## TN-0166

# Microelution SPE of Anticonvulsants from Serum for Fast, Sustainable Sample Preparation for Analysis by LC-MS/MS

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## Introduction

SPE requires more method development and processing time, but by optimizing the pretreatment, wash, and elution conditions to remove matrix interferences and maximize recovery of target analytes, it provides the cleanest samples for LC-MS/MS analysis. 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. Microelution SPE also uses less sample volume and reagents, reducing solvent usage and waste. 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 Anticonvulsant 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 Luna Omega 3 µm Polar C18 column was the better option for LC separation of Anticonvulsant analytes prior to LC-MS/MS analysis (TN-1357).

In this technical note, we outline optimal microleution SPE conditions for a panel of 11 Anticonvulsant drug analytes using the Strata-X-CW microlelution 96 well plate (**Table 1**). This was combined with a fast LC method using a Luna Omega 3 µm Polar C18 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

### *Microelution SPE Conditions*

| Step | Description |
|---|---|
| **Sample Pretreatment:** | 10 µL human serum was spiked with Anticonvulsants 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 (2:8, v/v). |
| **Dry:** | 1 minute at 20-25 in. Hg. |
| **Elute:** | 2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Methanol. |
| **Dry Down:** | Bypass. |
| **Reconstitution:** | Bypass. |
| **Dilute:** | Dilute with 200 µL mobile phase A (0.1 % Formic Acid in Water) before injection. |

## LC Conditions

**Column:** Luna™ Omega 3 µm Polar C18
**Dimensions:** 50 x 3.0 mm
**Part No.:** 00B-4760-Y0
**Mobile Phase:** A: 2 mM Ammonium Acetate
B: 2 mM Ammonium Acetate in Methanol

**Gradient:**

| Time (min) | % B |
|---|---|
| 0 | 20 |
| 0.5 | 20 |
| 1.5 | 40 |
| 2.5 | 80 |
| 3 | 95 |
| 3.5 | 95 |
| 3.51 | 20 |
| 5 | 20 |

**Flow Rate:** 0.8 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:** Dual Polarity
**Source Temperature:** 450° C
**GS1:** 55 psi
**GS2:** 60 psi
**CUR:** 35 psi
**IS:** +2500 V or -2500V

**Table 1 .** MS Transitions.

| Analyte | Q1 Mass (Da) | Q3 Mass (Da) |
|---|---|---|
| Pregabalin | 160.1 | 55 |
| Gabapentin | 172 | 137 |
| Levetiracetam | 171 | 126.1 |
| Lamotrigine | 256.1 | 211.1 |
| Felbamate | 178.1 | 117.1 |
| Lacosemide | 251.1 | 108 |
| Zonisamide | 212.9 | 132 |
| Topiramate | 338 | 78 |
| Oxcarbazapine | 253 | 236 |
| Carbamazepine Epoxide | 253.1 | 180.1 |
| Carbamazepine | 237.1 | 194.1 |

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## Results and Discussion

A Strata™-X-CW 2 mg/well 96-well microelution plate was employed for extraction of Anticonvulsants in serum under AB conditions (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 that bypasses dry-down and reconstitution. Although the Ethyl Acetate / Isopropanol / Ammonium Hydroxide (7:2:1, v/v/v) elution solvent resulted in good recovery for Anticonvulsants extracted using the Strata-X-C 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 extraction. The elution conditions for the microelution plate was changed to Methanol treated with 5 % Ammonium Hydroxide and showed improved analyte recovery (by 70-80 %) especially for Zwitterionic compounds. As a protic solvent, Methanol was a stronger solvent for polar analytes which increased solubility and resulted in complete elution of Pregabalin and Gabapentin from the weak cation exchange SPE sorbent. The optimized microelution method is shown in **Figure 2** (with representative chromatogram of extracted samples shown in **Figure 1**), compared to the Method Development plate conditions that were previously established.

