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**Development of a Rapid Drug Detection Method for Insects Using
Paper Spray Ionization Mass Spectrometry (PSI-MS)**

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Duquesne URSS

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Introduction

Insects have the ability to reflect the chemical environment that they have developed in. Thus, necrogenous insects can reflect the chemical environment of cadavers they have fed on.

Entomotoxicology, the study of insects as toxicological specimens, was a term first used by Derrick J. Pounder in 1991. While entomotoxicology has since become a multidisciplinary term, Pounder coined this term in reference to forensic entomotoxicology, a novel branch of forensic science that combined aspects of both forensic entomology and forensic toxicology. Pounder proposed that by analyzing insects that had fed on cadavers in the same manner as a normal tissue sample, investigators could still reliably detect if the victim had drugs in their system at the time of death.¹

Forensic scientists can potentially utilize insect larvae as an alternate biological matrix to detect drugs in cadavers that are skeletonized, burned, or otherwise badly damaged.²⁻⁴ Analysis of insects using paper spray ionization mass spectrometry (PSI-MS), an analytical method requiring little to no sample preparation, could prove a rapid, cost-effective, and non-destructive alternative form of toxicological analysis.⁴ Traditional tissue analysis via GC and LC-MS often requires lengthy sample preparation, uses expensive reagents, and are destructive to cadaver tissues. The goal of this project is focused on exploring PSI-MS with insects as a rapid, cost-effective, and non-destructive way for analysts to detect the presence of drugs in damaged cadavers.

Because this study was largely a proof-of-concept experiment, a drug surrogate was chosen to optimize sample preparation and extraction techniques. Phenethylamine (PEA) is a monoamine compound that is structurally similar to methamphetamine and amphetamine, both of which are common drugs of abuse. PEA was

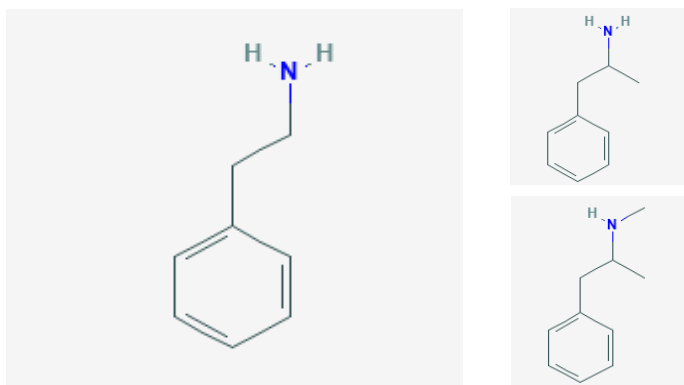


Figure 1: Phenethylamine (left) compared to amphetamine (top right) and methamphetamine (bottom right)

chosen as the drug surrogate for this reason. In addition, a model organism that was easy to raise and had a longer life cycle needed to be chosen for the early stages of this study; blowflies require more specific conditions for growth and would complete their life cycles too quickly for multiple rounds of method optimization.

Several different rapid and cheap extraction techniques for drug detection in the model insect *T. molitor* were explored during optimization, including methanol extraction, QuEChERS extraction, and a novel “bug-spray” technique. *T. molitor* insects fed on ground oat substrate spiked with PEA, and the effectiveness of each extraction method was investigated using PSI-MS with collision induced dissociation (CID). Over the course of three different rounds of mealworm rearing, the most time-efficient, cost-effective, and effective detection method was developed.

Materials and Methods

Mealworms were purchased from Uncle Jim’s Worm Farm (Spring Grove, PA) and kept refrigerated until use. In order to compare and contrast a wide variety of methods, three rounds of mealworms were raised in this study, denoted Round 1, Round 2, and Round 3. An overview of method development is summarized in Table 1.

Table 1: Summary of the differences between Rounds 1, 2, and 3 during method optimization.

