Application of Bio-SPME for the Enrichment of Illicit Phenethylamine and Cathinone Compounds from Biological Samples

Craig R. Aurand[1], Robert Shirey[1], Leonard Sidisky[1]
Janusz Pawliszyn[2], and Yong Chen[1]

[1]Supelco, Div. of Sigma-Aldrich
Bellefonte, PA 16823 USA

[2]Department of Chemistry
University of Waterloo
Waterloo, Ontario, Canada

sigma-aldrich.com

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Introduction

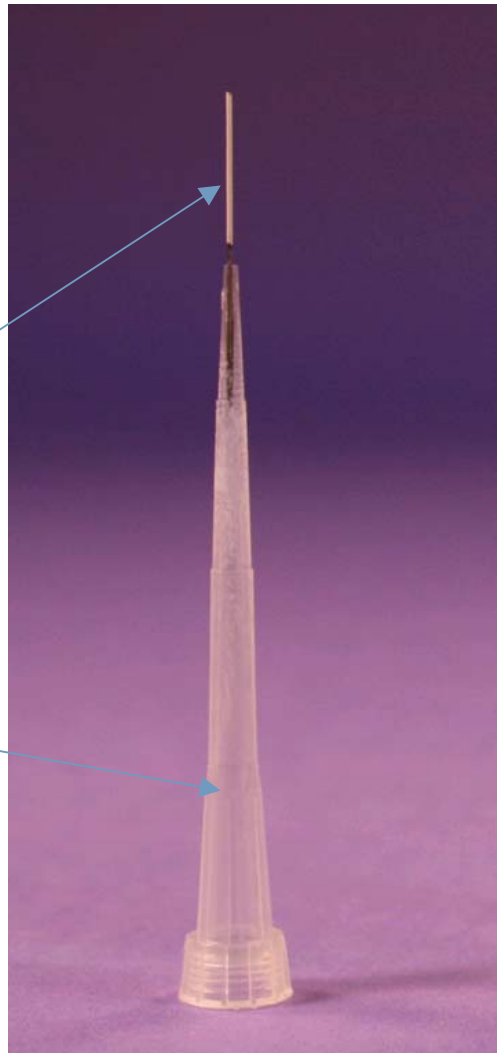
Recently the field of illicit drug testing has been met with a changing environment with the rapid development of unregulated designer and synthetic compounds. Of most concern has been the development of a class of phenethylamine and cathinone compounds being marketed as “Bath Salts, Jewelry Cleaner or Plant Food”. Though sold as not for human consumption, these compounds are reported to generate stimulating effects similar to that of methamphetamine, heroin and MDMA. For a period of time, these compounds could be acquired legally through the internet and head shops because of no direct legal control. In the US, both state and local governments have instituted bans on the sale of these Bath Salt compounds. The difficulty in testing of the Bath Salts for forensic testing facilities is the fact these compounds are not detected under normal ELISA testing methods; additional more specific LC/MS methods are necessary.

Introduction (contd.)

The following presentation demonstrates the implementation of Biocompatible Solid Phase Microextraction (Bio-SPME) for the enrichment of a set of illicit “Bath Salts” directly from plasma in an effort to explore the utility and unique selectivity of these newly developed sampling devices. The uniqueness of these devices derives from the embedded functionalized media of the Bio-SPME fibers for use in targeted small molecule enrichment from complex biological matrices.

Bio-SPME Tippets prepared in a 96-tip array were utilized for the enrichment of nine illicit “Bath Salts” from plasma samples. Bio-SPME fibers functionalized with mixed mode hydrophobic and cation exchange particles were employed for selective enrichment of the Bath Salts. After enrichment, the Bio-SPME Tippets were then directly desorbed in a 96-well plate for direct LC/MS TOF analysis. Key parameters that impact analyte extraction efficiency, such as sample pH and extraction time were explored along with the impact of biological sample. In addition to extracted analyte, overall sample matrix was also monitored and compared with typical dilution/precipitation techniques.

