Analysis of Nicotine and Cotinine in *Drosophila melanogaster* Raised on Supplemented Media

David J. Hill[1], Tyler E. Douglas[2], Rebecca D. Tarvin[2] and Richard W. Fitch[1]
[1]Department of Chemistry and Physics, Indiana State University, Terre Haute, IN 47809.
[2]Department of Integrative Biology, University of California Berkeley, Berkeley, CA 94720.

Background & Significance

In the evolution of chemical defense in organisms, toxic compounds acquired from food sources could be an initial step in protecting naive species from predators. To test this hypothesis, four populations of *Drosophila melanogaster* were evolved through different environments for six generations. One group that underwent no selection, a population that evolved on nicotine laced food, a population exposed to parasitic *Leptopilina heterotoma* wasps every generation, and a population exposed to wasps and fed nicotine laced food every generation.[1] Flies were examined at developmental stages from larvae to pupae to adult. Nicotine and cotinine were measured.

Drosophila Melanogaster[2*]

Leptopilina heterotoma[3**]

*actual size (~2-3 mm)
**actual size (~2 mm)

Sample Preparation

A total of 78 samples arrived frozen, were allowed to thaw, and extracted with 100µL of MeOH (Figure 1). The MeOH was spiked with 1µM d4-nicotine and 10µM d3-cotinine as internal standards. Samples were equilibrated 32 hours at room temperature. The extract was separated and transferred to new vials for analysis (Figure 2).

Figure 1

Figure 2

Gas Chromatography-Mass Spectrometry

The first analysis we performed was GC-MS. This instrument system is comprised of an autosampler, GC, MS, and a computer for control and data processing (Figure 4). The autosampler collects sample (1µL) by syringe and injects into a 250ºC port in the GC. This flash evaporates volatile compounds, which them pass into the chromatographic column. The column is a fused silica capillary about 30m in length and sits in the oven that increases in temperature from 100-280ºC. Different compounds pass through the GC at different rates, exiting at a characteristic retention time (RT). Compounds then enter the MS, a 4-component instrument (Figure 5). The ion source produces high energy (70eV) electrons which knock off an electron from the compound producing a radical ion which then fragments.

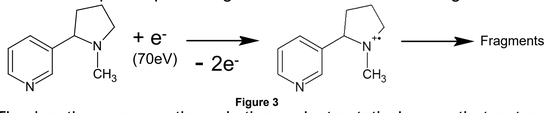

Figure 3

The ion then passes through three electrostatic lenses that act as funnel focusing ions into a quadrupole ion trap. In the trap, ions begin to orbit inside at characteristic radio frequencies (RF) according to their mass to charge ratio (*m/z*). Ions are then ejected by scanning RF across the energy range of the ions. Once ejected, they enter an electron multiplier and impact the walls, shedding multiple electrons per ion which then go on to impact and shed further electrons. Repeated events ultimately multiply the detector signal from one ion into ~10,000 electrons which can be readily measured.

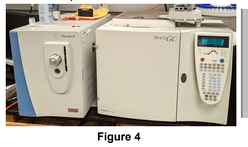

Figure 4

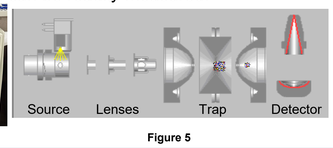

Figure 5

Liquid Chromatography-Mass Spectrometry

The Liquid Chromatograph-Mass Spectrometer (LC-MS) is another instrument used to separate and identify molecules similar to the GC-MS, but the LC-MS identifies both volatile and nonvolatile molecules. The LC is a four-piece device, the autosampler collects 10µL of a sample and is placed into the column. Unlike the GC-MS column that is 30m long, the LC-MS column may vary, we used a 10cm long column. The column separates compounds through the liquid phase and the retention time is dependent on the polarity of the molecule as well as the solubility. After the molecule passes through the column, it will go through the Taylor Cone that will electrospray the molecules. The Taylor Cone adds a charge (3-4kV) to the liquid, the droplets will evaporate and separate, and protonate the molecule.

Figure 6

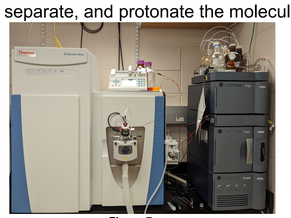

Figure 7

After the LC the ion will enter the MS through a capillary to guide the compound to the quadrupole. The quadrupole is a set of four rods with an alternating electric current, this allows ion masses to pass through one at a time. After the quadrupole, the ion is trapped in the C-Trap and released into the Orbitrap to be analyzed.

