# Method Development Strategies Using Polymer-based SPE for the Analysis of Peptides Prior to UPLC-MS/MS Analysis

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## Introduction

Two peptides, produced in the hypothalamus, oxytocin a 9-residue neuropeptide associated with parturition (childbirth) and lactation and vasopressin a peptide hormone associated with fluid retention and vasoconstriction were analyzed, ***Figure 1***. This poster investigates the selectivity of polymer-based solid phase extraction sorbents and rapid extraction of clinical matrices, matrix interference reduction and improved analyte detection.

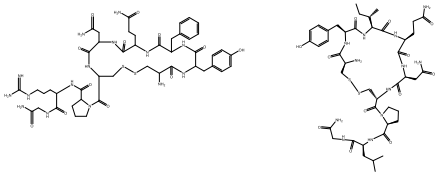


***Figure 1.*** *Structure of vasopressin (left) and oxytocin (right).*

## Experimental

### Reagents

Oxytocin and vasopressin were obtained from Phoenix Peptide. Ammonium hydroxide, ammonium acetate, formic acid and LC/MS grade solvents were purchased from Sigma-Aldrich Chemical Co. (Poole, UK). Water (18.2 MΩ.cm) was drawn fresh daily from a Direct-Q 5 water purifier (Merck Millipore, Watford, UK). Human serum was purchased from Golden West Biologicals Inc. (Ca, USA.). All materials for gel electrophoresis were obtained from ThermoFisher Scientific.

### Sample Preparation

Extractions were performed using the 30 mg 96-well plate format.

**SPE Optimization:** SPE investigated polymer-based non-polar retention with corresponding mixed-mode strong and weak cation exchange mechanisms using the EVOLUTE® EXPRESS sorbent family. The final SPE protocols are detailed in ***Table 1.*** All solvent/buffer steps were optimized to 1 mL while elution solvent volumes were minimized to 200 µL.

**Oxytocin and vasopressin:** 200 µL of serum spiked at 50 ng/mL.

***Table 1.*** *Optimized SPE procedures.*

| Step | EVOLUTE EXPRESS® ABN | EVOLUTE® EXPRESS CX | EVOLUTE® EXPRESS WCX |
|---|---|---|---|
| Condition | MeOH | MeOH | MeOH |
| Equilibration | 0.1% Formic acid | 50 mM $NH_4OAc$ pH6 | $H_2O$ |
| Pre-treatment | 1:1 1% Formic acid | 1:1 0.1%NH4OH | 1:1 1%NH4OH |
| Sample load | 400 µL | 400 µL | 400 µL |
| Wash 1 | 0.1% Formic acid | 0.1%NH4OH | 1%NH4OH |
| Wash 2 | – | 90:10 H2O: MeOH | 90:10 H2O: MeOH |
| Elution | 5% FA 80/20 $H_2O$/MeCN | 2%NH4OH in MeOH | 2%FA 30:70 $H_2O$/MeCN |

**Post extraction:** Extracts were either evaporated to dryness using an SPE Dry unit at 40 °C and reconstituted in 200 µL 0.1% FA 90/10 aq/MeCN or direct injected.

### Gel Electrophoresis Procedure

Details available on request.

### UPLC Conditions

**Instrument:** Waters ACQUITY I-Class UPLC equipped with a 15 µL flow-through needle (Waters Assoc., Milford, MA, USA)
**Column:** ACE C18-300: 50 mm x 2.1 mm id, 1.7 µm, (ACT, UK)
**Mobile Phase: A:** 0.1% FA aq : **B:** 0.1% FA MeCN
**Flow Rate:** 0.4 mL/min
**Gradient:** Linear ramp 10-33% B over 1.5 min; resume initial starting conditions at 1.6 min
**Column Temperature :** 40 °C **Injection Volume:** 10 µL

### Mass Spectrometry

**Instrument:** Xevo TQ-S triple quadrupole mass spectrometer (Waters Assoc., Manchester, UK) equipped with an electrospray interface. Positive ions were acquired in the multiple reaction monitoring (MRM) mode as shown in ***Table 2***.
**Desolvation Temperature:** 500 °C: **Ion Source Temperature:** 150 °C
**Collision Gas Pressure:** 3.6 x $10^{-3}$ mbar

***Table 2****. MS conditions.*

| Compound | MRM Transition | Cone Voltage (V) | Collision Energy (eV) |
|---|---|---|---|
| Oxytocin (Qual) | 1007.3 > 285.2 | 30 | 25 |
| Oxytocin (Quant) | 504.2 > 285.2 | 30 | 15 |
| Vasopressin (Qual) | 542.8 > 757.2 | 30 | 10 |
| Vasopressin (Quant) | 542.8 > 328.2 | 30 | 15 |

## Results

### LC-MS/MS Optimization

Oxytocin and vasopressin demonstrated precursor ions at 504.2 and 542.8 m/z, respectively, corresponding to $[M + 2H]^{2+}$ multiple charging. Chromatographic performance was compared using various phase chemistries as shown in ***Figure 2***. Optimum chromatography was obtained using the 50 mm ACE C18-300 Å phase.

