# Analysis of Nicotine and Metabolites in *Drosophila melanogaster* Raised on Nicotine-Containing Media

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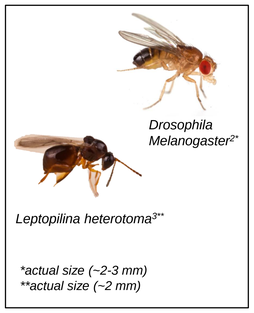
## Background & Significance

In the evolution of chemical defense in organisms, toxic compounds acquired from food sources could be an initial step, protecting undefended species from predators. To test this, Nicotine-sensitive (A3) and resistant (A4) strains of *Drosophila melanogaster* flies were cultured on nicotine-containing media and exposed to parasitic *Leptopilina heterotoma* wasps.[1] Flies were examined at developmental stages from larvae to pupae to adult. Nicotine and several metabolites were measured.




## Gas Chromatography-Mass Spectrometry

A Thermo Trace GC Ultra capillary gas chromatograph and iTQ 1100 ion trap mass spectrometer were used. The GC separates compounds in the gas phase. The compounds elute at characteristic retention times, then pass through a transfer line, and finally to the mass spectrometer (MS). The MS ionizes the molecules, then separates and detects the product ions. As seen in **Figure 2** below, the source produces ions at the beginning of the process and pushes them toward the lenses. The lenses focus the beam of particles using electric field gradients and progressively smaller paths so they can enter the ion trap. Once the trap is filled, the ions are scanned out using radio frequency voltage into the detector, where they are measured. This produces a chromatogram and a mass spectrum.

Figure 1

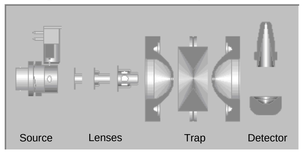



Figure 2

The most common ionization technique used in GC-MS is electron ionization, also called electron impact (EI).[4] A current passes through the filament in the source, which produces high energy (70 eV, ~1600 kcal/mol) electrons, which are then directed in a beam across the source using magnets. When analyte molecules pass through this beam, a valence electron is knocked out producing an unstable radical cation (molecular ion, M+). The molecular ion fragments into smaller ions and radicals. Only ions are seen on the mass spectrum because uncharged particles cannot be trapped and scanned. As shown in **Figure 3** below**,** nicotine fragments from its molecular ion of mass-to-charge ratio (*m/z*) 162 into an *m/z* 84 fragment. Nicotine is oxidized by many organisms to cotinine, likely using chromosome P450. This metabolic pathway is also shown below. Cotinine follows the same fragmentation pathways as nicotine but with different ion masses due to the change in structure.

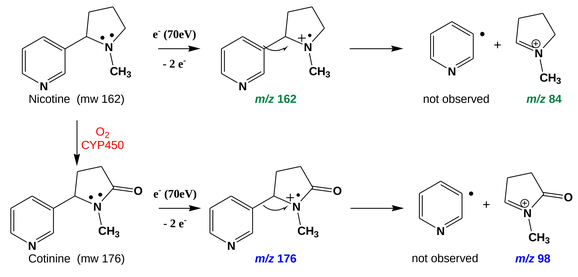



Figure 3

## Sample Preparation

Our analysis protocol is a dilute-and-shoot keeping with QuEChERS principles.[5] Flies were collected at each developmental stage (5 organisms each) and placed into individual vials containing limited volume inserts (LVI) and frozen until analysis as shown in **Figure 4.** Methanol (100 μL) was added and the samples soaked for 72 hours at room temperature to reach equilibrium. Extracts were then transferred it into fresh LVI vials.

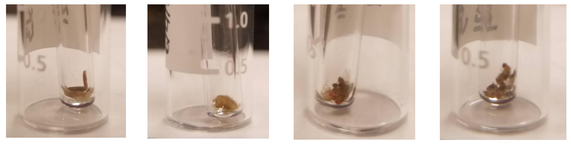



Figure 4

Crude extracts (1μL) were injected into the GC and separated on an RTX-5MS (5% phenylsilicone/95%methylsilicone) column at 1 mL/min flow at 100ºC (held 1 min) and ramped at 15ºC/minfrom to 280ºC (held 10 min). The MS was autotuned with perfluorotributylamine prior to analysis and used 70 eV energy, with automatic gain control (25 ms max ion time) and solvent delay of 3 min. Nicotine standards were used for calibration using serial dilutions from 100 μM to 1 μM and blanks at the start, end and between sets of samples to correct for instrument drift.

## Data Analysis

For selectivity, extracted ion chromatograms were used for integration of nicotine (6.6 min, *m/z* 84) and cotinine (9.6 min, *m/z* 98). **Figure 5** below depicts the mass spectrum of a pupal sample (N3A3P1), containing both compounds. Fatty acid methyl ester lipids are also identified by comparison of mass spectra to NIST reference spectra. These were integrated as follows: myristic (14:0), 9.5 min, *m/z* 43; palmitic (16:0), 10.8 min, *m/z* 55; palmitoleic (16:1, 10.9 min, *m/z* 39, and linolelaidic (18:2, 12.1 min, *m/z* 67).