	Round 1	Round 2	Round 3
[PEA] of feeding substrate	0, 100, and 1000ppm	0, 6, 6000ppm	0, 6, 6000ppm
Feeding conditions	Ventilated meal prep containers in the refrigerator	Ventilated meal prep containers on the benchtop	Ventilated meal prep containers on the benchtop
Euthanization Method	Submerging in boiling ethanol for ~30s	Flash-freezing with liquid nitrogen	Submerging in boiling water for ~15s
Homogenization Method	Manual crushing with a stir rod	Vigorously shaking frozen insects in 15 mL centrifuge tubes	Ceramic homogenizer beads from Agilent
QuEChERS Reagents	<p>(per 3 insects)</p> <ul style="list-style-type: none"> • 7.5 mL acetonitrile • 4 mL water • 3 g MgSO₄ • 0.75 g NaCl • 0.75 g Na₃ citrate • 0.375 g Na₂ citrate sesquihydrate 	<p>(per 1 insect)</p> <ul style="list-style-type: none"> • 5 mL acetonitrile • 2 g MgSO₄ • 0.5 g NaCl 	<p>(per 5 insects)</p> <ul style="list-style-type: none"> • 6 mL acetonitrile • 2 g MgSO₄ • 0.5 g NaCl <p>Additional d-SPE step using Agilent Bond Elut EMR Lipid kit</p>
Other Extraction Method	5 mL ethanol added to homogenized mealworms to extract	5 mL methanol added to homogenized mealworms to extract	6 mL methanol added to homogenized mealworms to extract
Paper spray solvent	Ethanol	Methanol	Methanol

Round 1:

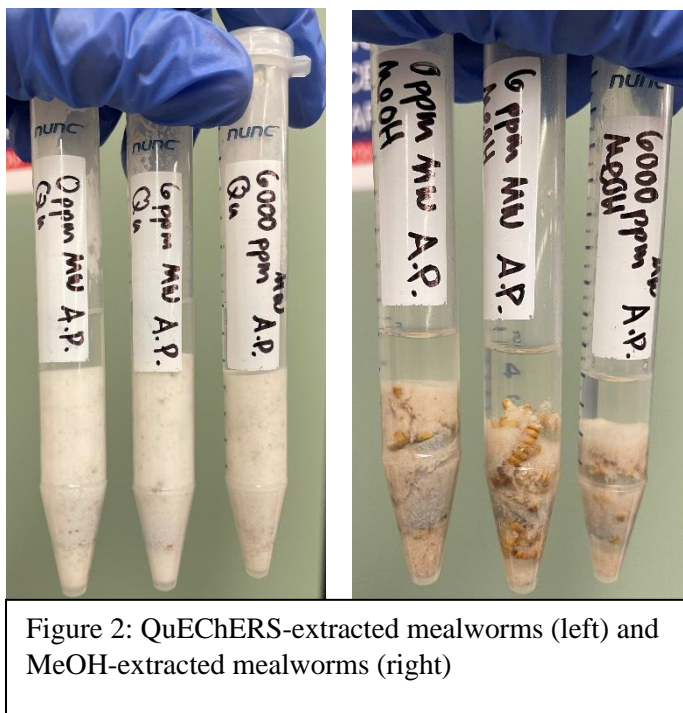
A 1000 ppm PEA stock solution was prepared by adding 52.0 μ L of > 99% PEA from Sigma-Aldrich to a 50 mL volumetric flask and diluting to the mark, and a 100 ppm PEA solution was created by adding 5 mL 1000 ppm solution to a second 50 mL volumetric flask and diluting to the mark. Three 10 g portions of ground oats were weighed out into beakers, and 10 mL of the 1000 ppm and 100 ppm solutions were added to two of the oat portions. A third control oat portion was created by adding 10 mL of deionized water to the beaker. The oats were placed in a 110°C oven for an hour until dry. Oats were then added to 28 oz ventilated plastic meal prep containers from NutriBox. 10 live mealworms were placed into each container, and containers were placed in the refrigerator.

Mealworms were harvested after 2 days and 5 days of feeding. In all cases, insects were euthanized three at a time by submerging in beakers full of 15 mL of boiling hot ethanol for 30 seconds.

From here, several dead, intact mealworms were set off to the side to perform bug-spray. The rest of the insects were then manually homogenized in the ethanol afterwards using a stir rod, and 2 mL aliquots were taken for paper spray. The rest of the ethanol was evaporated off under a fume hood, leaving only the solid sample behind. From here, QuEChERS extraction was performed as follows: 7.5 mL of acetonitrile and 4 g of water were added to the beakers and swirled. The solutions were poured into 50 mL centrifuge tubes, and 3 g MgSO_4 purchased from Fisher Scientific, along with 0.75 NaCl, 0.75 g $\text{Na}_3\text{citrate}$, and 0.325 g $\text{Na}_2\text{citrate}$ sesquihydrate purchased from Sigma Aldrich were added to each. Each tube was vortexed, then centrifuged for 5 minutes at 4,000 rpm. The supernatant was collected for analysis.

