TN-0164

LC-MS/MS Analysis of Antipsychotics in Serum Using Microelution SPE for Fast and More Sustainable Sample Preparation

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Introduction

Most of the work activity and operating cost in an analytical lab is spent in preparing and processing samples for injection. A simple and structured workflow can save time and cost. By optimizing pretreatment, wash, and elution conditions targeted to remove matrix interferences and maximize recovery of the analytes of interest, SPE provides maximum cleanliness of biological samples for LC-MS/MS analysis. However, SPE methods require more method development time than most other sample preparation methods and can result in longer sample processing times. Microelution SPE requires less solvent for the wash and elution steps. Typical wash volumes are 100-200 µL and typical elution solvent volumes are 50-100 µL. The low volume of elution solvent means extracted samples can often be simply diluted prior to analysis, skipping a time-consuming evaporation step that requires specialized equipment. These advantages can be used to create faster, more sustainable workflows.

First, a Strata™-X Method Development 96-well plate was used to determine the best sorbent and extraction conditions for a panel of Antipsychotic drugs and metabolites. The Strata-X-CW sorbent, under acidic load and wash conditions and basic elution conditions, provided the maximum % absolute recovery (TN-0163). Previously, we demonstrated that the Kinetex™ 2.6 µm Biphenyl column was the better option for LC separation of Antipsychotic analytes prior to LC-MS/MS analysis (TN-1355).

In this technical note, we outline optimal microleution SPE conditions for a panel of 14 Antipsychotic drug analytes using the Strata-X-CW microlelution 96 well plate (**Table 1**). This was combined with a fast LC method using a Kinetex 2.6 µm Biphenyl LC column to resolve all target analytes and determine absolute % recovery and % CV. Percent recovery for the extracted samples was calculated as follows:

$$\%\ Recovery = \left(\frac{Pre-spiked\ serum\ analyte}{Post-spiked\ serum\ analyte} X\ 100\right)$$

Precision was determined as % CV with N=4 replicates.

Sample Preparation

Initial Microelution SPE Conditions

Step	Description
Sample Pretreatment:	10 µL human serum was spiked with Antipsychotics standard mix and internal standards and then diluted with 200 µL of 25 mM Ammonium Formate, pH ~3.5 adjusted.
Condition:	Strata-X-CW Microelution 96-well plate, 2 mg/well (Part No.: 8M-S035-4GA) with 200 µL of Methanol.
Equilibrate:	200 µL Water.
Load:	200 µL diluted pre-treated sample.
Wash 1:	200 µL of Acidic Buffer 25 mM Ammonium Formate, pH ~3.5 adjusted
Wash 2:	200 µL Methanol / Water (1:1, v/v).
Dry:	1 minute at 20-25 in. Hg.
Elute Option 1:	2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Methanol.
Elute Option 2:	2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40).
Dilute:	Both fractions from Elute Option 1 and Elute Option 2 were diluted separately with 200 µL mobile phase A (0.1 % Formic Acid in Water) before injection.

Final Microelution SPE Conditions

Step	Description
Sample Pretreatment:	10 µL human serum was spiked with Antipsychotics standard mix and internal standards and then diluted with 200 µL of 25 mM Ammonium Formate, pH ~3.5 adjusted.
Condition:	Strata-X-CW Microelution 96-well plate, 2 mg/well (Part No.: 8M-S035-4GA) with 200 µL of Methanol.
Equilibrate:	200 µL Water.
Load:	200 µL diluted pre-treated sample.
Wash 1:	200 µL of Acidic Buffer 25 mM Ammonium Formate, pH ~3.5 adjusted
Wash 2:	200 µL Methanol / Water (1:1, v/v).
Dry:	1 minute at 20-25 in. Hg.
Elute:	2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40).
Dry Down:	Bypass.
Reconstitution:	Bypass.
Dilute:	Dilute with 200 µL mobile phase A (0.1 % Formic Acid in Water) before injection.

LC Conditions

Column: Kinetex 2.6 µm Biphenyl
Dimensions: 50 x 3.0 mm
Part No.: 00B-4622-Y0
Mobile Phase: A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Methanol

Gradient:

Time (min)	% B
0	20
1	40
2	80
3	95
3.5	95
3.5	20
5	20

Flow Rate: 0.7 mL/min
Injection Volume: 5 µL
Temperature: 40 °C
LC System: Agilent® 1260 Infinity
Detection: MS/MS
Detector: SCIEX® 6500 Triple Quad™

MS/MS Conditions

Ion Source: ESI
Polarity: Positive
Source Temperature: 350° C
GS1: 55 psi
GS2: 60 psi
CUR: 35 psi
IS: 2500 V

Table 1 . MS Transitions.