Figure 1. Bio-SPME Tippet Configuration



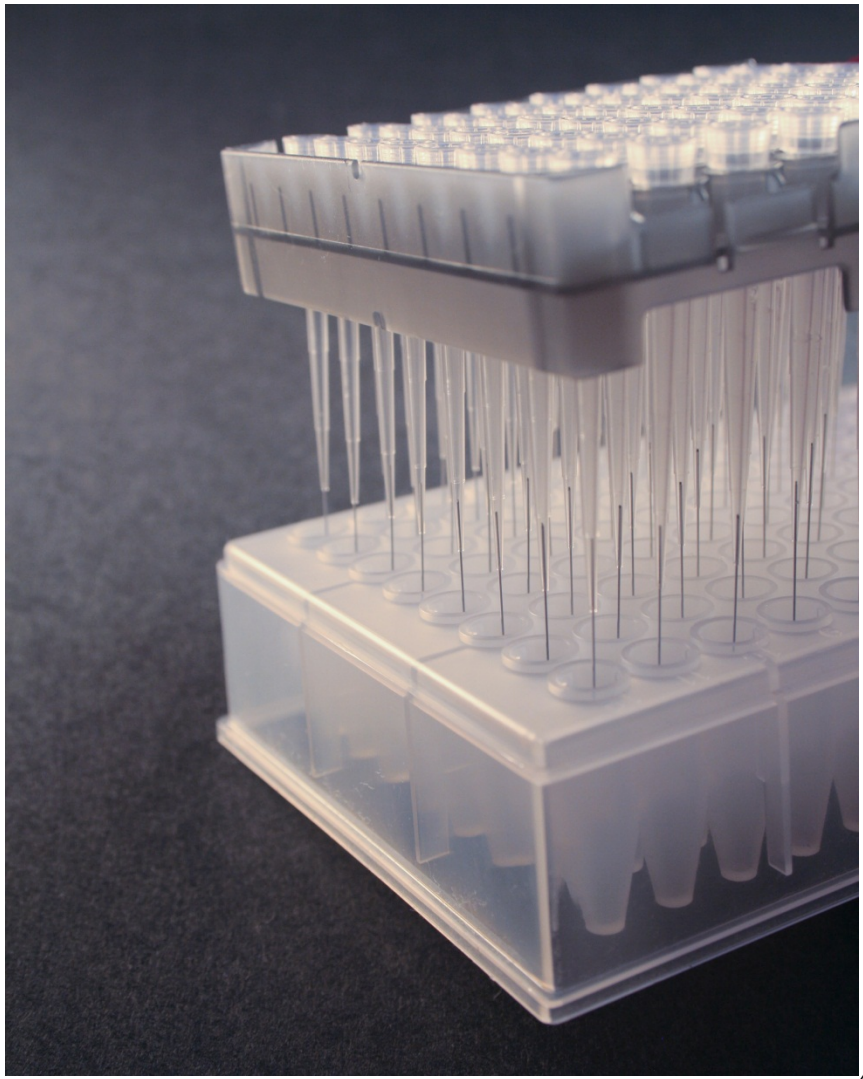
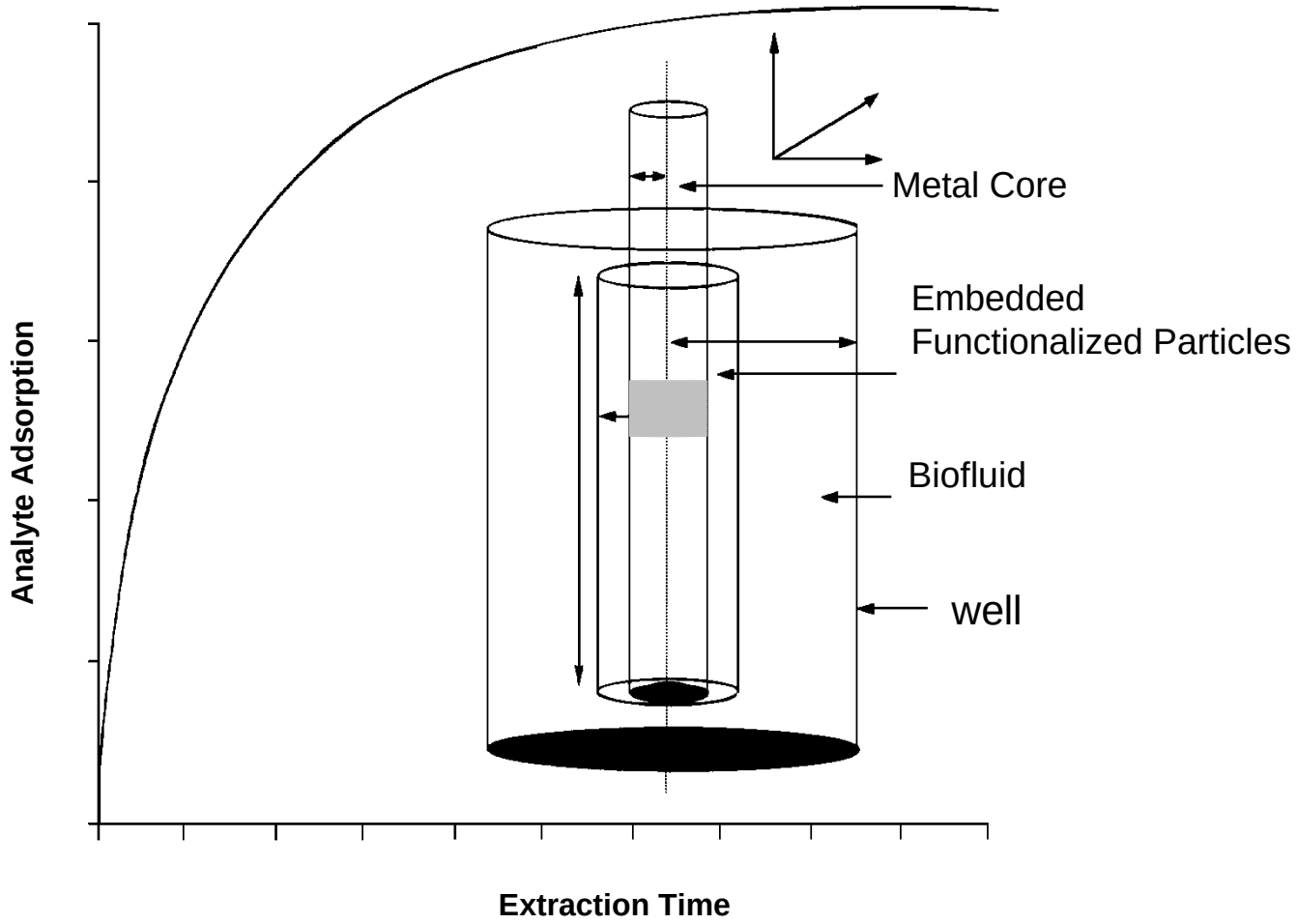


Figure 2. Adsorption Mechanism on Bio-SPME Extraction



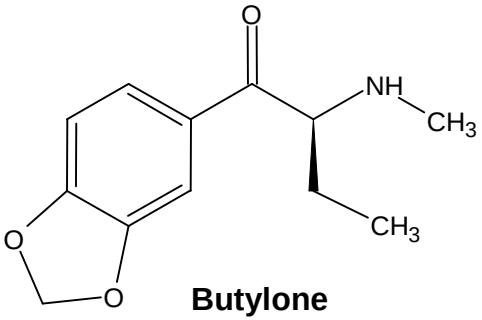
The adsorption mechanism for Bio-SPME is based upon Flick's Law, where differential migration exists between analytes in solution and analytes that partition into the fiber coating. Bio-SPME is not an exhaustive technique and extraction is governed by distribution constants dependent on an analytes affinity for the coating as compared to the sample matrix. After a given amount of time, an equilibrium is achieved between the concentration of analytes in the matrix and the fiber coating.