Round 2:

A 6000 ppm phenethylamine (PEA) stock solution was prepared by adding 156.0 μL of $\geq 99\%$ PEA from Sigma-Aldrich to a 25 mL volumetric flask and diluting to the mark, and a 6 ppm PEA solution was created by adding 25 μL of 6000 ppm solution to a second 25 mL volumetric flask and diluting to the mark. 10 mL of these stock solutions, along with 10 mL of deionized water, were used to make 0, 6, and 6000 ppm PEA spiked oat substrates using the same method as in



Round 1. Instead of being held in the refrigerator, mealworms were allowed to feed in the ventilated containers on the benchtop at room temperature.

Mealworms were harvested after 3, 5, and 7 days, and euthanized by flash-freezing with liquid nitrogen. After flash freezing, an attempt to homogenize frozen insects with a mortar and pestle was

made, but this was found to be too time-consuming and messy. The rest of the insects were frozen, individually placed into 15 mL centrifuge tubes, and shaken to break apart and homogenize. Methanol extraction was performed by adding 5 mL of MeOH to the 15 mL tubes, sealing and shaking them for 60 seconds, and placing them in the refrigerator to extract for 2 days. QuEChERS extraction was performed as follows: 5 mL of acetonitrile was poured into the 15 mL centrifuge tubes and swirled. 2 g MgSO₄ and 0.5 g NaCl were added to each of the tubes and immediately vortexed to incorporate. Each tube was vortexed, then centrifuged for 5 minutes at 4,000 rpm. As in Round 1, there was no additional d-SPE step and the supernatant was collected for analysis.

Round 3:

0, 6, and 6000ppm oat substrates were created exactly as described in Round 2. Organic baby carrots purchased from Aldi were washed thoroughly with water, broken in half, and soaked in 6 and 6000 ppm PEA solutions for 30 minutes. These carrots were added to the ventilated containers, and insects were allowed to feed on the benchtop. Mealworms for the two the extraction methods were harvested after 2 days, while bug-spray mealworms, bedding, and carrots were harvested after 7 days for analysis. All worms were euthanized by submerging in boiling deionized water for 15 seconds. After being dried off, five



Figure 3: “Bug-Spray” extraction technique, in which the insect is mounted on the clip electrode on a glass coverslip instead of a paper triangle.

insects per group were placed into 15 mL centrifuge tubes, and two ceramic homogenizer beads from Agilent was used per tube to homogenize the samples by shaking. In addition to mealworms, homogenization and extraction of each groups’ bedding and carrot halves were performed as well. 1.5 g

of bedding and two carrot halves from each feeding group were homogenized using the same methods as used for the mealworms. For bug-spray, 10 mealworms from each feeding group were preserved in vials filled with 5 mL of methanol.

Methanol extraction was performed as described in Round 2. QuEChERS extraction was performed as follows: 6 mL of acetonitrile was added to the tubes followed by 2 g MgSO₄ and 0.5 g of NaCl. The tubes were immediately sealed and vortexed, then centrifuged for 5 minutes at 4,000 rpm. While this was spinning down, 3 mL of HPLC-grade water and 1 g of proprietary lipid-removing sorbent from the Agilent Bond Elut EMR-Lipid dispersive SPE kit were added to fresh 15 mL centrifuge tubes and vortexed. 3 mL of supernatant from the samples were added to the water and Bond Elut mixture and vortexed. The tubes were then centrifuged for 5 minutes at 4,000 rpm and the supernatant was collected for further analysis.

Instrumentation

The PSI source was configured to a Linear Trap Quadrupole (LTQ) XL Linear Ion Trap Mass Spectrometer from ThermoFisher. PSI voltage was set to 3.33 kV to induce ionization from the filter paper and/or insect. LTQ parameters were as follows: positive ionization mode was utilized, capillary temperature was 250°C, capillary voltage was 30 V, and tube lens was 165 V. To perform collision induced dissociation (CID) on the samples, the collision energy was set to 30 arbitrary units.

To perform PSI-MS on extracted samples, triangular pieces of filter paper were cut and clamped

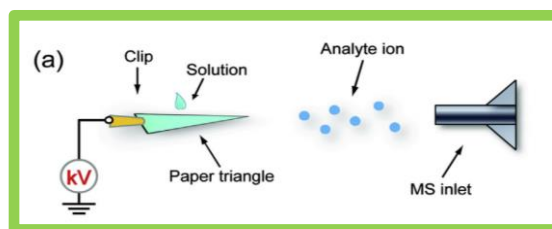


Figure 4: Example of PSI-MS schematic. A ThermoFisher LTQ XL Linear Ion Trap Mass Spectrometer was used.

via alligator clip onto the voltage source. 50 μL (about ~ 4 drops) of extract was pipetted onto the filter paper and allowed to dry. The carrier solvent of choice was then applied to the dried filter paper, the voltage was turned on, and data was collected continuously.⁵ In the case of the bug-spray samples, instead of clamping filter paper onto the voltage source, the whole insect was mounted and clamped on a glass coverslip as seen in Figure 3.