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	Analyte	Q1 Mass (Da)	Q3 Mass (Da)
Olanzapine	313.1	256.1	Quetiapine	384.1	253.1
Norclozapine	313	192.1	Ziprasidone	413	194
Clozapine	327	270.1	Dehydroariprazole	447	286.1
9-OH-Risperidone	427.2	207.2	Chlorpromazine	319	86
Haloperidol	376.1	165	Fluphenazine	439.2	171
Risperidone	411.2	191.1	Ariprazole	448	285.1
Promethazine	285	86.1	Lurasidone	493.2	166.1

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Results and Discussion

A Strata™-X-CW 2 mg/well 96-well microelution plate was employed for extraction of Antipsychotics in serum under AB condition (acidic load and basic elution) based on our initial SPE sorbent screening results (utilizing a Strata-X 30 mg/well 96-well SPE Method Development Plate, TN-0163). The optimized microelution method aimed toward higher recovery of analytes and direct injection of extracted samples bypassing time-consuming dry-down and reconstitution. Although the Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent resulted in good recovery for Antipsychotics extracted using the Strata-X-CW SPE sorbent from the Strata-X Method Development 96-well plate (**Table 2**), it wasn't ideal for direct injection of the extracted samples from the microelution SPE sorbent. Peak fronting and broadening of the analyte peaks observed because of a strong solvent effect, even though 3x dilution of the microelution extracted samples was made prior to injection. This effect was more pronounced with large volume sample injection and therefore required further optimization of the elution solvent. A number of different elution solvents were tested and 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40, v/v) elution was found to be more effective than 5 % Ammonium Hydroxide in Methanol by dislodging the more hydrophobic, late eluting analytes from the Strata-X-CW microelution plate (**Figure 1a** and **1b**). 5 % Ammonium Hydroxide in in Acetonitrile / Methanol (60:40, v/v), resulted in higher (blue) recovery for 50 % of the panel and lower (red) for other 50 %, compared to the Strata-X-CW sorbent of the Strata-X Method Development 96-well plate with the Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent (**Table 2**). The optimized microelution method is shown in **Figure 2**, compared to the Method Development plate conditions that were previously established. A representative chromatogram of extracted serum is shown in **Figure 3**.

The logP (1.8 to 4.9) and pK_a (7.6 to 9.2) range of the 14 Antipsychotics represents a panel of analytes with moderate to high hydrophobicity and basic functionality. Therefore, a strong 50 % organic wash resulted in 70-121 % analyte recovery at 10 ng/mL (**Table 2**). The only exception was Olanzapine, for which 58 % recovery was reported. Percent recovery for all analytes at three concentrations (10 ng/mL, 40 ng/mL, and 400 ng/mL) resulted in >60 % recovery and % CVs from 3.7-19.4 % for all analytes. All results were within ±20 % CV for all analytes, except for Chlorpromazine and Olanzapine (**Table 3**).

The process efficiency (PE) for most of the analytes tended to increase as the concentration increased over the 3 concentrations evaluated (**Table 3**). Matrix effect at all three concentrations for all drug analytes ranged from 34 % to 122 %. Matrix effect generally improved with increasing concentration, with values of 80 % to 103 % for all analytes at 400 ng/mL, except Olanzapine and Lurasidone (**Table 3**). Calibration curves over a 100-fold dynamic range (5 ng/mL to 500 ng/mL) with 1/x weighting demonstrated excellent linearity with R^2 values ≥0.992 for all target compounds, even those that had lower recovery or exhibited increased ion suppression or enhancement and lover process efficiency (**Table 3**, **Figure 4**).

Figure 1a. Representative Chromatogram of Antipsychotics in Serum Using Strata-X-CW Microelution SPE with 5 % Ammonium Hydroxide in Methanol as the Elution Solvent.