Concentration of analyte in stationary phase compared to concentration of analyte in solution:

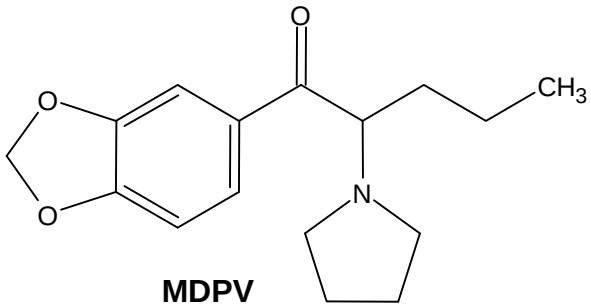
$$K = n_s/V_1C_2°$$

K = Distribution constant
n_s = Moles of analyte in stationary phase
V_1 = Volume of stationary phase
$C_2°$ = Final analyte concentration in solution

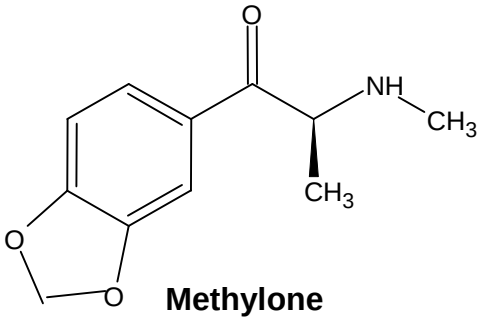
Experimental Bath Salt Analytes



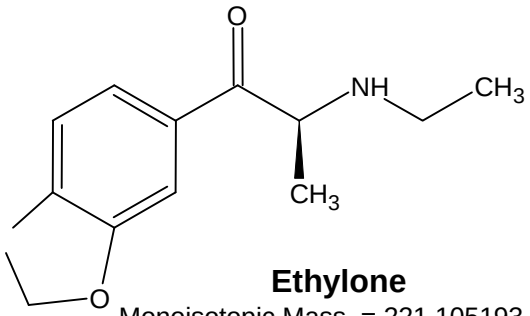
Butylone
Monoisotopic Mass = 221.105193 Da
Cerilliant B-045



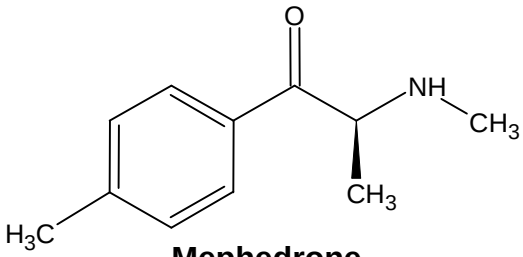
MDPV
Monoisotopic Mass = 275.152144 Da
Cerilliant M-146



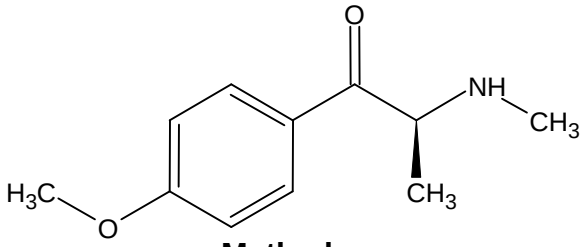
Methylone
Monoisotopic Mass = 207.089543 Da
Cerilliant M-140



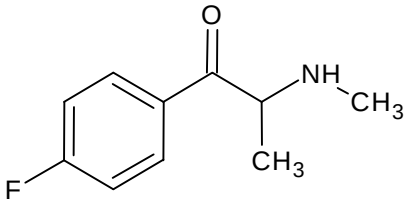
Ethylone
Monoisotopic Mass = 221.105193 Da
Cerilliant E-071



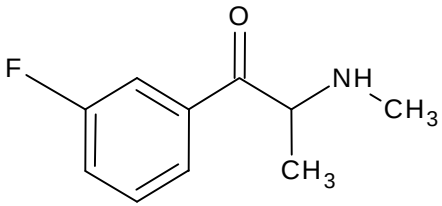
Mephedrone
Monoisotopic Mass = 177.115364 Da
Cerilliant M-138



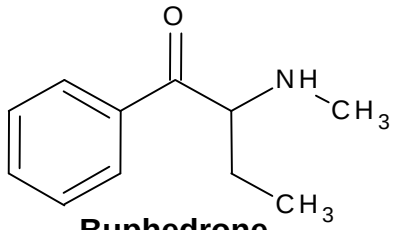
Methedrone
Monoisotopic Mass = 193.110279 Da
Cerilliant M-147



