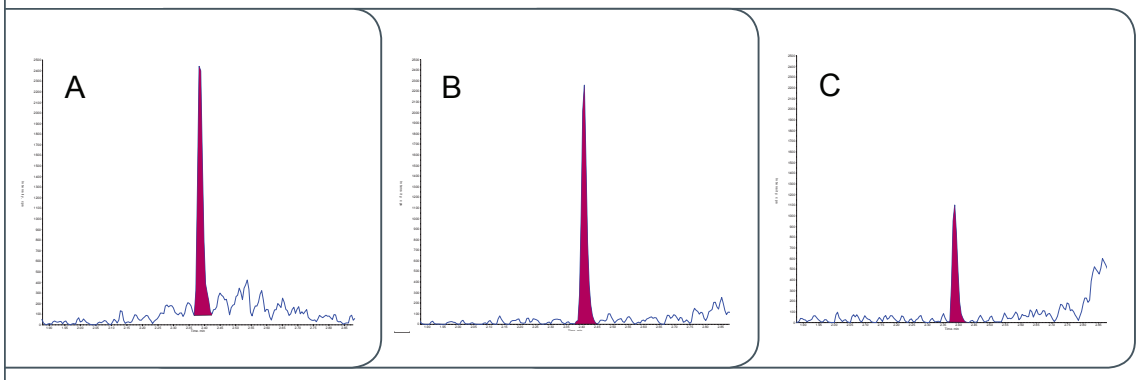


***Figure 3.*** *Example chromatograms*

*Xylometazoline, 30 pg/mL in Human plasma (EDTA) using different on-line extraction selectivities;*
*A: Turboflow® Cyclone, 50x1.0mm; B: Turboflow® Cyclone MCX, 50x1.0mm;*
*C: Turboflow® WCX, 50x1.0mm*

## Evaluation of Extraction Recovery and Extract Cleanliness

For the determination of extraction recovery and extract cleanliness, a third valve was incorporated to the system (as shown in Figure 4). An additional loop equivalent to the size of the autosampler loop was applied across this valve, allowing the addition of pure solution samples post extraction column. Pure solutions and plasma samples were then injected onto the system, whilst varying the solution injected post extraction column in a way described in Figure 5. Assessment of extraction recovery and ionisation recovery was performed on concentration levels of 30 pg/mL, 400 pg/mL and 4000 pg/mL. For evaluation, the mean analyte peak area response of five replicate injections was used to calculate specific recoveries according to formulas given in Figure 6.

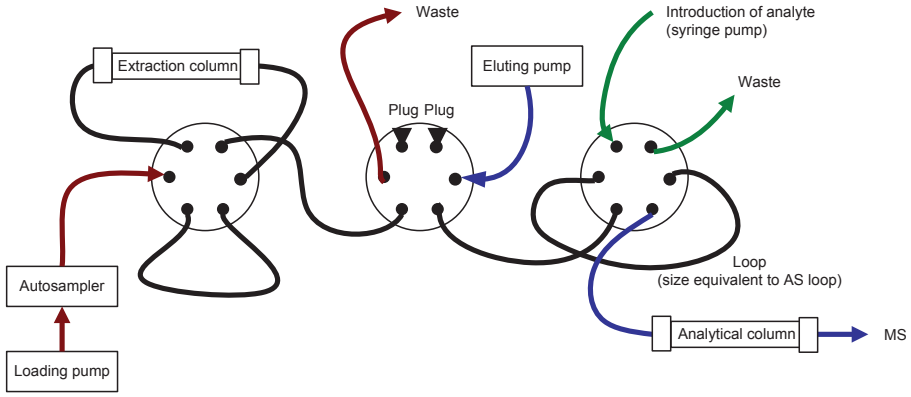


***Figure 4.*** *Turboflow® configuration for the evaluation of extraction recovery and extract cleanliness*

| | Sample injected | Sample post injected |
|---|---|---|
| pure solution sample | QC pure solution | Water |
| plasma sample | QC plasma | Water |
| pure solution post spiked sample | Water | QC pure solution |
| plasma post spiked sample | Plasma blank | QC pure solution |

***Figure 5.*** *Injection configuration for the evaluation of extraction recovery and extract cleanliness*

$$\text{Recovery in water [\%]} = \frac{\text{pure solution sample response}}{\text{pure solution post spiked sample response}} \times 100$$

$$\text{Recovery in human plasma [\%]} = \frac{\text{plasma sample response}}{\text{plasma post spiked sample response}} \times 100$$

$$\text{Ionisation recovery [\%]} = \frac{\text{plasma post spiked sample response}}{\text{pure solution post spiked sample response}} \times 100$$

$$\text{Total recovery [\%]} = \frac{\text{plasma sample response}}{\text{pure solution post spiked sample response}} \times 100$$