Results

Round 1:

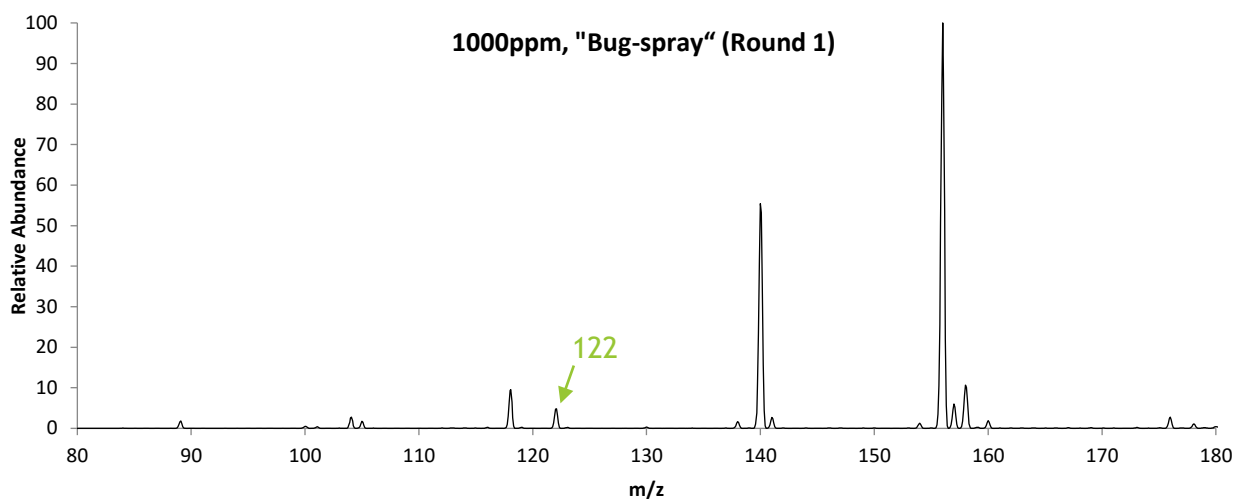


Figure 5: Bug-sprayed insect from the 1000ppm feeding group in Round 1.

Round 2:

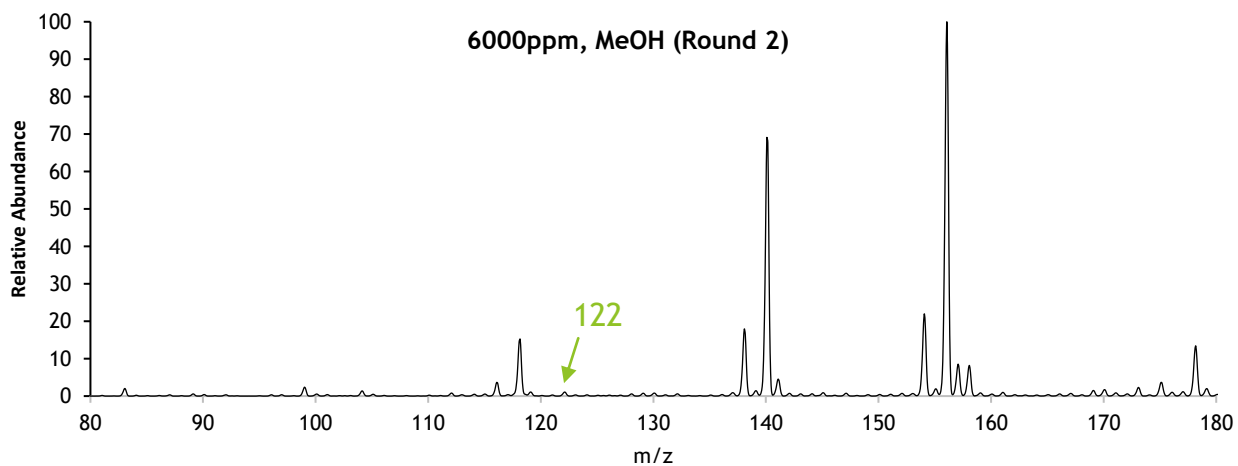


Figure 6: MeOH-extracted insect from 6000ppm feeding group in Round 2.