4-Fluoromethcathinone
Monoisotopic Mass = 181.090292 Da
Cerilliant F-015

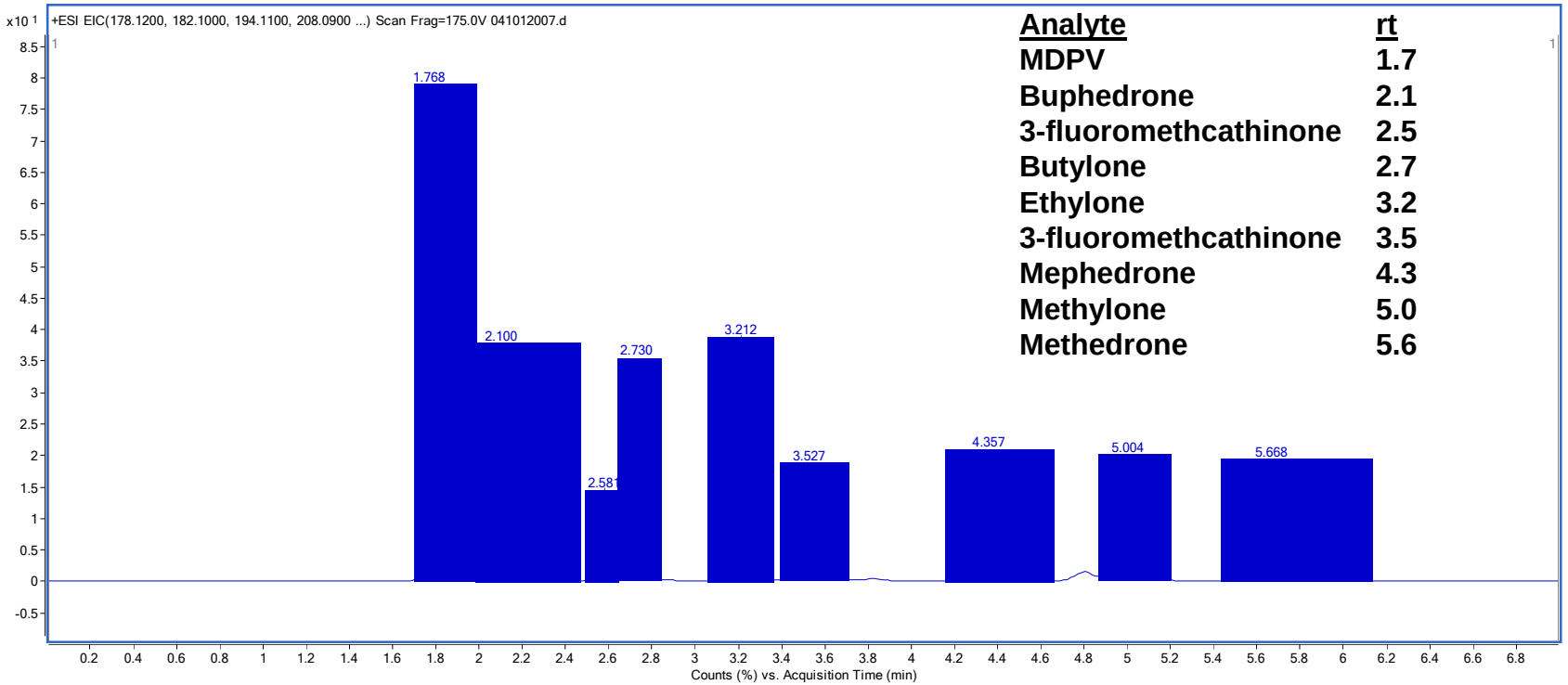


3-Fluoromethcathinone
Monoisotopic Mass = 181.090292 Da
Cerilliant F-016



Buphedrone
Cerilliant B-047

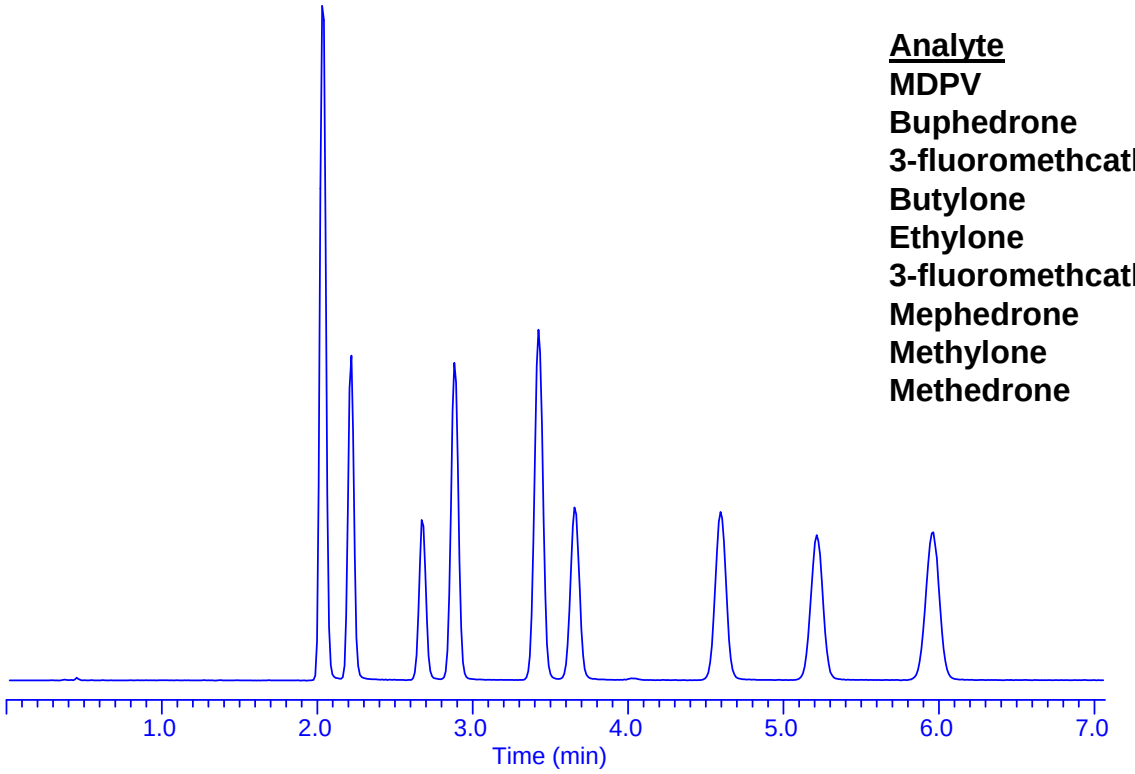
Figure 3. HILIC Separation of Bath Salts



Analyte	rt
MDPV	1.7
Buphedrone	2.1
3-fluoromethcathinone	2.5
Butylone	2.7
Ethylone	3.2
3-fluoromethcathinone	3.5
Mephedrone	4.3
Methylone	5.0
Methedrone	5.6

system:	Agilent 1290 Infinity with 6210 TOF
column:	Ascentis Express HILIC, 10 cm x 2.1 mm, 2.7 µm (53939-U)
mobile phase:	5 mM ammonium formate (98:2 acetonitrile:water)
flow rate:	0.6 mL/min.
temp.:	35 ºC
system pressure:	127 bar
injection:	1 µL
MS det.:	ESI+, 100-1000m/z ,

Figure 3. HILIC Separation of Bath Salts (contd.)