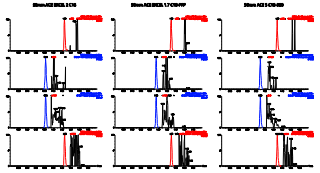


***Figure 2****. Column chromatography comparison.*

### Extraction Optimization

SPE strategies focused on extraction pH, wash and elution solvent combinations for optimal extraction recoveries and cleanliness. The CX chemistry demonstrated minimal extraction of vasopressin and around 60% recoveries of oxytocin. Further method development demonstrated no improvement. ABN and WCX chemistries delivered greater than 60% recoveries for both analytes as shown in ***Figure 3.***

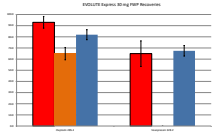


***Figure 3****. Extraction recovery profiles for SPE chemistry comparison.*

Pre-treatment and wash solvent optimization for ABN and WCX chemistries are demonstrated in ***Figure 4.***

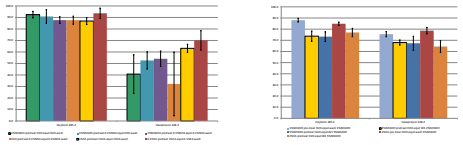


***Figure 4****. Extraction recovery profile for pre-treatment and wash solvent optimization using ABN (left) and WCX (right).*

Final modification to improve extract cleanliness involved pH modification and elution in high aq proportions. ***Figure 5.*** demonstrates acid comparison and maximum aq content for each ABN and WCX chemistries.

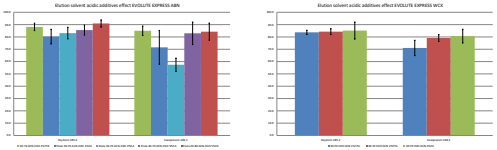


***Figure 5****. Extraction recovery profile for elution optimization using ABN (left) and WCX (right).*

The presence of water wettable components allows the possibility to eliminate plate conditioning steps prior to sample loading resulting in a simple load-wash-elute procedure. ***Figure 6.*** demonstrates final extraction recoveries for full method (documented in ***Table 1.)*** and L-W-E procedures combining traditional evaporation/reconstitution and direct injection using EVOLUTE® EXPRESS ABN.

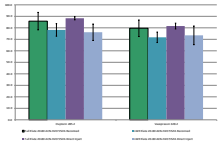


***Figure 6****. Extraction recovery profile for optimized extraction conditions using EVOLUTE® EXPRESS ABN.*

Final extraction protocols were investigated for protein removal and phospholipid content. ***Figure 7.*** demonstrate gel electrophoresis profile of ABN extracts using various elution volumes.

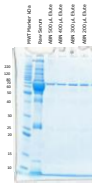


***Figure 7.*** ABN *Gel electrophoresis protein profile.*

***Figure 8.*** demonstrates the total ion chromatograms (TICs) of typical phospholipid MRMs obtained comparing ABN and WCX extracts.

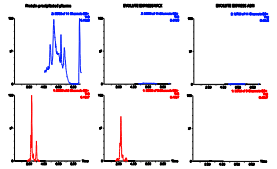
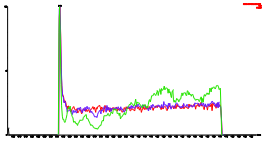


***Figure 8.*** *Phospholipid MRM TICs for final serum extracts for each chemistry.*

Final extract cleanliness was further investigated using post-column infusion (PCI) experiments. Oxytocin and vasopressin were infused post-column, directly prior to the ESI source to allow easy identification of regions of suppression throughout the assay, as shown in ***Figure 9***.

***Figure 9.*** *PCI baselines of the optimized ABN extraction protocol: blank solvent (red), extracted serum using full SPE (purple) and direct inject procedure (green).*



EVOLUTE® EXPRESS ABN demonstrated overall better performance. Spiked calibration curves constructed from 25-1000 pg/mL returned excellent coefficients of determination ($r^2$) and sensitivity. Typical calibration lines are demonstrated in ***Figure 10.***

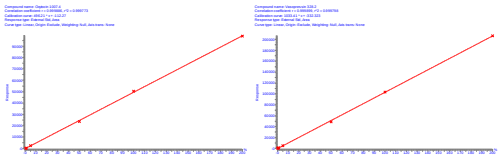


***Figure 10.*** *Serum calibration curves extracted using EVOLUTE EXPRESS® ABN.*

## Conclusion

- EVOLUTE® EXPRESS WCX provided good recoveries. However, better cleanliness was obtained using the optimized elution combinations with EVOLUTE® EXPRESS ABN.
- SPE optimization resulted in recoveries greater than 70% while demonstrating good removal of matrix components in the form of proteins and phospholipids.
- It was also possible to eliminate evaporation and move to direct inject using the selected chromatographic mobile phases, however, there was an increase in regions of suppression observed.
- Optimized SPE protocol still showed excellent protein removal whilst maintaining good peptide recoveries.



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