APPLICATIONS

Automated SPE Method Development Using Strata™-X 96-Well SPE Method Development Plates In Conjunction with a Tecan Freedom EVO® Liquid Handling System

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Introduction

The importance of clean sample extracts in maintaining analytical instrumentation cannot be overstated. Clean extracts will extend the life of costly chromatographic columns and replacement parts, as well as decrease the amount of money and time spent performing maintenance on downstream instruments. Despite the many benefits of proper sample preparation, the complexity of biological matrices can make the sample preparation method development process laborious and time intensive.

Because of the complexity of bioanalytical samples, we will be focusing on performing Solid Phase Extraction (SPE) as it results in ultra clean, concentrated samples, however the SPE method development process can be difficult. The goal of this publication is to offer an effective solution that will simplify the cumbersome SPE method development process. In order to develop an analytical SPE method for a wide range of compounds, we propose a fast and more structured approach to screen various SPE sorbents. This approach utilizes a Strata-X 96-Well SPE Method Development (MD) Plate (Phenomenex) packed with four unique polymer-based SPE sorbents; reversed phase (Strata-X), a mixed-mode strong cation-exchanger (Strata-X-C), a mixed-mode weak cation-exchanger (Strata-X-CW), and a mixed-mode weak anion-exchanger (Strata-X-AW). A Tecan Freedom EVO liquid handling system will be employed to screen the four sorbents to efficiently target the most effective SPE approach. This is a completely automated process that will free an analyst from the bottleneck of manual sample preparation.

SPE Phases and Conditions

Using the four different sorbents from the MD plate listed above, we will screen acidic, basic, and neutral conditions in each step of the SPE process including the load, wash, and elution, ultimately determining the best pH for retaining the analytes of interest (**Figure 2**). The polymeric SPE MD plate contains four sorbents in three different columns each, comprising a total of 24 wells for each material (**Figure 1**). Five rows will be used for replicate extractions of analyte spiked sample matrix, two rows will be used for post spiked blank samples extract (with analyte, at the same concentration as extracted samples) which are referred to as extracted reference (EREF), and one row will be used for blank matrix extractions. The average of the two EREF, will be used to quantitate recovery that will normalize the matrix effect. The configuration of the Strata-X sorbents from left to right in the MD plate are, Strata-X, Strata-X-C, Strata-X-CW, and Strata-X-AW. Each sorbent is packed at a bed mass of 30 mg.

Materials and Equipment Used for the SPE Automation

A Tecan Freedom EVO automated liquid handling system equipped with an eight channel disposable tip liquid handling arm (LiHa) to perform pipetting and a robotic manipulator arm (RoMa) to handle automated plate movement on a vacuum manifold (Te-VacS™) was used. Processing the MD plate is fully 'unattended', requiring no analyst intervention. All pipetting steps (dilution, plate conditioning, loading, washing, and elution) and vacuum control (duration and vacuum amount) are controlled by a Freedom EVOware® software script. This instrument configuration is appropriate not only for the MD plate, but for safely processing plates in high throughput once the best Phenomenex sorbent is selected.

Experimental Designs:

Table 1. Solvents and reagents required for three different load and elute conditions

Solvent	Solution composition
Buffer solution 1	25 mM Ammonium formate, adjusted to pH 2.5 with formic acid (called the acidic buffer)
Buffer solution 2	25 mM Ammonium acetate (pH ~ 6.9, without pH adjustment)
Elution solvent 1	Methanol containing 5 % Formic acid (acidic elution)
Elution solvent 2	Methanol containing 5 % Ammonium hydroxide (basic elution)
Elution solvent 3	Methanol (neutral elution)

Sample Preparation

Solid Phase Extraction Protocol

Sorbent: Strata-X 96-Well SPE Method Development Plate
Part No.: KS0-8209
Condition: 400 µL of Methanol
Equilibrate: 400 µL of Water or buffer solution 1 or 2
Load: 500 µL Plasma (spiked with 25 ng/mL analyte) diluted with 1 mL of water or buffer solution 1 or 2
Wash 1: 800 µL of Water or buffer 1 or 2
Wash 2: 800 µL of 70 % Methanol
Elute: Neutral Elution: 450 mL Methanol
Basic Elution: 450 mL Methanol with 5 % Ammonium hydroxide
Acidic Elution: 450 mL Methanol with 5 % Formic acid
Dry down: Samples are blown down to dryness under nitrogen at 45 °C
Reconstitute: 500 µL of initial mobile phase