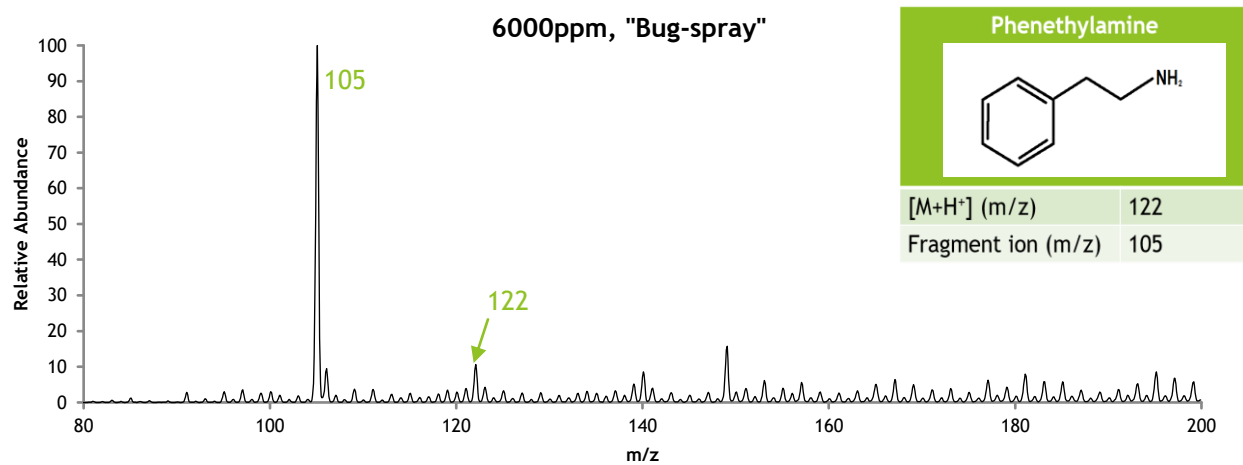


Figure 7: Spectra of “bug-sprayed” insect from 6000ppm feeding group before CID.

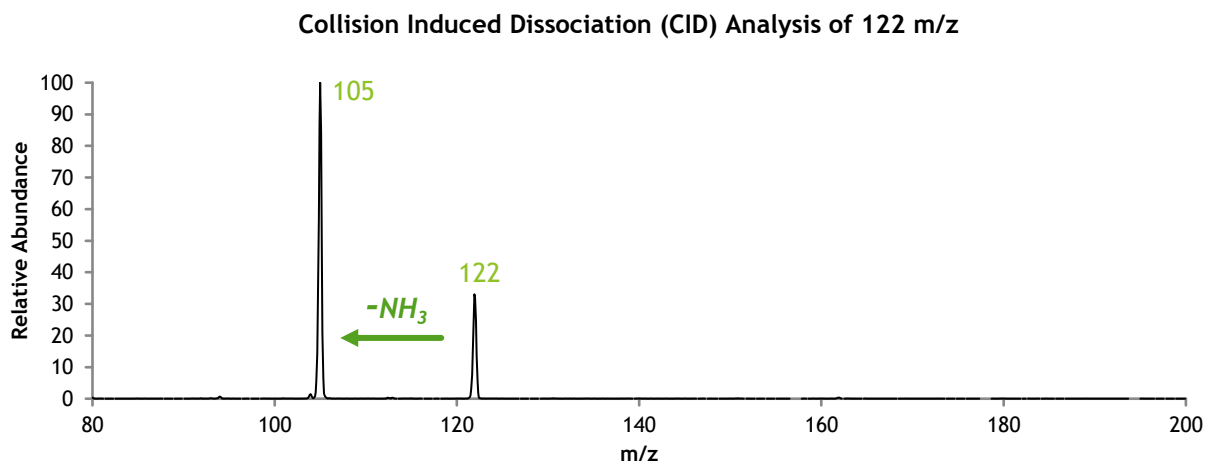


Figure 8: Spectra of insect from Figure 7 after application of CID. Applying energy to the PEA molecular ion via CID led to fragmentation of 122 m/z → 105 m/z, a fragmentation pattern characteristic to PEA. This is how 122 m/z peaks in all samples were verified to be caused by the presence of PEA.