***Figure 6.*** *Formulas as used for calculation of specific recoveries (as applied to Xylometazoline response)*

## Results

Results tabulated in Figure 7 demonstrate the way that the balance between extraction recovery and extract cleanliness affects the total recovery and therefore the final signal that may be achieved. Whilst the Turboflow® Cyclone column provides best extraction recovery, it additionally raises the amount of ion suppression effects, resulting in a loss in total recovery. Ion suppression effects are most likely related to insufficient extract cleanliness. Note that the Turboflow® Cyclone column provides a selectivity similar to the analytical column, therefore, the relatively high level of ion suppression is easily accountable. In contrast the Turboflow® Cyclone MCX and the Turboflow® WCX columns use a differing selectivity to the analytical column, providing a lower level of ion suppression. However, the Turboflow® WCX column provides a poor extraction recovery, resulting in the poorest total recovery that was achieved. The Turboflow® Cyclone MCX column provides acceptable extraction recovery and almost no signal suppression, which suggests that it provides best extract cleanliness. This is reflected by the LLOQ chromatograms shown in Figure 3. Here the Turboflow® Cyclone MCX column provides less background signal whilst keeping comparable signal intensity when compared to the Turboflow® Cyclone column. This results in the best S/N ratio of all three selectivities.

It is noted that the extraction recovery from plasma is generally higher than that observed from water. This suggests that the presence of matrix compounds is necessary to achieve sufficient retention on the extraction column.

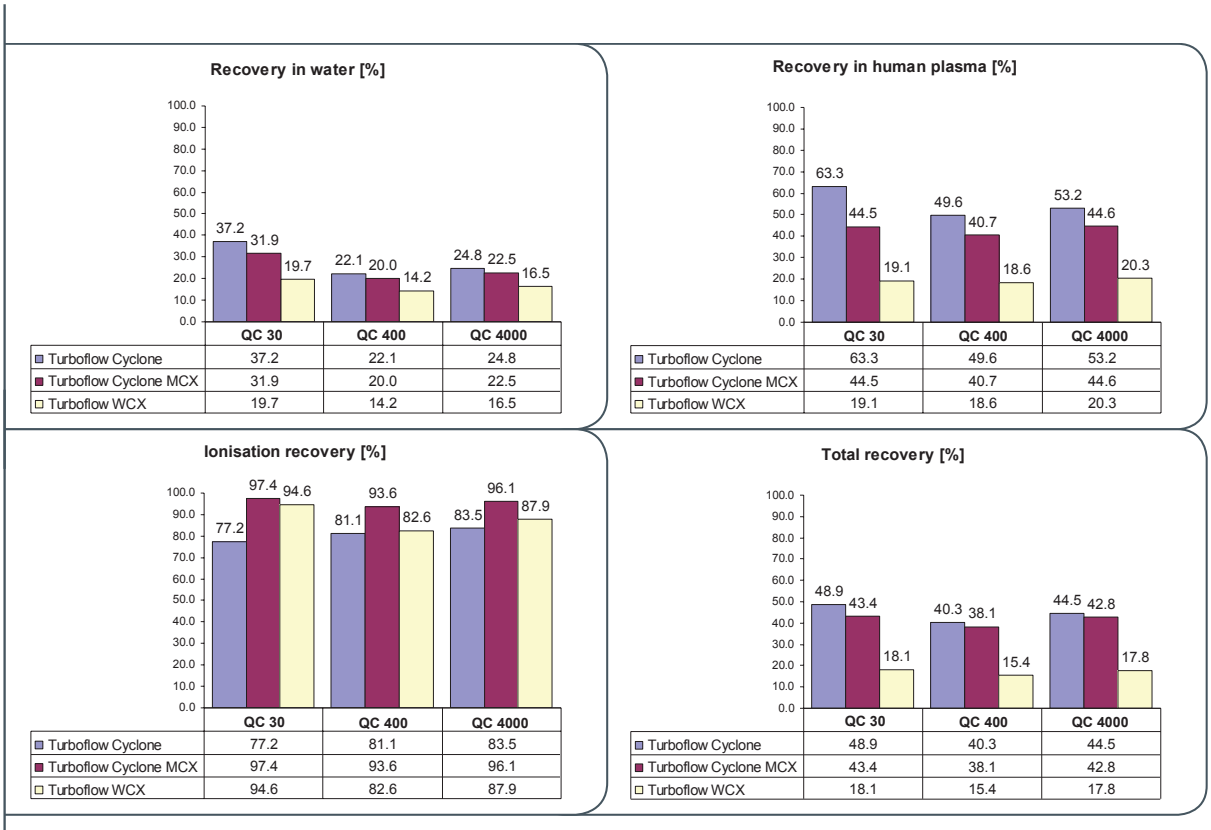


***Figure 7.*** *Results of extraction recovery and extract cleanliness evaluation*

*Specific recoveries, calculated according to formulas given in Figure 6 using the mean analyte peak area response of five replicate injections*

## Post Column Infusion Experiments

For additional qualitative ionisation recovery evaluation, post analytical column infusion experiments were performed. Blank pure solution samples and blank plasma samples were injected onto the system whilst applying a constant infusion of analyte to the MS post analytical column. Thereby differences in signal intensity at expected analyte retention time indicate the amount of ion suppression and therefore ionisation recovery. Values obtained are tabulated in Figure 9. Example chromatograms for post column infusion experiments are shown in Figure 8. Values obtained must be taken as qualitative only but do correlate with results as shown in Figure 7.