LC/MS/MS Conditions

Column: Kinetex® 2.6 µm C18
Dimensions: 50 x 2.1 mm
Part No.: 00B-4462-AN
SecurityGuard ULTRA Holder: AJ0-9000
SecurityGuard ULTRA Cartridge: AJ0-8782
Mobile Phase: A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Acetonitrile

Gradient:

Time (min)	B (%)
0.0	5
2.0	95
3.0	95
3.1	5
5.0	5

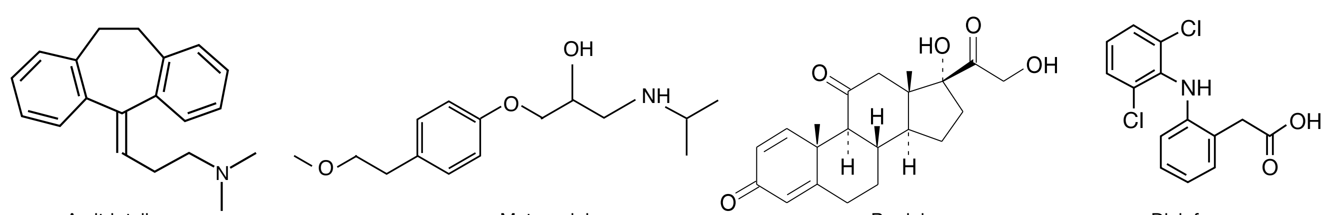
Flow Rate: 0.4 mL/min
Injection: 10 µL
Temperature: Ambient
Detection: Triple Quad™ 4500 MS/MS (AB SCIEX)

Figure 1. Strata™-X 96-Well Method Development Plate sorbent configuration and assigned extraction conditions for individual sorbents

Strata-X			Strata-X-C			Strata-X-CW			Strata-X-AW		
X Sample NN	X Sample AB	X Sample BA	C Sample NN	C Sample AB	C Sample BA	CW Sample NN	CW Sample AB	CW Sample BA	AW Sample NN	AW Sample AB	AW Sample BA
X Sample NN	X Sample AB	X Sample BA	C Sample NN	C Sample AB	C Sample BA	CW Sample NN	CW Sample AB	CW Sample BA	AW Sample NN	AW Sample AB	AW Sample BA
X Sample NN	X Sample AB	X Sample BA	C Sample NN	C Sample AB	C Sample BA	CW Sample NN	CW Sample AB	CW Sample BA	AW Sample NN	AW Sample AB	AW Sample BA
X Sample NN	X Sample AB	X Sample BA	C Sample NN	C Sample AB	C Sample BA	CW Sample NN	CW Sample AB	CW Sample BA	AW Sample NN	AW Sample AB	AW Sample BA
X Sample NN	X Sample AB	X Sample BA	C Sample NN	C Sample AB	C Sample BA	CW Sample NN	CW Sample AB	CW Sample BA	AW Sample NN	AW Sample AB	AW Sample BA
X EREF NN	X EREF AB	X EREF BA	C EREF NN	C EREF AB	C EREF BA	CW EREF NN	CW EREF AB	CW EREF BA	AW EREF NN	AW EREF AB	AW EREF BA
X EREF NN	X EREF AB	X EREF BA	C EREF NN	C EREF AB	C EREF BA	CW EREF NN	CW EREF AB	CW EREF BA	AW EREF NN	AW EREF AB	AW EREF BA
X BLK NN	X BLK AB	X BLK BA	C BLK NN	C BLK AB	C BLK BA	CW BLK NN	CW BLK AB	CW BLK BA	AW BLK NN	AW BLK AB	AW BLK BA