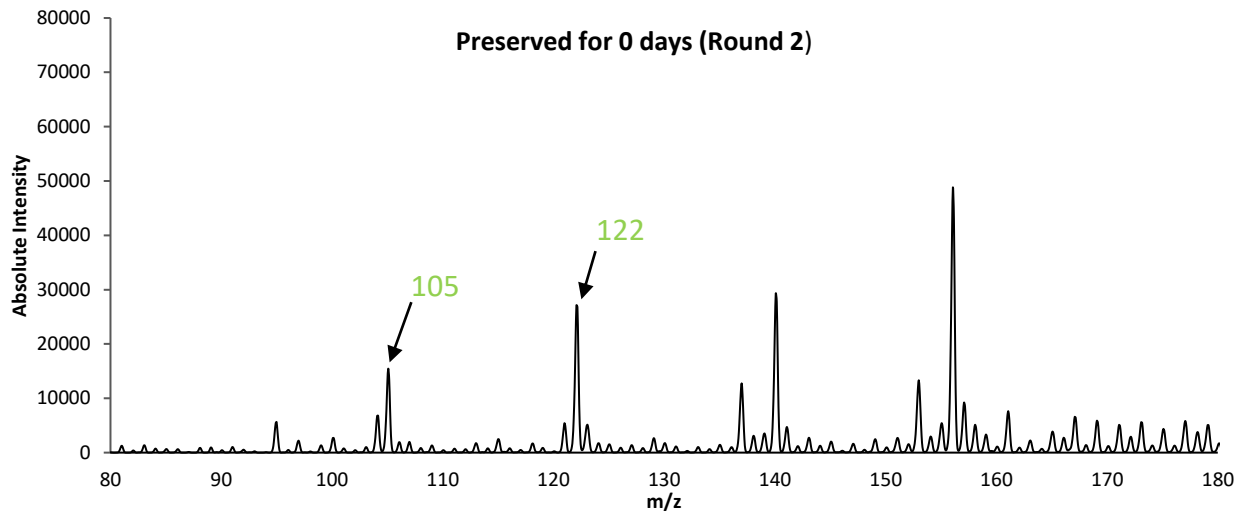


Figure 9: Insect from the 6000ppm feeding group that was euthanized after 5 feeding days and bug-sprayed immediately after euthanization.

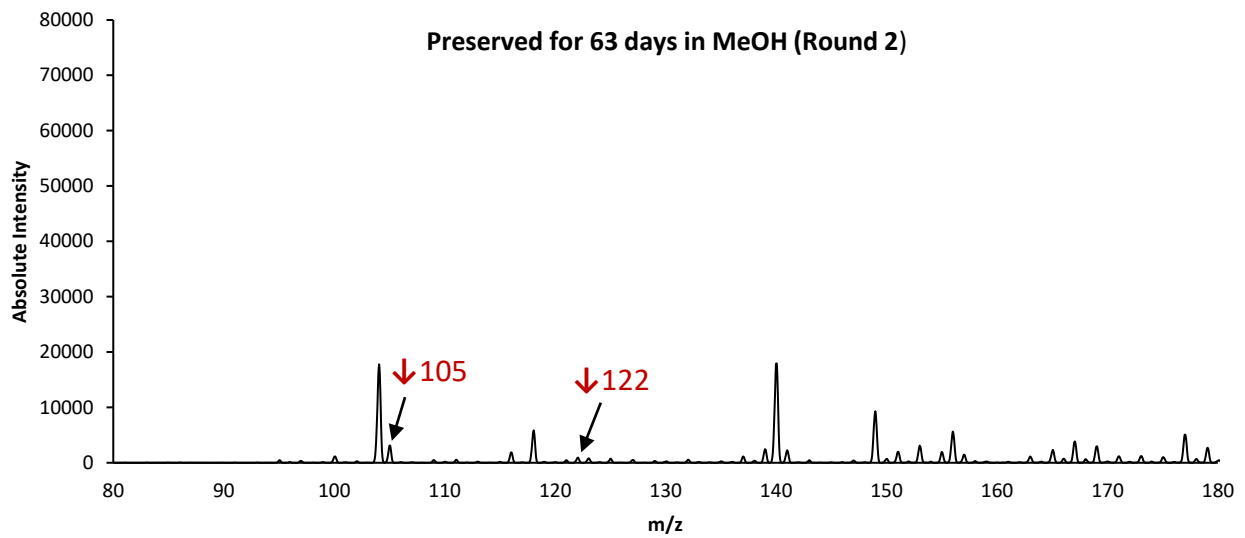


Figure 10: Insect from the 6000ppm feeding group that was raised under the same conditions as that in Figure 9 and bug-sprayed after being preserved for 63 days in MeOH. The characteristic 105 and 122 peaks of PEA decreased greatly in intensity.