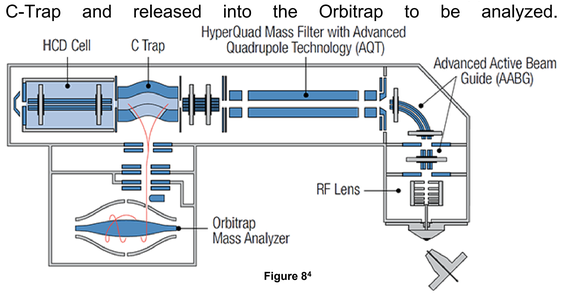

Figure 8[4]

GC-MS Analysis

The first step of GC-MS analysis is integration of the standard solutions at 1mM, 100µM, 10µM, 1µM, and 100nM of both nicotine and cotinine on the chromatogram, allowing the standard to be quantified for a calibration curve of Area vs Concentration. The Mass Chromatogram for nicotine's base peak at m/z 84 and cotinine's base peak at m/z 98 (**Figure 9**) can be integrated the same as the standard solutions, then compared to the calibration curve (**Figure 10** and **Figure 11**) to quantify the concentration of nicotine and cotinine.

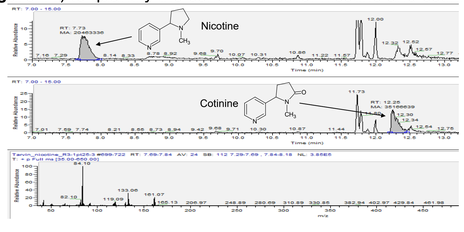

Figure 9

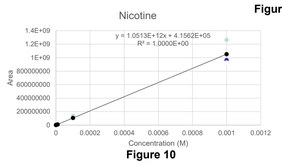

Figure 10

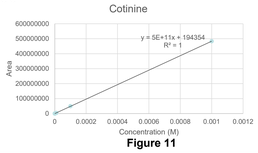

Figure 11

LC-MS Analysis

The LC-MS data analysis followed similar steps to determine concentrations of nicotine and cotinine. The same standard solutions were used other than 1mM nicotine and cotinine because in previous studies using the LC-MS nicotine and cotinine concentrations did not exceed 100µM. Calibrations curves were generated using the standard solutions' integration of the Mass Chromatogram of nicotine and cotinine ratio to the internal standard of d4-nicotine and d3-cotinine respectively. The LC-MS is a more sensitive instrument that allows the quantification of d4-nicotine and d3-cotinine unlike the GC-MS.

In GC-MS, only volatile compounds can be analyzed unlike the LC-MS that can analyze both volatile and nonvolatile compounds. Nicotine glucuronide is a common metabolite after nicotine consumption, and is a nonvolatile compound with a m/z 339 in the LC-MS. However, when injected into the GC-MS at 250ºC, the bond attached to the glucuronic acid and nicotine breaks (**Figure 12**). This will result in a higher yield of recovered nicotine in the GC-MS.

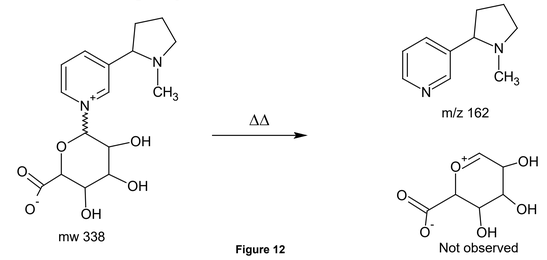

Figure 12

The areas of four metabolites in the LC-MS were calculated, Metabolite A, Metabolite B, Glucuronide A, and Glucuronide B. Metabolite A and B shown peaks at m/z 163 similar to nicotine, Glucuronide A and B shown peaks at m/z 339 (**Figure 13**). We did not have standards of nicotine glucuronide available so, we normalized all four metabolites by dividing the area of the metabolite by the area of d3-cotinine.

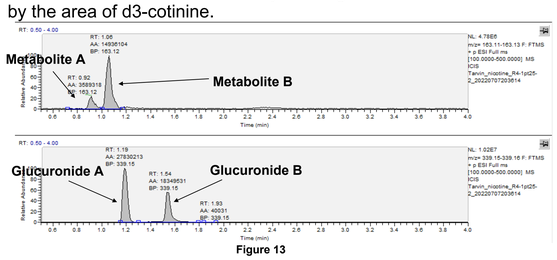

Figure 13

The areas of nicotine and cotinine were divided by that of the internal standard d3-cotinine in the GC-MS. Unlabeled cotinine has an EI base peak of *m/z* 98 while d3-cotinine is at *m/z* 98, while nicotine and d4-nicotine share a base peak at m/z 84. Concentrations were calculated from the regression equation (Figures 10 and 11).