(X = Strata-X; C = Strata-X-C; CW = Strata-X-CW; and AW = Strata-X-AW)
NN = Neutral load/neutral elution
AB = Acid pH load/basic pH elution
BA = Basic pH load/acidic pH elution
BLK = Blank (unspiked plasma extract)
EREF = Blank plasma extract post spiked with analytes
Sample = Spiked plasma extract

Figure 2. Structure of test probes used for SPE sorbent screening



Amitriptyline
log P = 5.1
pK_a = 9.67

Metoprolol
logP = 1.8
pK_a = 9.67

Prednisone
logP= 2.07
pK_a = 12.58

Diclofenac
logP = 4.98
pK_a = 4.15

Results

Figure 3. Recovery data for Metoprolol resulting from SPE phase screening

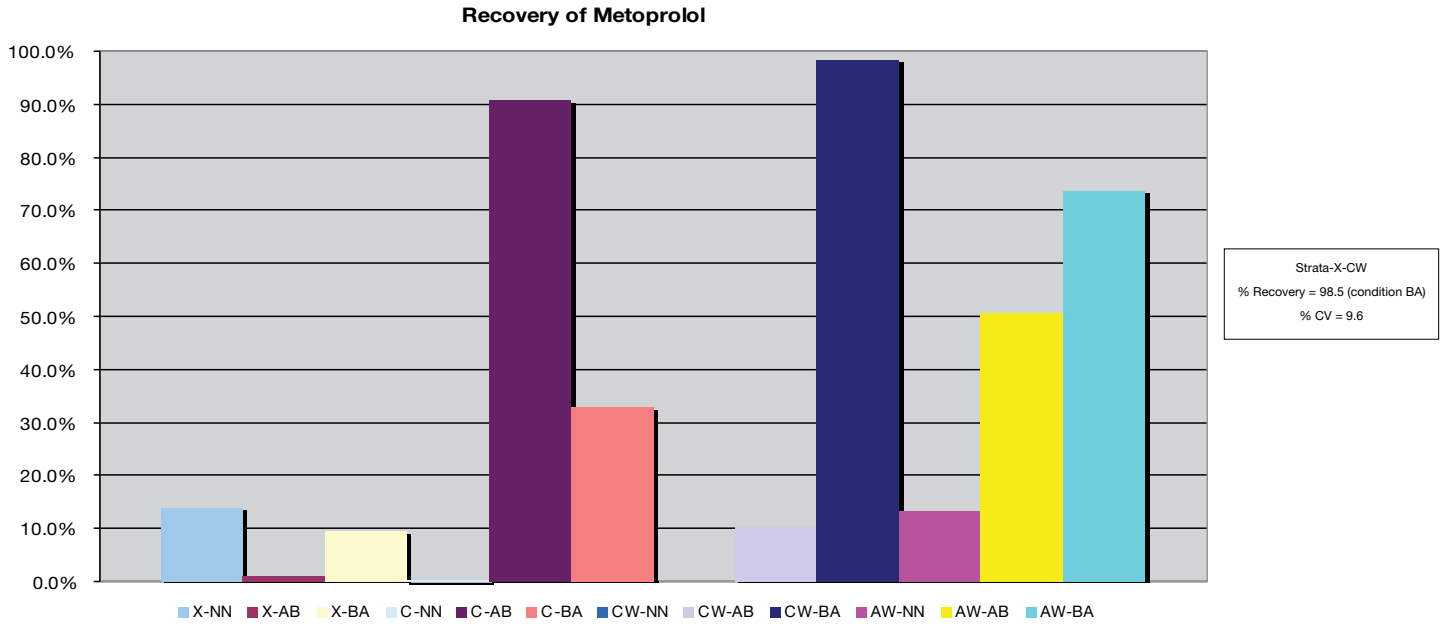


Figure 4. Recovery data for Amitriptyline resulting from SPE phase screening

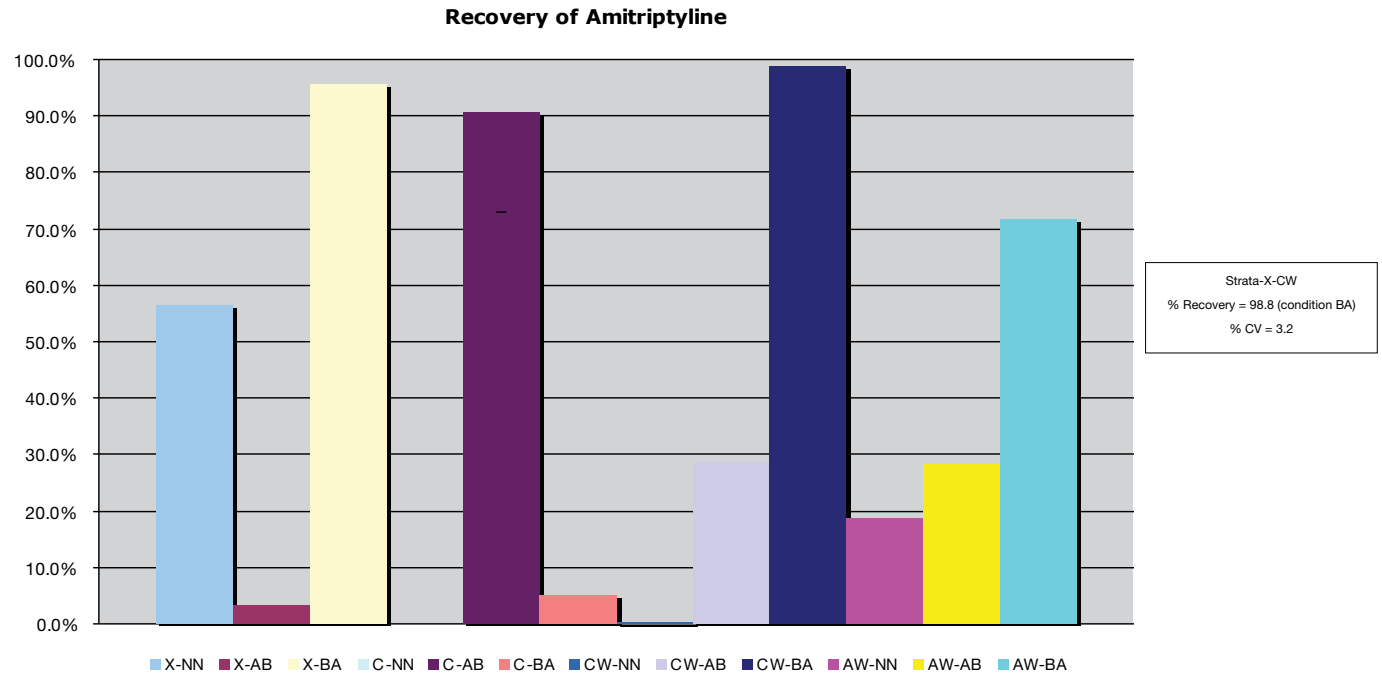


Figure 5. Recovery data for Diclofenac resulting from SPE phase screening

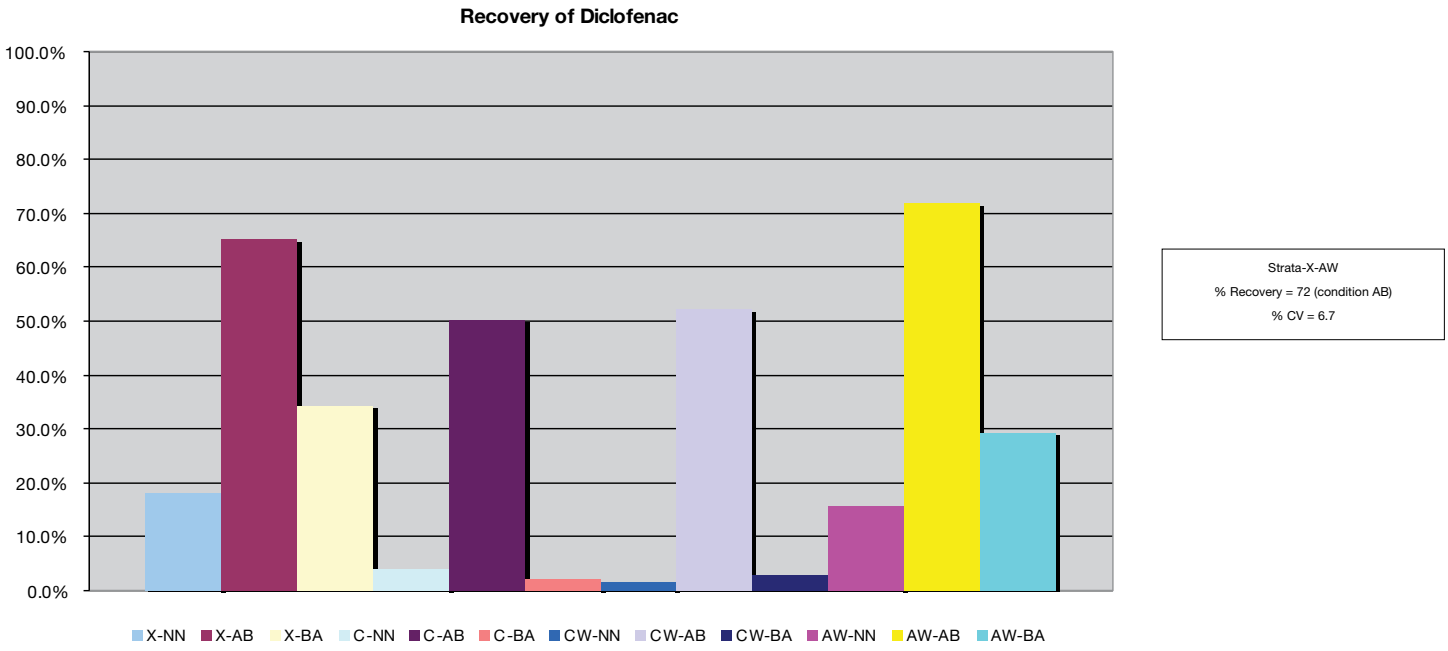
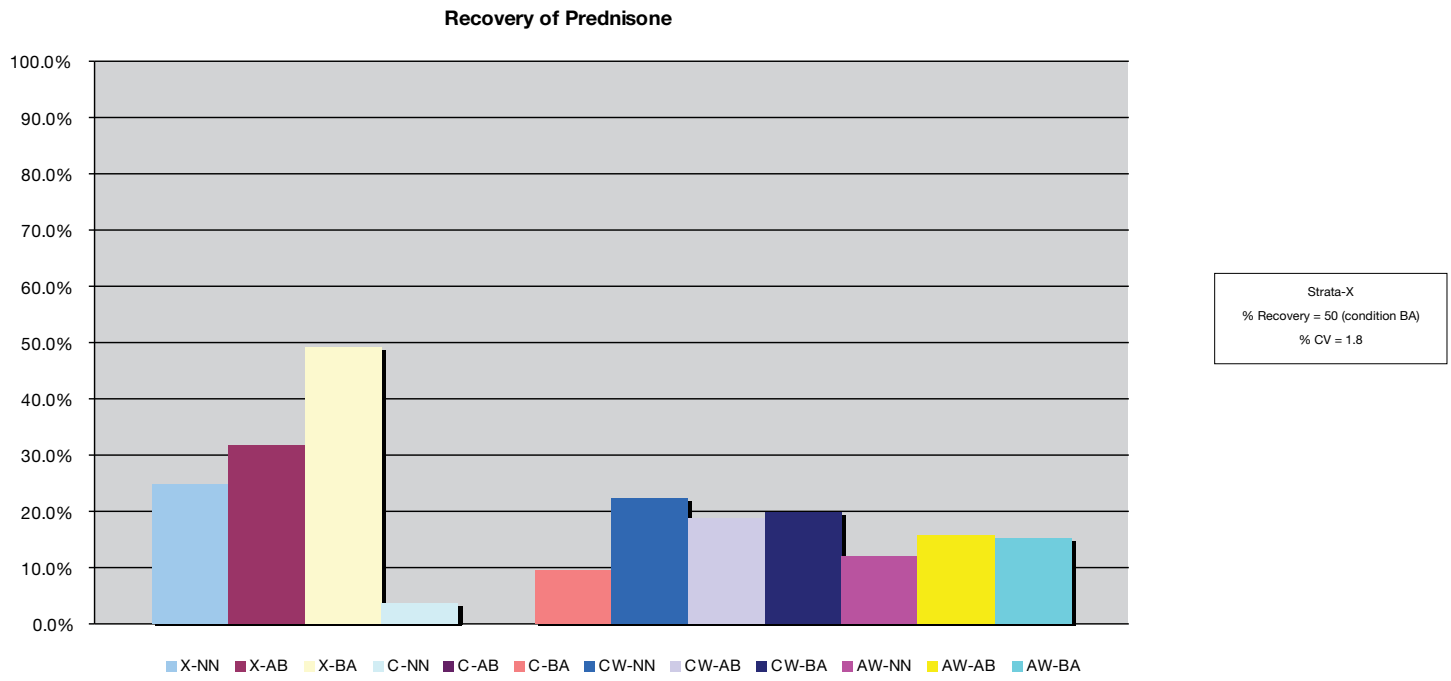