Round 3:

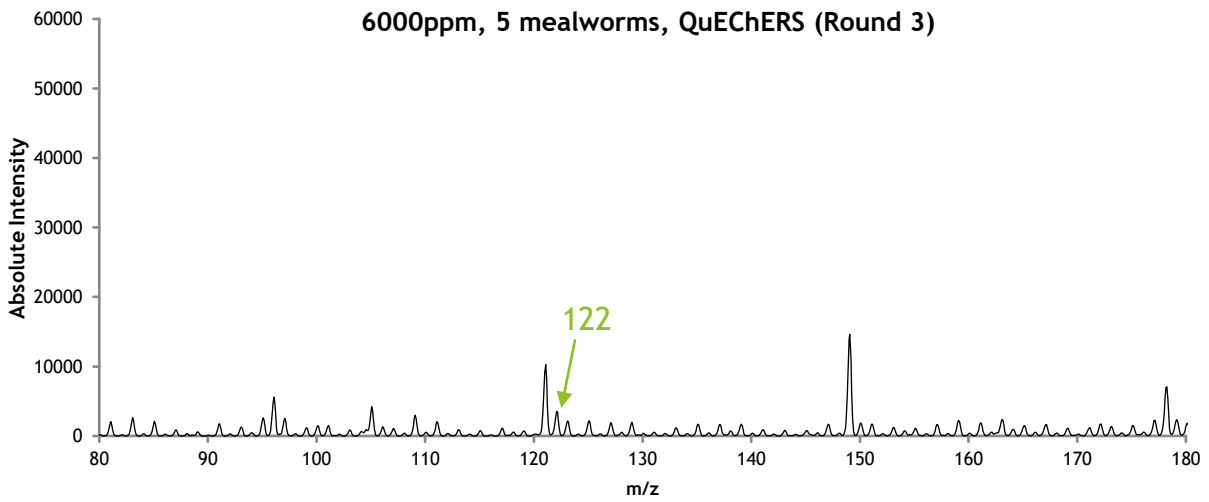


Figure 11: QuEChERS-extracted mealworm from the 6000ppm feeding group in Round 3.

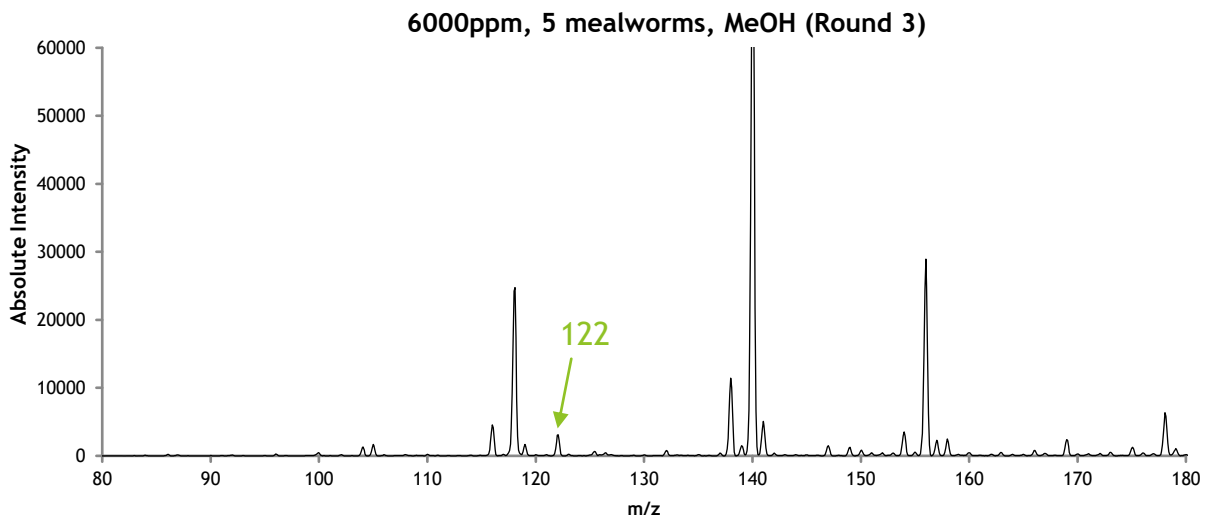


Figure 12: MeOH-extracted mealworm from the 6000ppm feeding group in Round 3.