Analyte	rt
MDPV	2.0
Buphedrone	2.2
3-fluoromethcathinone	2.7
Butylone	2.9
Ethylone	3.4
3-fluoromethcathinone	3.7
Mephedrone	4.6
Methylone	5.2
Methedrone	6.0

Table 1. Log D and pKa 1 Values for Bath Salts Evaluated

Analyte	Log D pH 3.0	Log D pH 7.0	pKa 1
Methylone	-3.49	-1.21	7.74
Ethylone	-2.98	-0.71	7.75
Butylone	-2.98	-0.7	7.74
Methedrone	-2.55	-0.07	7.48
Mephedrone	-2.4	0.12	7.41
3-fluoromethcathinone	-2.46	0.22	7.14
4-fluoromethcathinone	-2.24	0.4	7.14
Buphedrone	-2.2	0.49	7.14
MDPV	-0.67	1.01	8.41

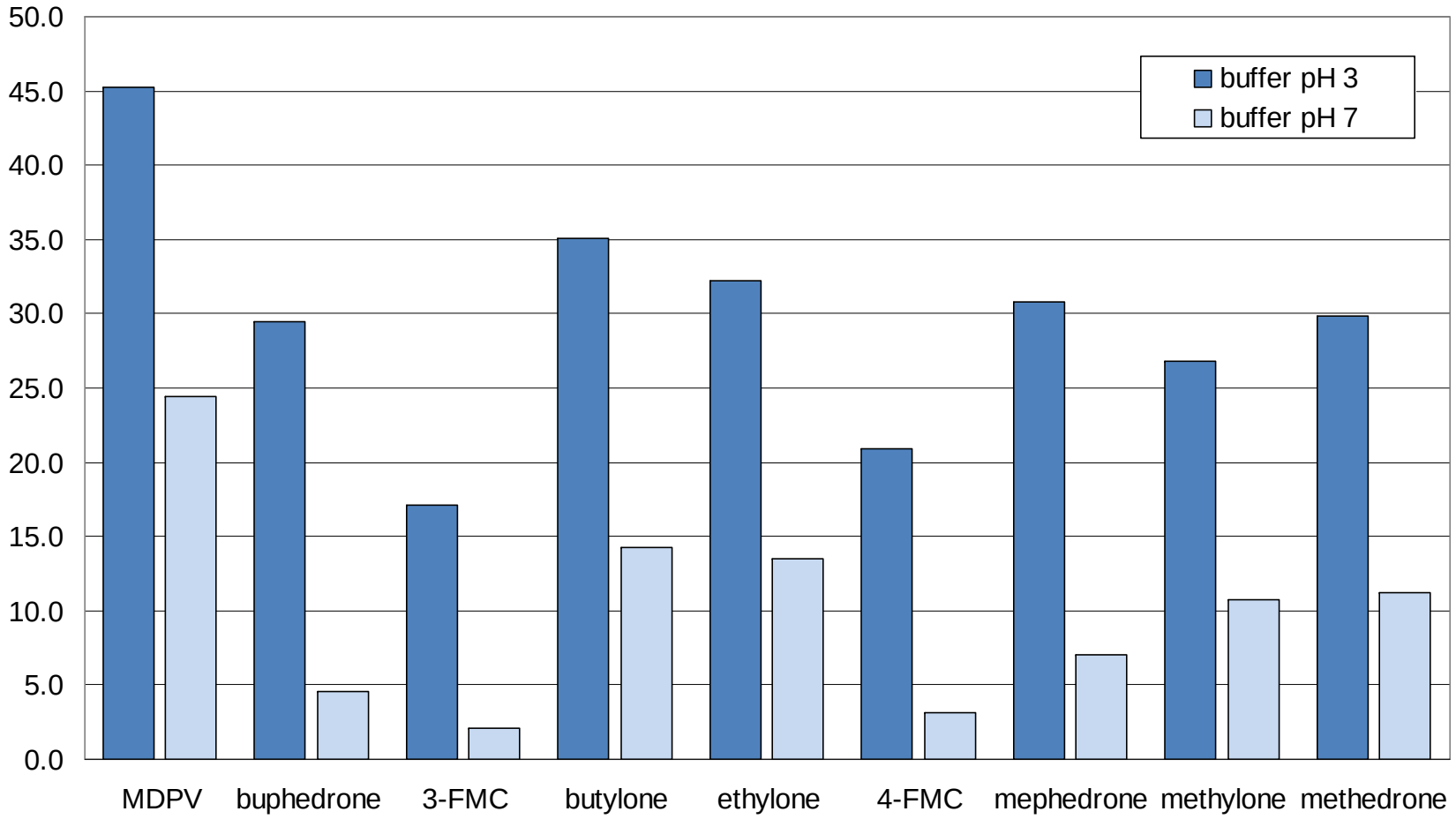
The charge state of an analyte will impact the efficiency of the extraction, particularly when using a mixed mode ion-exchange material. It is important the analytes are fully ionized in solution to increase the availability for ion-exchange to take place with the ionic site of the stationary phase. Table 1 depicts the affect of pH on the Log D value of the analytes, as pH decreases all of the Bath Salts become more hydrophilic, thus making them less likely to partition from an aqueous environment. At lower pH, these analytes are less likely to extract by partitioning as in standard reversed-phase modes.

The first portion of this study is to evaluate the pH affect on analyte extraction using the Bio-SPME mix mode fiber. Here pH controlled buffer solutions were spiked with Bath Salts and extracted as described in Table 2.

Table 2. Bio-SPME Buffer Extraction Conditions

Fiber :	Mixed mode Lot 1738-77, Strong Cation Exchange/C18 silica in tippets
Conditioning:	15 – 30 min. in 50% methanol-water
Sample pH3	Buffer solution pH 3 – water pH adjusted to pH 3.2 with formic acid, spiked to 200 ng/mL with Bath Salts
Sample pH7	Buffer solution pH 7 –10 mM ammonium formate pH 7, spiked to 200 ng/mL with Bath Salts
Extraction:	15 min. from 96-well plates 1 mL volume, 750 µL of sample per well
Desorption:	60 min. in 150µL methanol with 0.5% NH_4OH (28% solution) in 300 µL volume 96 well plate
Drying step:	60 min. at 40 °C under a blanket of nitrogen
Reconstitution:	50 µL of methanol and mixed for 2 min. on plate shaker, rapid speed

Figure 4. Extraction from Buffered Water, pH Effect



pH Study Summary

As described in Figure 4, the sample pH dramatically influences the extraction efficiency of the Bath Salts. Under neutral pH7 condition, the analytes are partially charged resulting in a greater influence from hydrophobic extraction with the mixed mode surface. Under the acid pH3 conditions, Bath Salts are in a fully ionized form, thus greatly increasing the ability to ion-exchange with the mixed mode surface of the Bio-SPME fiber. At this pH the Bath Salts are also much more hydrophilic and have a higher tendency not to partition with a purely hydrophobic surface. The acid functionality of the mixed mode surface also acts to decrease the surface barrier of the alkyl C18 chain, thus making it a more accessible surface for the hydrophilic Bath Salts. Extraction time of 15 minutes demonstrated reproducible recovery results, while being pre-equilibrium condition.