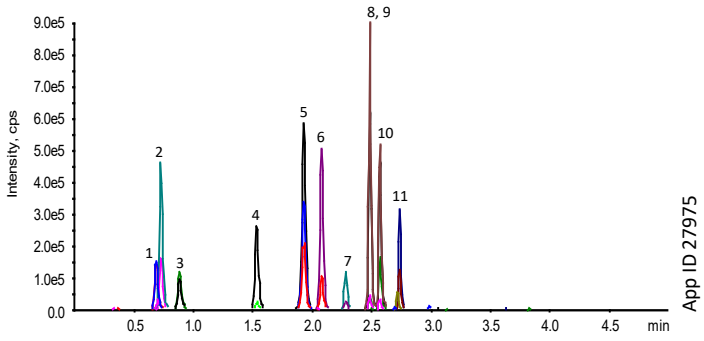
The 11 anticonvulsants studied here have a wide range of hydrophobicities and acid-base characteristics. The decrease in organic strength in the second wash from 50 to 20 % Methanol in the microelution method significantly improved recovery of the polar and neutral Topiramate (logP = 0.12, $pK_a$ = 11.09) Felbamate (logP = -0.7, $pK_a$ = 3.82) by 2- to 3-fold. However, the more polar Levetiracetam with a negative logP of -0.6 and a $pK_a$ of 2.6 couldn't sustain the 20 % organic wash and had poor recovery (<25 %).

Percent recovery for the optimized microelution method at 10 ng/mL was between 80-124 % (**Table 2**) except the polar compounds Topiramate (55 %) and Levetiracetam (<25 %). Percent recovery at 3 different concentrations (10 ng/mL, 40 ng/mL, and 400 ng/mL) was >80 % for all analytes except Levetiracetam at all three concentrations, Oxcarbazepine at 40 ng/mL, and Topiramate at 10 ng/mL. Percent CVs were within ±20 % for all analytes (**Table 3**). A 100-fold recovery improvement was observed for Topiramate from the lowest concentration (10 ng/mL) to the highest concentration (400 ng/mL). However, this loss of recovery may be an artifact of poor MS signal at the lower concentrations.

Matrix Effect was 34 % to 68 % at 10 ng/mL. It improved to 80±5 % at 40 ng/mL, except Topiramate (95 %) and Carbamazepine Epoxide (112 %). At 400 ng/mL, Matrix Effect was 100±10 % except Carbamazepine Epoxide (251 %). Process Efficiency was 33 % to 59 % at 10 ng/mL for all analytes except Carbamazepine Epoxide (85 %). Process Efficiency was 50±10 % at 40 ng/mL except Oxcarbazepine (62 %), Topiramate (86 %), and Carbamazepine Epoxide (164 %). At 400 ng/mL, Process Efficiency was 100±20 % except Oxcarbazepine (74 %) and Carbamazepine Epoxide (382 %).

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 ≥0.993 for all target compounds including the Zwitterions Pregabalin and Gabapentin (**Figure 3**). The only exception Levetiracetam ($R^2$ = 0.976) with <25 % recovery (**Table 2**).

**Figure 1.** Analysis of Anticonvulsants Extracted from Serum Using Strata-X-CW Microelution Plate, Under Acidic Load and Basic Elution, on a Luna™ Omega 3 µm Polar C18 Column. Spiked Concentration of Antipsychotics in Serum is 30 ng/mL.




| Peak No. | Analyte | Retention Time (min) | Polarity |
|---|---|---|---|
| 1 | Pregabalin | 0.7 | Positive |
| 2 | Gabapentin | 0.8 | Positive |
| 3 | Levetiracetam | 0.88 | Positive |
| 4 | Zonisamide | 1.53 | Positive |
| 5 | Lacosamide | 1.93 | Positive |
| 6 | Felbamate | 2.08 | Positive |
| 7 | Lamotrigine | 2.28 | Positive |
| 8 | Carbamazepine Epoxide | 2.5 | Positive |
| 9 | Topiramate | 2.5 | Negative |
| 10 | Oxcarbazepine | 2.6 | Positive |
| 11 | Carbamazepine | 2.8 | Positive |