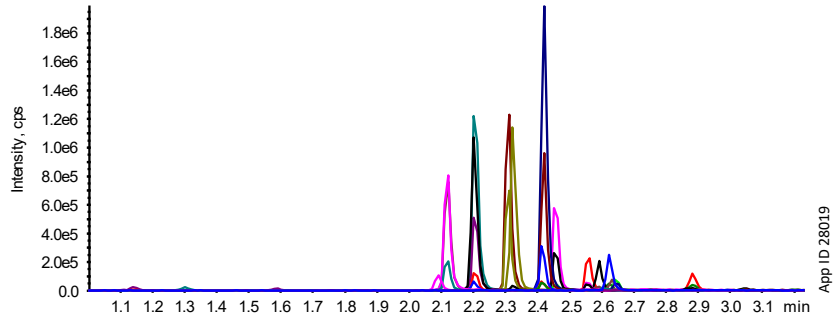

Figure 1b. Representative Chromatogram Showing Higher Detector Response for Late Eluting Antipsychotics in Serum Using Strata-X-CW Microelution SPE Eluting with 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40, v/v), versus 5 % Ammonium Hydroxide in Methanol (**Figure 1a**).

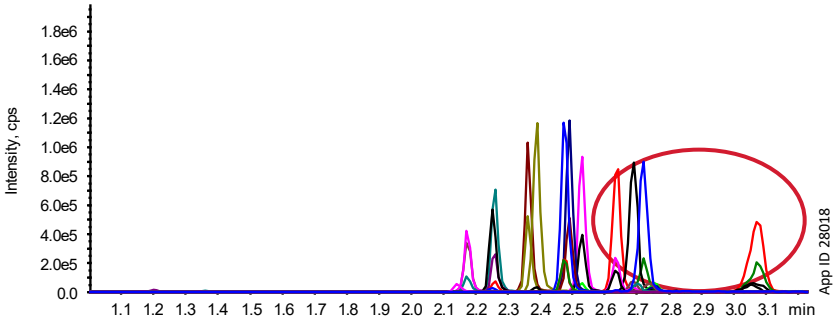

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Table 2. Recovery Comparison of Antipsychotics Extracted from Serum Using the Strata™-X-CW SPE of the Strata-X Method Development 30 mg/well 96-Well Plate and the Strata-X-CW Microelution 2 mg/well 96-Well Plate. Increased Recovery in Blue and Decreased Recovery in Red.

	Strata-X-CW SPE Sorbent of the Strata-X Method Development 30 mg/well 96-Well Plate *Spiked conc. of serum = 1 ng/mL		Strata-X-CW Microelution 2 mg/well 96-Well Plate *Spiked conc. of serum = 10 ng/mL	
	Elution Solvent: Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v)		Elution Solvent: 5 % Ammonium Hydroxide in in Acetonitrile / Methanol (60:40, v/v)	
Analyte	% Recovery	% CV (N = 4)	% Recovery	% CV (N = 4)
Olanzapine	80	8.9	58	6.8
Norclozapine	90	10.3	79	11.6
Clozapine	91	5.5	121	6.1
9-OH-Risperidone	97	12.3	103	7.1
Haloperidol	91	1.5	95	12.4
Risperidone	95	8.4	109	10.5
Promethazine	97	14.4	70	9.3
Quetiapine	91	14.5	70	9.3
Ziprasidone	85	7.9	88	6.8
Dehydroariprazole	97	9.2	74	12.8
Chlorpromazine	59	15.8	70	10.6
Fluphenazine	95	22.6	74	16
Aripiprazole	85	2.9	101	5.3
Lurasidone	95	9.2	74	9.3

Figure 2. SPE Method Comparison of Antipsychotics from Serum Using the Strata-X-CW SPE of the Strata-X Method Development 30 mg/well 96-Well Plate (Left) and the Strata-X-CW Microelution 2 mg/well 96-Well Plate (Right).

Step	Description
Sample Pretreatment:	500 µL human serum was spiked with Antipsychotic standard mix at a concentration of 1 ng/mL (except Fluphenazine at 3 ng/mL) and then diluted with 1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted.
Condition:	Strata-X Method Development 96-well plate, 30 mg/well (Part No.: KS0-8209) with 1 mL of Methanol.
Equilibrate:	1 mL Water.
Load:	About 1.5 mL of pre-treated sample.
Wash 1:	1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted.
Wash 2:	1 mL Methanol / Water (1:1, v/v).
Dry:	5-8 minutes at 20-25 in. Hg.
Elute:	2 aliquots of 300 µL of Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v).
Dry Down:	15-20 minutes at 40 °C under a gentle stream of Nitrogen. Time ≅ 25 min Total Dry Time ≅ 35 min
Reconstitution:	500 µL on initial mobile phase spiked with 5 ng/mL Internal Standard mix (Lurasidone-D_8, Aripiprazole-D_8, Fluphenazine-D_8, Olanzapine-D_8). Time ≅ 1 min
Total Evaporation and Reconstitution Time:	≅ 36 min/plate
Total Reagent Volume:	6.1 mL/well ; 585.6 mL/plate