A second study was performed on the extraction of the Bath Salts from plasma samples. For this portion of the study, both analyte extraction along with matrix monitoring was conducted for the plasma samples. Table 3 details the Bio-SPME plasma extraction conditions.

Table 3. Bio-SPME Plasma Extraction Conditions

Plasma pH3	Rat plasma pH adjusted with phosphoric acid, spiked to 50 ng/mL bath salts
Plasma pH7	Rat plasma pH adjusted with 1 M sodium phosphate buffer pH 7 (10 mM final), spiked to 50 ng/mL bath salts
Extraction:	15 min. from 96-well plates 1 mL volume, 750 µL of sample per well
Desorption:	60 min. in 150 µL methanol with 0.5% NH_4OH (28% solution) in 300 µL volume 96-well plate
Drying step:	60 min. at 40 °C under a blanket of nitrogen

Figure 5. Plasma Extraction pH Effect

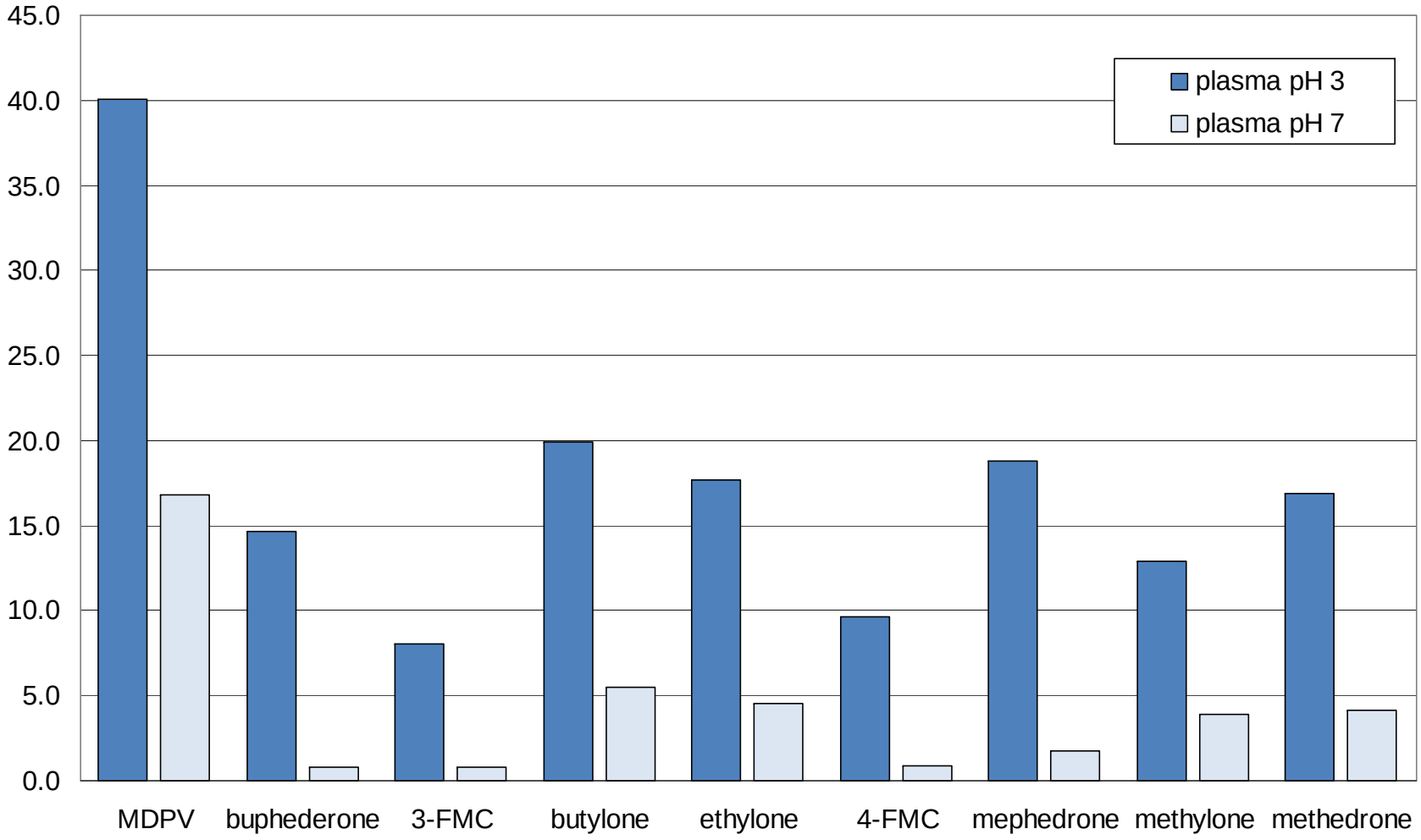
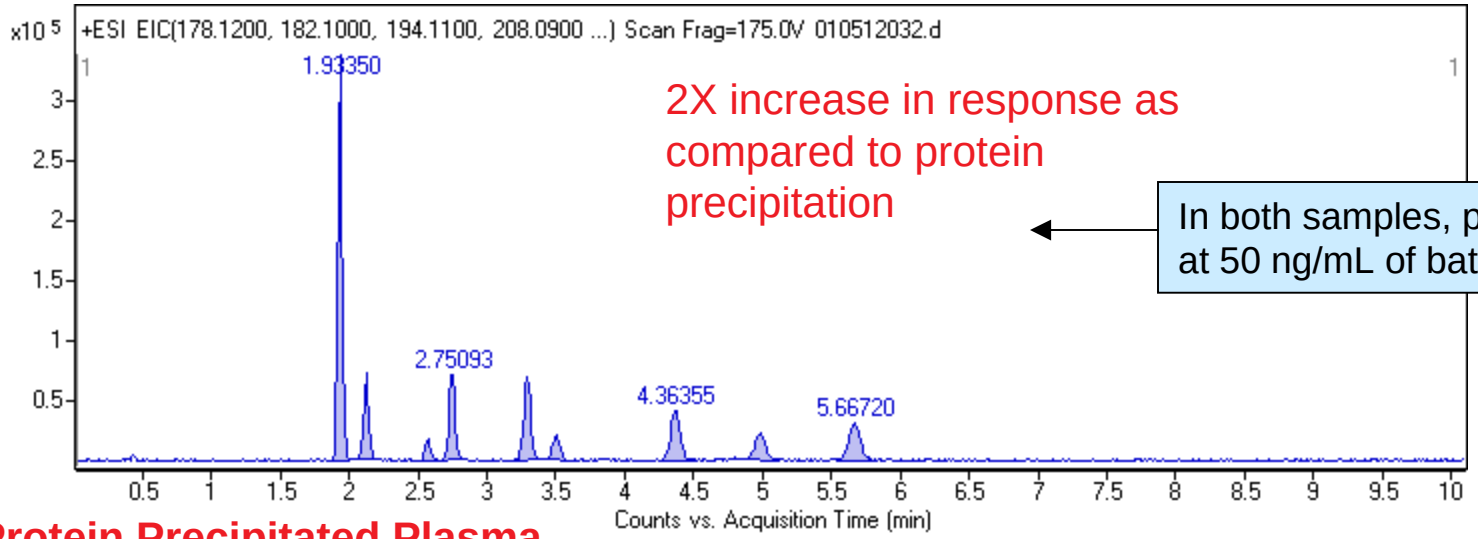