## Representative Gas Chromatogram and Mass Spectra for Nicotine and Cotinine

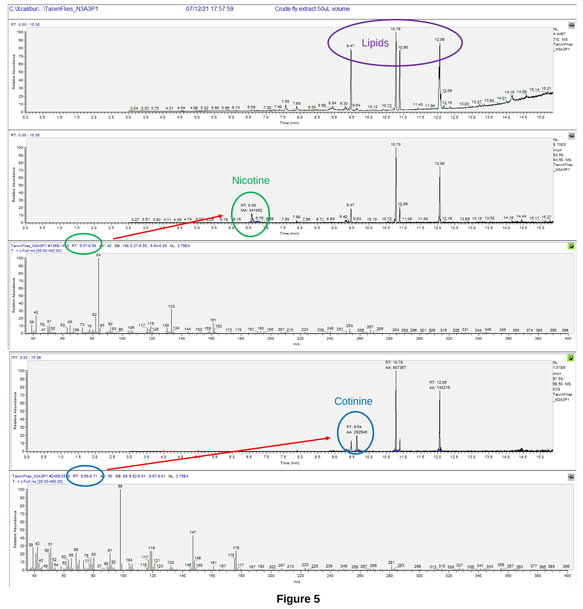



Figure 5

## Results

The quantity of nicotine per fly were compared for sensitive (A3) and resistant (A4) lines. According to **Figure 6** below, the resistant line has significantly more nicotine ng/fly than the sensitive line across all developmental stages. Cotinine was also observed in all stages, indicating metabolism of nicotine. Other possible metabolites nornicotine and myosmine were not observed. The ratio of cotinine to nicotine is important as it gives a measure of the rate of metabolism. **Figure 7** shows the ratios of the cotinine to nicotine areas for each of the two groups. Nicotine was not detected in 3/5 of larval samples for A3, but significant cotinine was observed in all larvae.

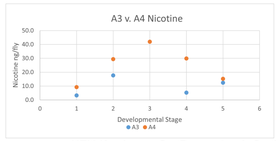
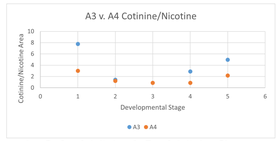



Figure 6




KEY (**1** = Larva, **2** = Pupae day 1, **3** = Pupae day 3, **4** = Adult day 1, **5** = Adult day 3).

Figure 7

## Lipid Normalization

In the absence of an added internal standard, we examined fly lipids as possible compounds that might control for variation in mass. Four lipids unique to the flies were identified in the extracts (see **Figure 8**, below); and integrated as follows: methyl myristate (14:0) *m/z* 43; methyl palmitate, (16:0) *m/z* 55; methyl palmitoleate (16:1) *m/z* 39, and methyl linolelaidate (18:2) *m/z* 67.

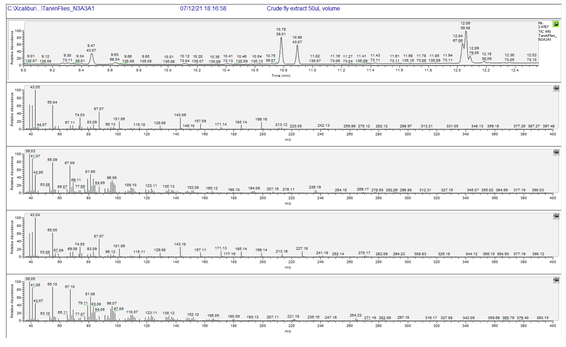


Figure 8

We then calculated the ratio the area of nicotine to that of each of the four lipids based on the ions selected previously. This is shown in **Figure 9** below. While the plots show somewhat similar trends, it appears that lipid profiles vary through developmental stages. Thus it is not clear that this form of internal normalization is viable.

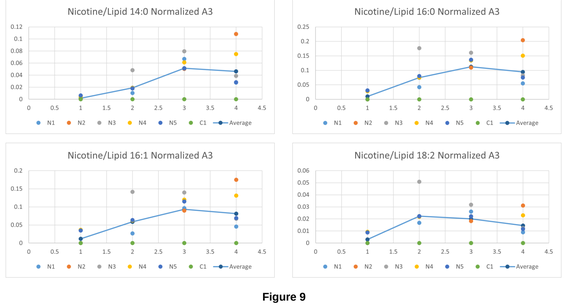



Figure 9

## Conclusions

We have determined the levels of nicotine in sensitive and resistant strains of *D. melanogaster* flies cultured on nicotine-supplemented media during development. A QuEChERS approach was found to be satisfactory for analysis. Unfortunately, the lack of an internal standard limited the method. Internal normalization to fly lipids was examined, but variation of lipids with developmental phase limited its utility. Nicotine levels were found to be consistently higher in the resistant strain. We also looked for nicotine metabolites cotinine, nornicotine and myosmine. The latter two were not found, but cotinine was observed. Curiously, resistant flies, while having higher nicotine, have a lower cotinine/nicotine ratio than the sensitive flies studied. We hypothesize that this is because resistant flies ingest more nicotine-containing food on the media. Other experiments have suggested that nicotine increases the lifespan of the flies in the presence of wasps, suggesting passively acquired nicotine may be protective. Further experiments will further evaluate the value of internal lipids for un-normalized analyses and will use isotopically labeled nicotine and cotinine as internal standards to obtain more precise data.

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