**Table 2.** Recovery Comparison of Anticonvulsants Extracted from Serum Using the Strata-X-C 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.

| | Strata-X-C 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 Methanol | |
| Analyte | % Recovery | % CV (N = 4) | % Recovery | % CV (N = 4) |
| Pregabalin | 53 | 9.0 | 95 | 17.3 |
| Carbamazepine | 82 | 3.0 | 88 | 12.5 |
| Felbamate | 31 | 17.6 | 91 | 11.2 |
| Gabapentin | 50 | 8.9 | 87 | 13.6 |
| Lacosamide | 31 | 21.6 | 80 | 13.8 |
| Lamotrigine | 76 | 7.6 | 98 | 17.3 |
| Zonisamide | 25 | 17.1 | 85 | 14.3 |
| Oxcarbazepine | 37 | 17.4 | 97 | 19.4 |
| Carbamazepine Epoxide | 96 | 2.9 | 124 | 15.8 |
| Topiramate | 26 | 54.1 | 54.9 | 20.1 |
| Levetiracetam | <25 | N/A | <25 | N/A |

**Figure 2.** SPE Method Comparison of Anticonvulsants from Serum Using the Strata™-X-C 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).

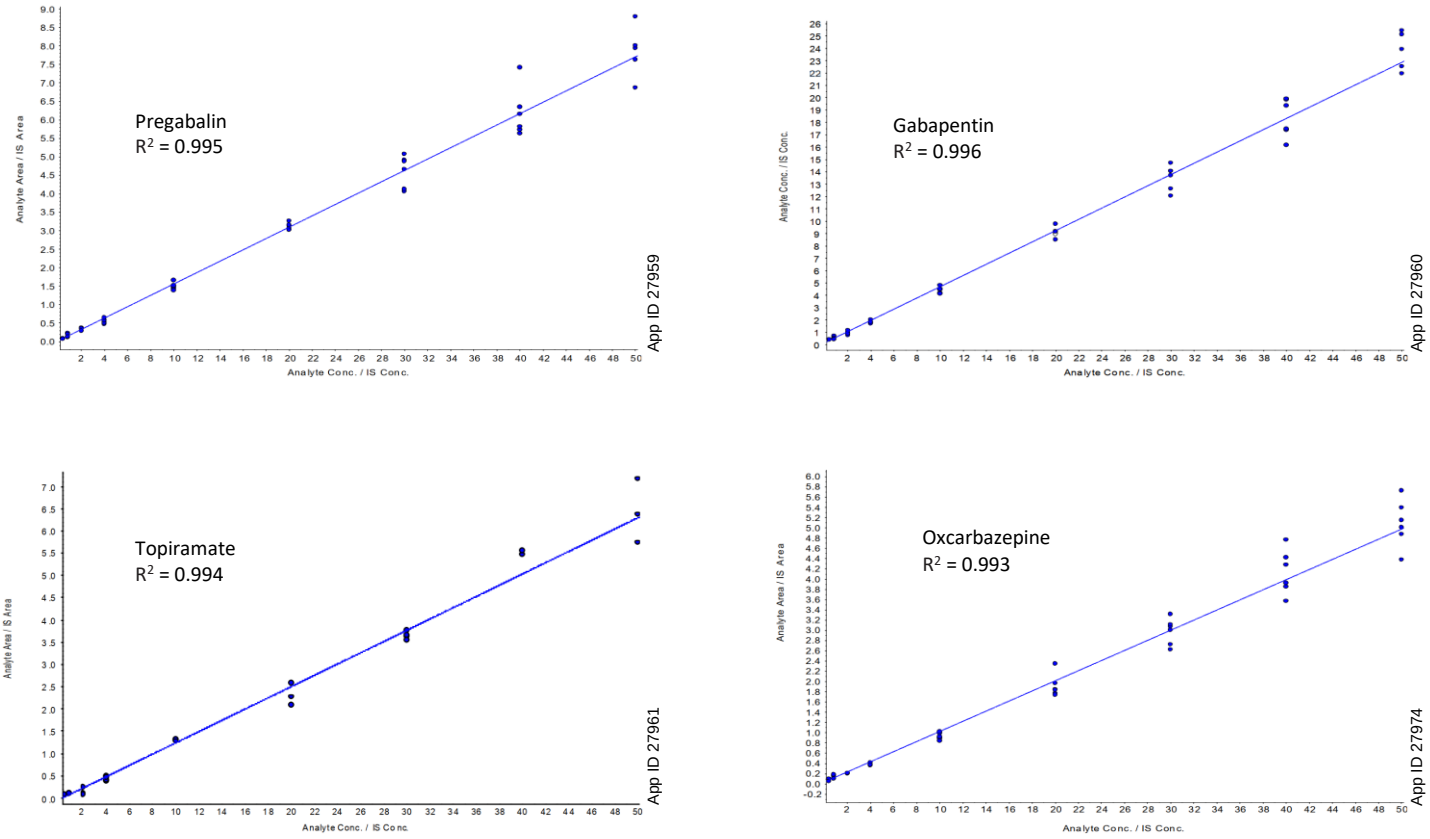
| Step | Description |
|---|---|