Figure 5 depicts the extraction efficiency of Bath Salts from the pH adjusted plasma samples. As anticipated, sample matrices can impact the extraction efficiency of the analytes. Plasma adjusted to pH 3 demonstrated similar extraction efficiencies to pH 3 buffer conditions. The plasma pH 7 extraction efficiencies were notably lower than from the pH 7 buffer conditions. Additional experiments confirmed protein binding effects of the bath salts in the plasma sample were the cause for the lower extraction efficiencies at pH 7. At pH 3 conditions, the low pH disrupted the protein binding effect of the Bath Salts, thus enhancing the extraction efficiencies on the Bio-SPME fiber.

A separate protein precipitation technique was performed on plasma samples for comparison of analyte response and matrix monitoring. Protein precipitation was conducted using 3:1 ratio of 1% formic acid acetonitrile to plasma, this centrifuged solution was then analyzed directly. Figures 6 and 7 depict the comparison of analyte response and extracted matrix between the Bio-SPME technique and protein precipitation.

Figure 6. Analyte Response: Sample Prep Technique

Bio-SPME Mixed Mode pH 3.0



2X increase in response as compared to protein precipitation

In both samples, plasma spiked at 50 ng/mL of bath salts

Protein Precipitated Plasma

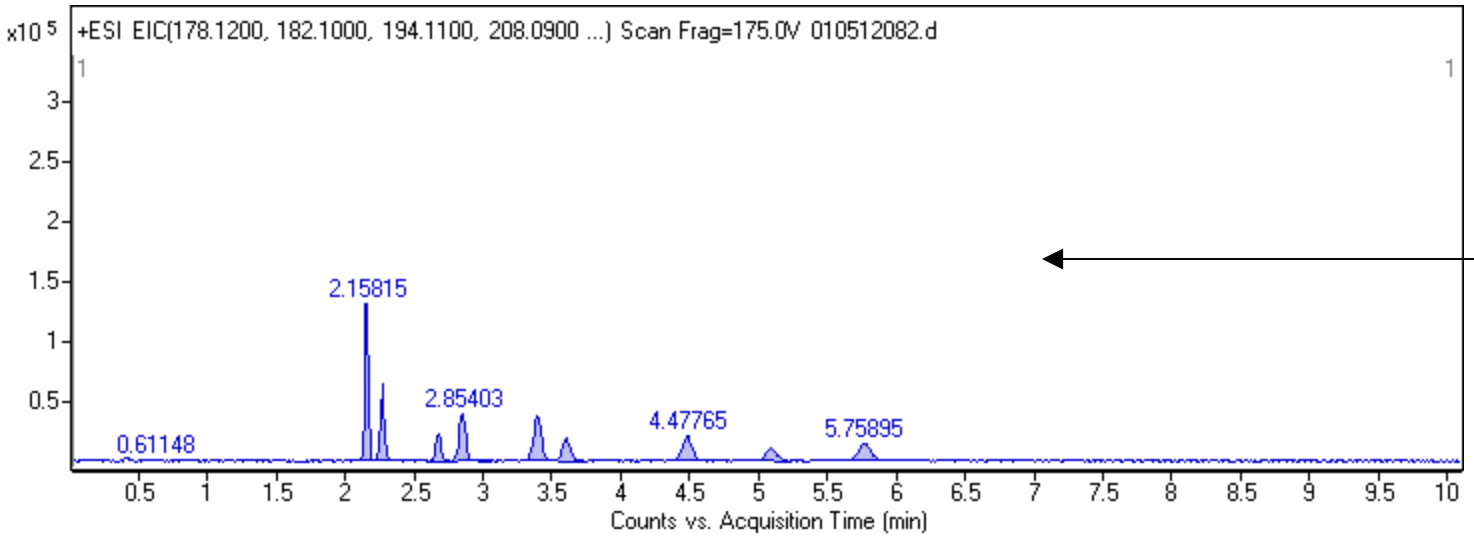


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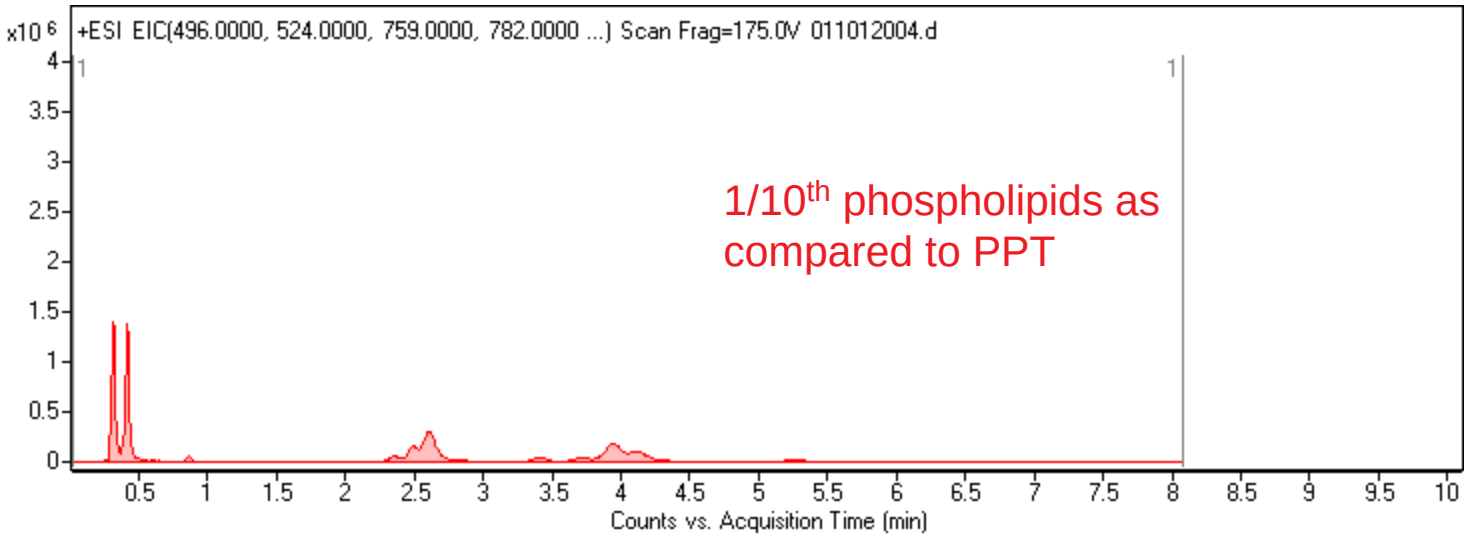
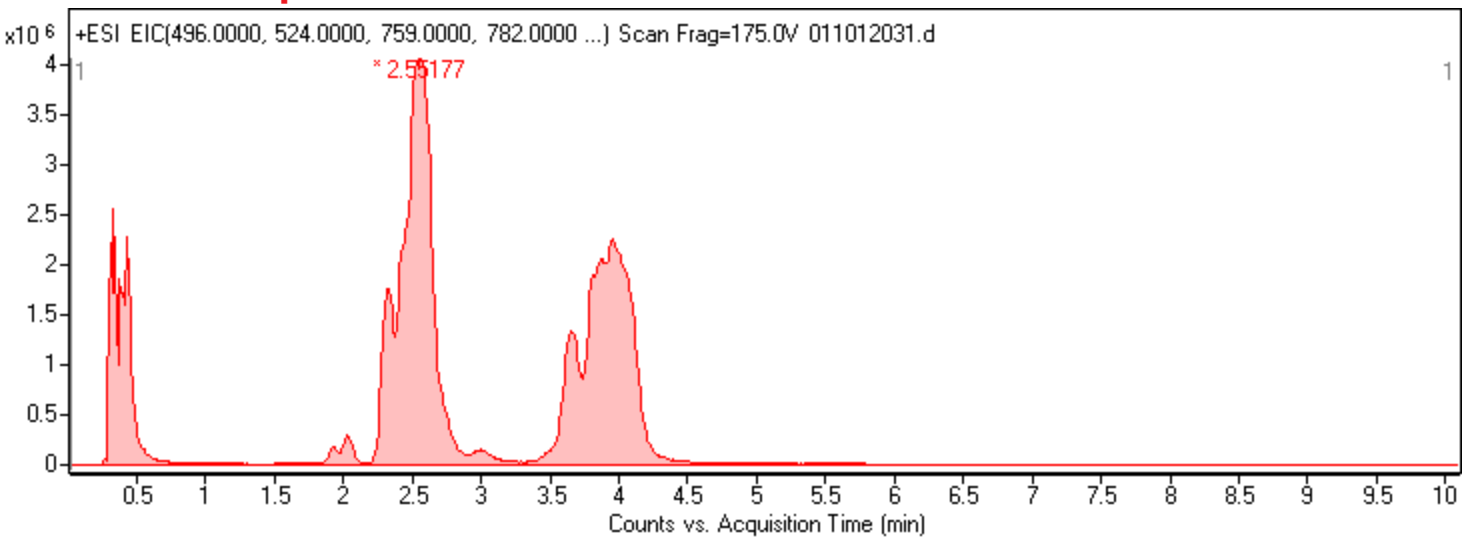
Figure 7. Phospholipid Matrix: Sample Prep Technique