## Results

Results confirm that the Turboflow® Cyclone MCX extraction column provides best ionisation recovery, whilst the Turboflow® Cyclone provides highest signal suppression at expected analyte retention. Significant suppression of signal was observed towards the top of the analytical gradient at 2.85 min and 2.95 min on all types of extraction column. These retention times are possibly related to the elution of phospholipids, one of the known potential major suppressing species found in plasma. This was confirmed by monitoring some common phospholipid transitions while injecting blank plasma. It was observed that phospholipids could be detected even after several subsequent gradient cycles whilst solely injecting blank pure solution.

| Extraction column used | Ion suppression estimation | Ionisation recovery* |
|---|---|---|
| Turboflow® Cyclone | 45% | 55% |
| Turboflow® Cyclone MCX | 20% | 80% |
| Turboflow® WCX | 35% | 65% |

*Ionisation recovery [%] = 100 – Ion suppression [%]

***Figure 9.*** *Post column infusion experiment results*

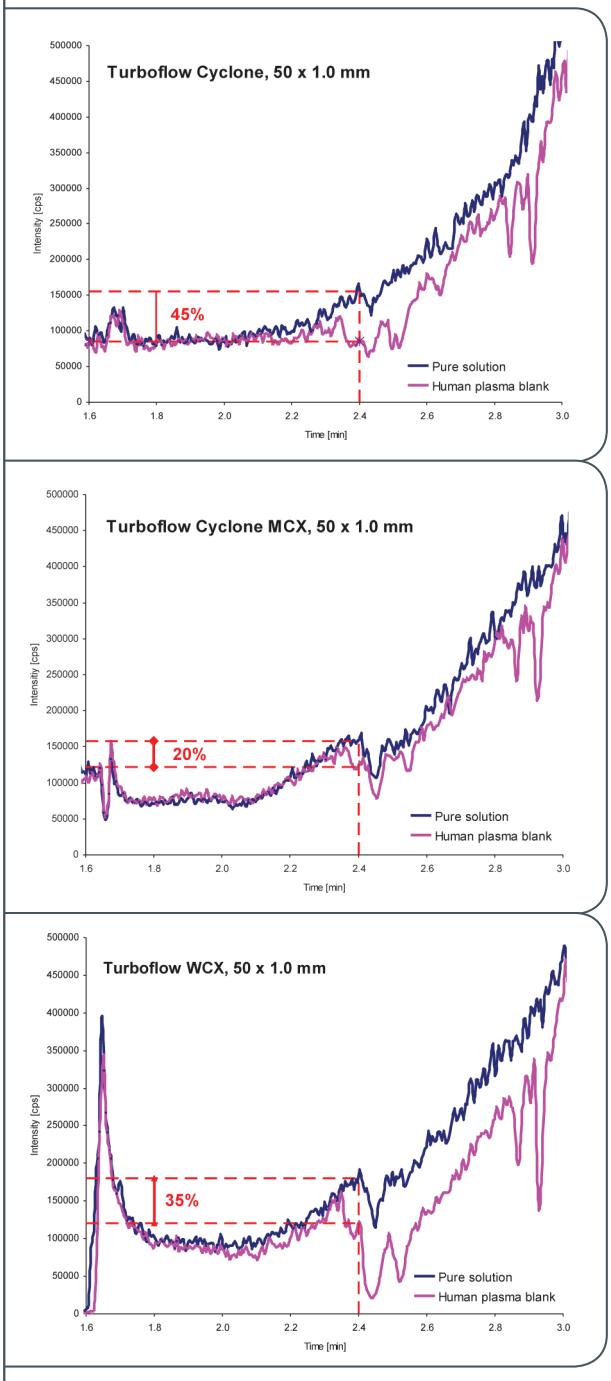


***Figure 8.*** *Example chromatograms for post column infusion experiments*

*Estimated ion suppression is reflected by percental difference in signal intensity between blank plasma and blank pure solution injections at expected analyte retention time*

## Conclusions

The results presented here indicate that applying different modes of selectivity to the sample extraction procedure results in different qualities of extract cleanliness. Therefore, choosing the right selectivity may help to effectively improve sensitivity by reducing ion suppression effects, whilst keeping extraction recovery acceptable. For the case illustrated here, the mixed mode cation exchange gave best results in S/N due to significantly lower signal suppression, whilst giving satisfactory extraction recovery. With the reversed phase polymer extraction column better extraction recovery could be obtained but extracts were not clean enough to prevent signal suppression and significantly higher background. Finally, the weak cation exchange mode gave poor extraction recovery and moderate extract cleanliness resulting in the poorest total recovery.

## Comments

Based on optimised conditions, a TFC-MS/MS method was successfully qualified. A d4-labeled internal standard was used for quantification, covering a concentration range of 30.0 – 5000 pg/mL of Xylometazoline in human plasma (EDTA). The results presented here show that the Turboflow® Cyclone MCX column was first choice to use, however, unexpected extraction column carry-over did not allow this extraction mode to be validated. Therefore the Turboflow® Cyclone polymer column was applied for validation, providing no carry over issues and acceptable sensitivity.