| **Sample Pretreatment:** | 500 µL human serum was spiked with Anticonvulsants standard mix at a concentration of 1 ng/mL then diluted with 1 mL of 25 mM Ammonium Formate, pH 4-5 adjusted. |
| **Condition:** | Strata-X Method Development 96-well plate, 30 mg/well (Part 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.<br><br>Time ≅ 25 min<br>Total Dry Time ≅ 35 min |
| **Reconstitution:** | 500 µL on initial mobile phase spiked with 5 ng/mL Internal Standard mix (Carbazepine-$D_{10}$, Gabapentin-$D_{10}$, Topiramate-$D_{12}$).<br><br>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 Anticonvulsants 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 (2:8, v/v). |
| **Dry:** | 1 minute at 20-25 in. Hg. |
| **Elute:** | 2 aliquots of 50 µL of 5 % Ammonium Hydroxide in Methanol. |
| **Dry Down:** | Bypass.<br><br>Total Dry Time ≅ 1 min |
| **Reconstitution:** | Bypass. |
| **Dilute:** | Dilute with 200 µL mobile phase A (2 mM Ammonium Acetate) before injection. |
| **Total Evaporation and Reconstitution Time:** | ≅ 1 min/plate |
| **Total Solvent Volume:** | 1.3 mL/well ; 124.8 mL/well |