Figure 6. Recovery data for Prednisone resulting from SPE phase screening



Discussion

One of the goals of this method development scheme is to maximize sample cleanup in order to minimize background noise and increase column lifetime. We used an aggressive 70 % organic wash along with unique sets of load, wash, and elution conditions, to manipulate the retention and elution of the analytes, as a result of selectivity differences specific to each of these four SPE phases. A representative chromatogram of the neat standard (comprising all four test analytes at 25 ng/mL) using a Kinetex® 2.6 µm, C18, 50 x 2.1 mm column is outlined in **Figure 7**.

A replicate of 5 samples were extracted to evaluate the reproducibility of each method tested. The obtained recoveries from screening are summarized in **Table 2**. For the basic analytes, amitriptyline and metoprolol, the best extraction conditions were on Strata-X-CW under BA (basic load and acidic elution) conditions (**Figures 3** and **4**). Strata-X-CW is a polystyrene-divinylbenzene based polymer functionalized with a carboxylic acid moiety. The ion-exchange bonding capacity between the analyte and the carboxylic acid was strongly held under high pH load (basic load) and subsequent 70 % methanol in wash 2. The acidic elution of 5 % formic acid in methanol was sufficient to neutralize the ionic interactions, giving almost quantitative recovery.

For the neutral drug, prednisone, the best extraction conditions were obtained on Strata-X under BA (basic load and acidic elution) conditions. The neutral polymer-based sorbent was able to retain prednisone via hydrophobic interactions under basic load conditions. The analyte was then dislodged from the sorbent, resulting in recoveries of 50 % under acidic elution conditions. The interaction of the moderately polar prednisone (logP = 2.07) was not strongly sustained during the 70 % organic wash. By lowering the % organic in the wash, analyte recovery can be improved further.

The best conditions for the acidic analyte, diclofenac, were found using the Strata-X-AW sorbent under acidic load and basic elution conditions, resulting in recoveries of 72 % (**Table 2**). Strata-X-AW is a weak anion-exchanger with primary and secondary amine functionalities. Under acidic load conditions the amines are positively charged to engage in an ion-exchange interaction between the sorbent and diclofenac. Diclofenac was then eluted from the phase by applying an ammoniated methanol elution.

Conclusion

The purpose of this work is to develop a robust and fast structured approach to screen numerous SPE phases with different selectivities in order to rapidly and reproducibly target the most reliable SPE method that can be used for the quantitation of samples comprising a wide range of analytes.