Results

The GC-MS data and LC-MS data showed substantially different nicotine levels for each sample. Below, for example the R2 group of flies shows 2-10 fold higher nicotine in GC-MS as LC-MS.

GC-MS Nicotine

Sample	# flies	L	(M)	ng	ng/fly
R2-0-1	8	1.00E-04	-3.1E-07	-5.07	-0.63
R2-0.5-1	8	1.00E-04	4.24E-06	68.81	8.60
R2-0.5-2	8	1.00E-04	6.2E-06	100.52	12.56
R2-0.5-3	8	1.00E-04	6.82E-06	110.66	13.83
R2-1.25-1	8	1.00E-04	9.68E-06	157.00	19.63
R2-1.25-2	8	1.00E-04	1.06E-05	172.60	21.57
R2-1.25-3	8	1.00E-04	8.35E-06	135.48	16.93

Table 1

LC-MS Nicotine

Sample	# Flies	L	M	ng	ng/fly
R2-0-1	8	1.00E-04	1.6E-07	2.60	0.32
R2-0.5-1	8	1.00E-04	4.24E-07	6.87	0.86
R2-0.5-2	8	1.00E-04	1.82E-07	2.96	0.37
R2-0.5-3	8	1.00E-04	3.3E-06	53.57	6.70
R2-1.25-1	8	1.00E-04	3.73E-07	6.05	0.76
R2-1.25-2	8	1.00E-04	5.61E-07	9.10	1.14
R2-1.25-3	8	1.00E-04	6.9E-07	11.20	1.40

Table 2

Conclusions

When comparing LC-MS and GC-MS analysis of nicotine and cotinine the concentrations vary. In **Figure 14**, a graph of Area vs Sample that displays Nicotine in GC-MS, Nicotine in LC-MS, Metabolite A, Glucuronide A, and Glucuronide B. It is clear that the areas for nicotine in GC-MS follows the same pattern as the glucuronides in LC-MS. This supports injector decomposition as the probable cause of the apparent higher nicotine levels in GC-MS.

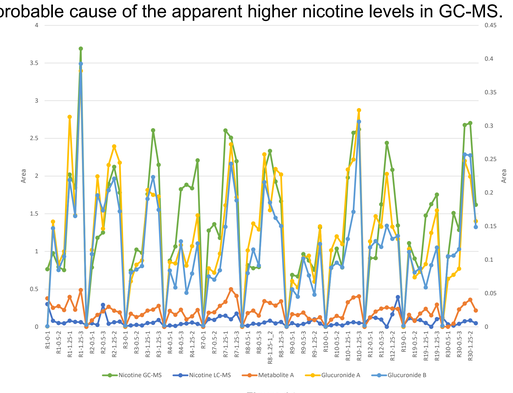

Figure 14

Acknowledgements

This work was performed as part of the Summer Undergraduate Research (SURE) program of 2022 at Indiana State University. We would like to thank Rheannah Donald and Sarah Moreau at Indiana State University, the Tarvin Lab for collaborative *D. melanogaster* work and the Department of Chemistry and Physics at Indiana State University for logistical support.

Funding

National Science Foundation
DUE-092345 (GC-MS)
CHE-1531972 (LC-MS)
IOS-1556982 (Collaborative)

University of California at Berkeley (RDT, TED)
Miller Institute for Basic Research in Science
Hellman Fellows Program

Indiana State University (RWF, DJH)
Center for Student Research and Creativity
Department of Chemistry and Physics
College of arts and Sciences
Office of the Provost

References

1. Douglas, T. E., Tamsil, K. E., Beskid, S. G., Tarvin, R. D., Nirtaut, B. E., Gernand, C. E., & Fitch, R. W. (2021). *Toxin breakdown does not preclude the potential for defensive toxin use in a fruit fly*. bioRxiv, https://doi.org/10.1101/2021.07.23.453507.
2. *D. melanogaster* photo. https://e-insects.wageningenacademic.com/drosophila_ melanogaster
3. *L. heterotoma* photo. https://www.flickr.com/photos/hans_smid/42086438181/
4. Schematic of the Q Exactive Plus mass spectrometer. https://www.creative-proteomics.com/support/q-exactive-plus-hybrid-quadrupole-orbitrap-mass-spectrometer.htm

Indiana State University