Step	Description
Sample Pretreatment:	10 µL human serum was spiked with Antipsychotics standard mix and internal standards and then diluted with 200 µL of 25 mM Ammonium Formate, pH ~3.5 adjusted.
Condition:	Strata-X-CW Microelution 96-well plate, 2 mg/well (Part No.: 8M-S035-4GA) with 200 µL of Methanol.
Equilibrate:	200 µL Water.
Load:	200 µL diluted pre-treated sample.
Wash 1:	200 µL of Acidic Buffer 25 mM Ammonium Formate, pH ~3.5 adjusted
Wash 2:	200 µL Methanol / Water (1:1, v/v).
Dry:	1 minute at 20-25 in. Hg.
Elute:	2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Acetonitrile / Methanol (60:40).
Dry Down:	Bypass. Total Dry Time ≅ 1 min
Reconstitution:	Bypass.
Dilute:	Dilute with 200 µL mobile phase A (0.1 % Formic Acid in Water) before injection.
Total Evaporation and Reconstitution Time:	≅ 1 min/plate
Total Reagent Volume:	1.3 mL/well ; 124.8 mL/well

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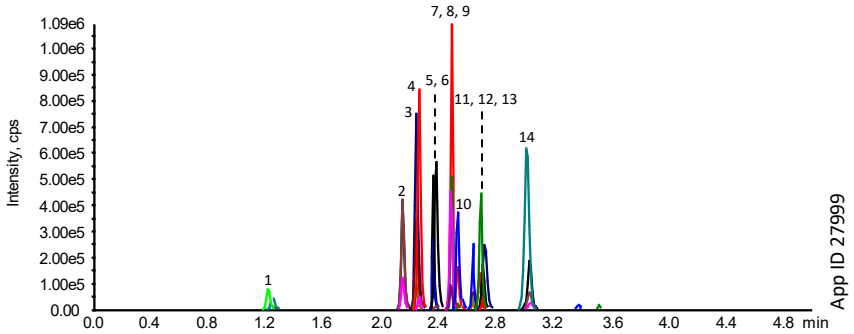
Figure 3. Analysis of Antipsychotics Extracted from Serum Using Strata™-X-CW Microelution Plate, Under Acidic Load and Basic Elution, on a Kinetex™ 2.6 µm Biphenyl Column. Spiked Concentration of Antipsychotics in Serum is 5 ng/mL.



Peak No.	Analyte	Retention Time (min)
1	Olanzapine	1.25
2	Norclozapine	2.14
3	Clozapine	2.24
4	9-OH-Risperidone	2.3
5	Haloperidol	2.4
6	Risperidone	2.4
7	Promethazine	2.53
8	Quetiapine	2.5
9	Ziprasidone	2.5
10	Dehydroariprazole	2.6
11	Chlorpromazine	2.7
12	Fluphenazine	2.7
13	Ariprazole	2.7
14	Lurasidone	3.1

Table 3. Method Performance and Qualification Data Utilizing the Finalized Optimized Conditions for Antipsychotics from Serum Using Strata-X-CW Microelution Plate.