Figure 7. Representative chromatogram of the neat standard at 25 ng/mL (analytes in retention order: 1. Metoprolol, 2. Prednisone, 3. Amitriptyline, 4. Diclofenac)

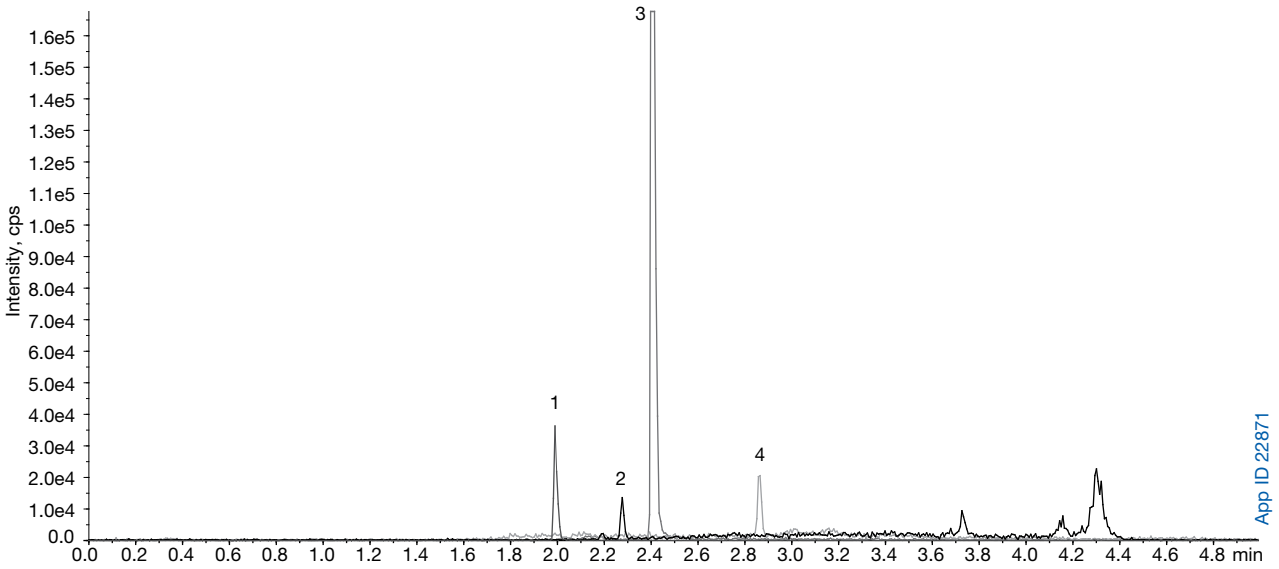


Table 2. Recovery data for each analyte for the targeted method upon screening

Analyte	% Recovery	% CV	Targeted SPE Phase	Targeted Condition
Metoprolol	98.5	9.6	Strata-X-CW	BA
Amitriptyline	98.8	3.2	Strata-X-CW	BA
Diclofenac	72	6.7	Strata-X-AW	AB
Prednisone	50	1.8	Strata-X	BA

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Configuration of Freedom EVO for Processing Phenomenex Plate-based SPE Chemistries

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Ordering Information

Strata™-X 96-Well SPE Method Development Plates

Part No.	Description	Unit
KS0-8241	Strata-X 10 mg/well (3 rows) Strata-X-C 10 mg/well (3 rows) Strata-X-CW 10 mg/well (3 rows) Strata-X-AW 10 mg/well (3 rows	ea
KS0-8209	Strata-X 30 mg/well (3 rows) Strata-X-C 30 mg/well (3 rows) Strata-X-CW 30 mg/well (3 rows) Strata-X-AW 30 mg/well (3 rows)	ea

96-Well Plate Accessories

Part No.	Description	Unit
Collection Plates (deep well, polypropylene)		
AH0-7192	96-Well Collection Plate, 350 µL/well	50/pk
AH0-7193	96-Well Collection Plate, 1 mL/well	50/pk
AH0-7194	96-Well Collection Plate, 2 mL/well	50/pk
AH0-8635	96-Well Collection Plate, 2 mL/well Square/Round-Conical	50/pk
AH0-8636	96-Well Collection Plate, 2 mL/well Round/Round, 8 mm	50/pk
AH0-7279	96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifold		
AH0-8950	96-Well Plate Manifold, Universal with Vacuum Gauge	ea

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