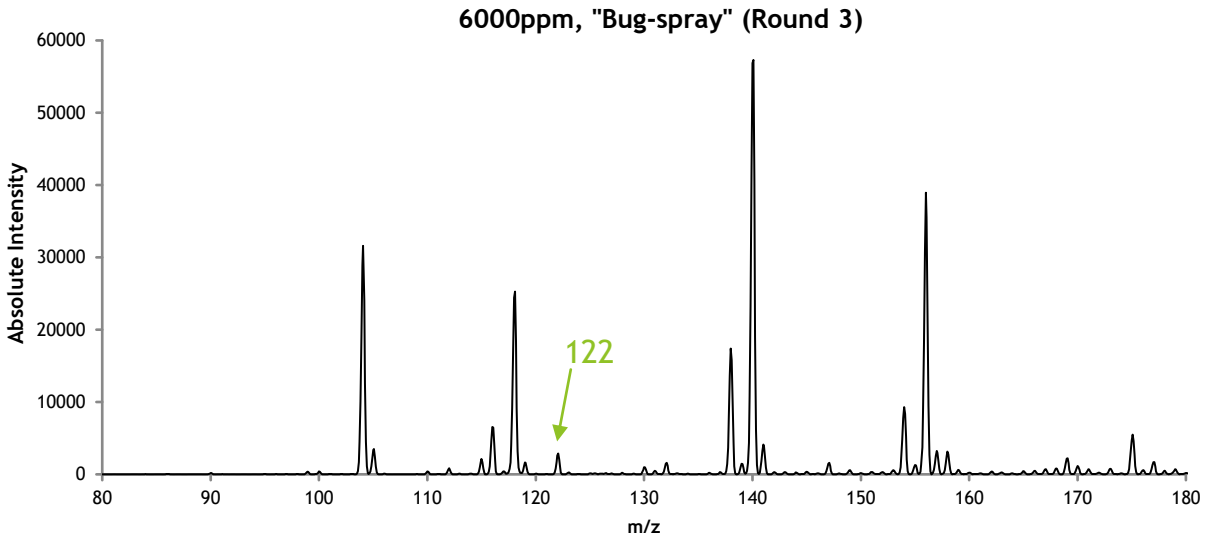


Figure 13: Bug-sprayed mealworm from the 6000ppm feeding group in Round 3.

Discussion

In Round 1, QuEChERS and EtOH extraction proved to be unsuccessful; PEA was unable to be detected in both the 100 and 1000ppm PEA groups across both extraction methods. The bug-spray extraction, however, was successful at the 1000ppm feeding level (Figure 5). Moving into Round 2, the QuEChERS method was altered and a MeOH extraction was employed instead of an EtOH.

In Round 2, bug-spray was successful at the 6000ppm level (Figures 7-8); however, QuEChERS failed to extract PEA once again and MeOH only showed very weak PEA signal at the 6000ppm level (Figure 6). Because only one mealworm was utilized per extraction method, it was hypothesized that this weak signal simply meant that more mealworms would need to be sampled at once. Therefore, in Round 3, five mealworms were extracted via QuEChERS and MeOH per sample instead of one. Additionally, the effect of insect preservation in MeOH was investigated in Round 2. 63 days of preservation in MeOH were shown to significantly decrease the absolute intensity of the PEA 122 m/z peak in insects from the same feeding group that were bug-sprayed (Figures 9-10).

In Round 3, bug-spray, MeOH, and QuEChERS extractions all showed similar absolute intensities of the 122 m/z peak at the 6000ppm level, signifying that all three extractions of PEA were successful (Figures 11-13). The improvement of the MeOH and QuEChERS extractions could be attributed to the homogenization of five insects at once instead of just one (Round 2) or three (Round 1). Despite this success at the 6000ppm level, PEA was not able to be reliably detected at the 6ppm level in any of the samples.

Over the course of all three Rounds, bug-spray was by far the most rapid, easy, and cheap method employed, making it the most practical sample preparation method for rapid detection of drugs using PSI-MS. This is because bug-spray allows for immediate analysis after euthanization. Although MeOH and QuEChERS showed similar PEA extraction levels in Round 3, the advantage of skipping extraction step altogether while only utilizing one insect is.

Future Directions

Because this proof-of-concept experiment showed success with PEA as a surrogate, future directions for this study involve raising mealworms on structurally-similar drugs such as methamphetamine and applying the successful extraction methods described here. In addition, a long-term goal is to eventually spike flesh with the drug of choice, place the flesh outdoors, and perform the rapid detection methods on blowfly larvae (maggots) instead of mealworms.

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