Bio-SPME Mixed Mode pH 3.0



Protein Precipitated Plasma



Ascentis Express C18,
5 cm x 2.1 mm,
5 mM ammonium formate
(95:5 methanol:water)
0.3 mL/min., 55 °C

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Conclusions

- The Bio-SPME extraction studies demonstrated the capability of analyzing sub 10 ng/mL concentration levels of Bath Salts in plasma samples.
- The mixed-mode chemistry of the Bio-SPME enable unique selectivity for enrichment of the polar basic compounds as the Bath Salts.
- Plasma samples prepared using the Bio-SPME technique had 10X reduction in detected phospholipid matrix as compared to standard precipitation techniques, while demonstrating increased analyte response.
- The Bio-SPME Tippet device enables a unique approach for micro extraction and concentration of analytes from biological matrices, requiring minimal sample volumes. As with other sample prep techniques, parameters such as sample pH and matrix can have an impact on extraction efficiencies.

Conclusions (contd.)

- The binding process used to embed the functionalized media facilitates a shielding affect to prevent binding of proteins and higher molecular endogenous matrix. By doing so, this sampling technique enables the differentiation of free form analytes in the sample form protein bound analytes.
- This approach demonstrates the ability for quantitative analyte enrichment from limited sample volumes. These tippets can be configured for singular or multi tippet processing, greatly increasing the capability for high throughput applications.