Analyte	Concentration (ng/mL)	% Recovery	% CV	% Matrix Effect	PE	Precision	Accuracy	Linear regression R^2	Regression Equation
Olanzapine	10	58	6.8	58	43	15	90	0.994	y = 0.0648 x + -0.586
	40	46	9.4	53	23	7.8	89		
	400	40	19.2	34	29	9.2	85		
Norclozapine	10	79	11.6	79	40	11	98	0.995	y = 0.188 x + 0.244
	40	92	9.4	94	54	9.7	100		
	400	76	14.3	102	83	10.7	85		
Clozapine	10	121	6.1	122	87	5.2	101	0.994	y = 0.076 x + 0.0969
	40	122	3.7	116	116	6.4	99		
	400	116	5.3	99	111	6.7	99		
9-OH-Risperidone	10	103	7.1	102	75	6.3	102	0.995	y = 0.293 x + 0.892
	40	120	6.8	107	94	7.2	115		
	400	97	13	103	111	12.5	88		
Haloperidol	10	95	12.4	94	61	15	101	0.994	y = 0.431 x + 0.995
	40	110	6.7	105	78	7.1	109		
	400	91	13.7	103	97	8.8	85		
Risperidone	10	109	10.5	62	86	4.7	85	0.995	y = 0.206 x + 1.51
	40	115	8.2	78	78	8.5	105		
	400	100	15.4	95	95	13	88		
Promethazine	10	70	9.3	77	60	8.2	88	0.997	y = 0.202 x + -0.0321
	40	78	6.0	86	82	2.4	85		
	400	62	11.4	102	73	11	84		
Quetiapine	10	70	9.3	94	59	12.3	102	0.992	y = 0.432 x + 1.2
	40	78	6	105	67	4.9	111		
	400	62	11.4	103	81	9.5	85		
Ziprasidone	10	88	6.8	83	42	9.4	104	0.995	y = 0.197 x + 0.35
	40	82	7.2	82	64	7.5	109		
	400	75	14.4	80	72	7	87		
Dehydroaripiprazole	10	74	12.8	74	61	14	99	0.998	y = 0.0292 x + 0.0292
	40	79	8.8	89	99	9.1	109		
	400	71	15.4	101	92	8.9	92		
Chlorpromazine	10	70	10.6	63	108	5	88	0.992	y = 0.0921 x + -0.167
	40	65	6.2	76	98	4.7	85		
	400	50	11.7	100	60	2.6	90		
Fluphenazine	10	74	16	74	62	15.9	89	0.993	y = 0.00822 x + -0.000277
	40	78	9.5	88	87	9.5	89		
	400	68	12.9	102	83	9.7	85		
Aripiprazole	10	101	5.3	101	62	5.2	101	0.995	y = 0.0167 x + -0.00437
	40	112	6.4	106	112	6.4	99		
	400	93	5	97	115	6.7	99		
Lurasidone	10	74	9.3	60	75	12.9	113	0.998	y = 0.0972 x + 0.2
	40	99	6.7	63	107	6.9	110		
	400	82	11.9	56	82	9.8	90		

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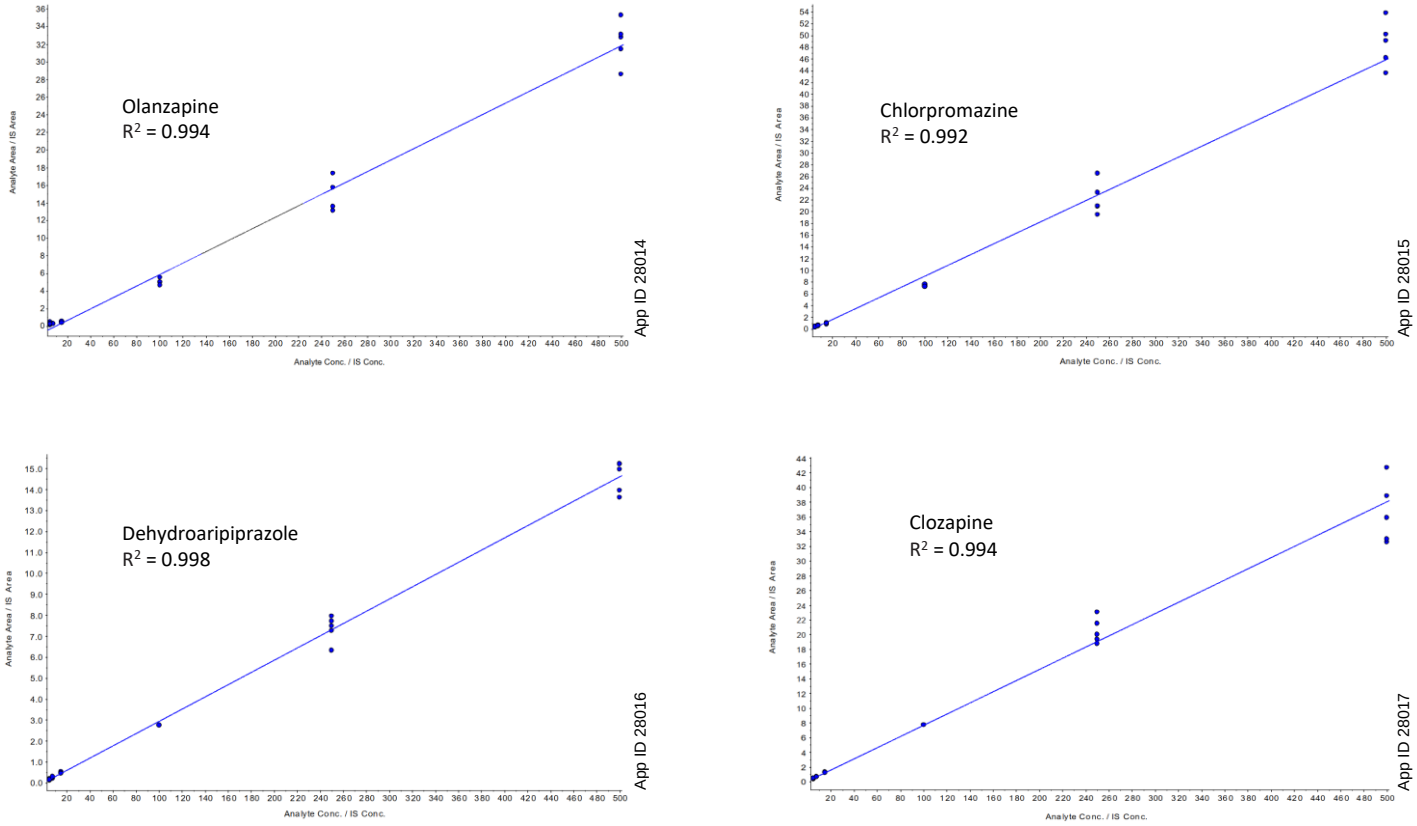
Figure 4. Calibration Curves for Four Selected Antipsychotics Over 100-Fold Dynamic Range (5 ng/mL to 500 ng/mL).