**Table 3.** Method Performance and Qualification Data Utilizing Optimized Conditions and the Strata-X-CW Microelution Plate for the Extraction of Anticonvulsants from Serum.

| Analyte | Concentration (ng/mL) | % Recovery | % CV | % Matrix Effect | PE | Precision | Accuracy | Linear regression $R^2$ | Regression Equation |
|---|---|---|---|---|---|---|---|---|---|
| Pregabalin | 10 | 95 | 17.3 | 52 | 49 | 12.8 | 102 | 0.995 | y = 0.154 x + 0.0246 |
| | 40 | 98 | 3 | 84 | 50 | 3.1 | 101 | | |
| | 400 | 105 | 5.1 | 98 | 100 | 10.8 | 101 | | |
| Carbamazepine | 10 | 88 | 12.5 | 54 | 48 | 13.8 | 91 | 0.997 | y = 0.162 x + 0.0656 |
| | 40 | 87 | 6.6 | 83 | 51 | 7.5 | 98 | | |
| | 400 | 101 | 5.4 | 103 | 104 | 5.9 | 103 | | |
| Felbamate | 10 | 91 | 11.2 | 56 | 51 | 5.4 | 85 | 0.996 | y = 0.39 x + 0.14 |
| | 40 | 88.2 | 9.6 | 83 | 52 | 9.8 | 97 | | |
| | 400 | 106.2 | 8.3 | 107 | 116 | 8.2 | 105 | | |
| Gabapentin | 10 | 87 | 13.6 | 58 | 51 | 15.5 | 95 | 0.996 | y = 0.454 x + 0.168 |
| | 40 | 94 | 4.5 | 85 | 47 | 5.8 | 99 | | |
| | 400 | 98.7 | 8.5 | 102 | 98 | 8.6 | 101 | | |
| Lacosamide | 10 | 80.2 | 13.8 | 57 | 45 | 5.9 | 86 | 0.995 | y = 0.498 x + 0.173 |
| | 40 | 86.2 | 11.7 | 83 | 50 | 11.7 | 98 | | |
| | 400 | 101.7 | 7 | 107 | 112 | 6.5 | 104 | | |
| Lamotrigine | 10 | 98 | 17.3 | 60 | 59 | 14.1 | 95 | 0.994 | y = 0.09 x + 0.040 |
| | 40 | 90 | 14.3 | 85 | 55 | 14.7 | 99 | | |
| | 400 | 105 | 8.5 | 110 | 119 | 7.7 | 104 | | |
| Zonisamide | 10 | 85 | 14.3 | 58 | 49 | 20 | 78 | 0.995 | y = 0.236 x + 0.0888 |
| | 40 | 89 | 6.2 | 82 | 50 | 6.3 | 101 | | |
| | 400 | 106 | 8.2 | 104 | 112 | 7.6 | 107 | | |
| Oxcarbazepine | 10 | 96.5 | 19.4 | 34 | 33 | 4 | 85 | 0.993 | y = 0.0987 x + 0.0404 |
| | 40 | 71 | 11.8 | 77 | 62 | 12 | 95 | | |
| | 400 | 85 | 8.5 | 78 | 74 | 10.7 | 104 | | |
| Carbamazepine Epoxide | 10 | 124.1 | 15.8 | 68 | 85 | 14.7 | 97 | 0.994 | y = 0.502 x - 0.0569 |
| | 40 | 133 | 11.8 | 112 | 164 | 11.8 | 103 | | |
| | 400 | 152 | 8.6 | 251 | 382 | 7.8 | 105 | | |
| Topiramate | 10 | 54.9 | 51 | 68 | 37 | 78 | 70 | 0.994 | y = 0.126 x + 0.0245 |
| | 40 | 92 | 31.7 | 95 | 86 | 36 | 109 | | |
| | 400 | 109 | 22.2 | 109 | 106 | 20 | 105 | | |
| Levetiracetam | 10 | <25 | | | | | | 0.9766 | y = 0.124 x + 0.159 |
| | 40 | <25 | | | | | | | |
| | 400 | <25 | | | | | | | |

**Figure 3.** Calibration Curves for Four Selected Anticonvulsants Over 100-Fold Dynamic Range (5 ng/mL to 500 ng/mL).




## Conclusions

The method using the Strata™-X-CW SPE Microelution plate resulted in a simple, rapid extraction for identification and quantitation of 10 Anticonvulsants from human serum. The method reduces time and solvent usage and can be automated for a high throughput, more sustainable sample preparation workflow for LC-MS/MS analysis.

## 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 |

## Luna™ Omega Ordering Information

| 3 µm MidBore™ Columns (mm) | | | | SecurityGuard™ Cartridges (mm) |
|---|---|---|---|---|
| **Phases** | **50 x 3.0** | **100 x 3.0** | **150 x 3.0** | **4 x 2.0*/10pk** |
| Polar C18 | 00B-4760-Y0 | 00D-4760-Y0 | 00F-4760-Y0 | AJ0-7600 |
| PS C18 | 00B-4758-Y0 | 00D-4758-Y0 | 00F-4758-Y0 | AJ0-7605 |
| C18 | 00B-4784-Y0 | 00D-4784-Y0 | 00F-4784-Y0 | AJ0-7611 |
| SUGAR | — | — | 00F-4775-Y0 | AJ0-4496 |

for ID: 2.0 – 3.0 mm

*SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282

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