Conclusions

The described SPE method utilizing the Strata™-X-CW SPE Microelution plate resulted in a simple, rapid extraction for identification and quantitation of 14 Antipsychotics from human serum which is cost effective and can efficiently incorporated into a clinical research workflow. The simplified microelution SPE method provides a fast sample extraction that can be automated and uses less solvent than a traditional SPE method. The no evaporation option reduces total preparation time by 30-40 minutes. If elution solvent evaporation is preferred, 20-25 minutes is still saved because of the low volume of elution solvent. Combined with the fast LC method using the Kinetex™ 2.6 µm Biphenyl column, this microelution SPE method provides the ideal combination of a streamlined and more sustainable workflow for LC-MS/MS analysis.

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SPE Ordering Information

Strata™-X Method Development 96-Well Plate		
Part No.	**Description**	**Unit**
KS0-8209	Strata-X, -X-C, -X-CW, and -X-AW 30 mg/well each	ea

Strata-X Microelution 96-Well Plates (ea)	
Phase	**2 mg**
Strata-X-AW	8M-S038-4GA
Strata-X-A	8M-S123-4GA
Strata-X	8M-S100-4GA
Strata-X-C	8M-S029-4GA
Strata-X-CW	8M-S035-4GA

Kinetex™ Ordering Information

2.6 µm Midbore™ Columns (mm)						SecurityGuard™ ULTRA Cartridges (mm)‡
Phases	**30 x 3.0**	**50 x 3.0**	**75 x 3.0**	**100 x 3.0**	**150 x 3.0**	**3/pk**
EVO C18	00A-4725-Y0	00B-4725-Y0	—	00D-4725-Y0	00F-4725-Y0	AJ0-9297
PS C18	00A-4780-Y0	00B-4780-Y0	—	00D-4780-Y0	00F-4780-Y0	AJ0-8950
Polar C18	—	00B-4759-Y0	—	00D-4759-Y0	00F-4759-Y0	AJ0-9531
Biphenyl	—	00B-4622-Y0	—	00D-4622-Y0	00F-4622-Y0	AJ0-9208
XB-C18	00A-4496-Y0	00B-4496-Y0	00C-4496-Y0	00D-4496-Y0	00F-4496-Y0	AJ0-8775
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777
HILIC	00A-4461-Y0	—	—	00D-4461-Y0	00F-4461-Y0	AJ0-8779
Phenyl-Hexyl	—	00B-4495-Y0	—	00D-4495-Y0	00F-4495-Y0	AJ0-8781
F5	—	00B-4723-Y0	—	00D-4723-Y0	00F-4723-Y0	AJ0-9321

for 3.0 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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